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Animal Nutrition and Productions

Series II

Edited by
Daniel Simeanu and Daniel Mierlita

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About the Editors

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Daniel Simeanu, PhD, University hab. professor, Iași University of Life Sciences, Romania, acquired all of his degrees in higher education in Romania, from his preparatory degree in 1999 to becoming a tenured professor in 2021. He defended his doctoral thesis in 2003 and his habilitation thesis in 2021 to become a PhD supervisor. Research areas: nutrition and feeding; the influence of feeding on animal production quality. Publishing activity: 200+ papers published in national and international journals, with 54 published in Web of Science journals. He has managed four research projects and participated as a member of 25 grant teams. He has published five books as the sole or first author and co-authored 15 university books and handbooks. He has been awarded two prizes, one by the Romanian Academy (2004) and one by the EURO INVENT Exhibition of Creativity and Innovation (2019) for two referential books in the field of animal sciences engineering (Treaty of Aviculture—coord. by Vacaru-Opriș I. and Animal productions—Simeanu D., Doliș M.G.).

Daniel Mierlita

Daniel Mierlita graduated in Animal Science and has a PhD in Animal Nutrition. Currently, he is a professor with tenure at the Animal Science Department of the University of Oradea and is a PhD supervisor on Animal Nutrition at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania. His current research interests include animal nutrition, feeding management, feed efficiency, animal product quality (fatty acid profiles, health lipid indices, bioactive compounds, and antioxidants), and alternative feed ingredients. Daniel has participated in 19 scientific projects, out of which 8 were coordinator projects. Daniel Mierlita is an associate editor of refereed journals, is a scientific committee member of conferences, and has collaborated with a number of companies on projects and consulting, some of them having been granted by the European Commission. To date, Daniel has contributed to 230 scientific publications, 44 of which are in international, refereed journals.

Preface

When we started working on this Special Issue, we assumed that the research results to be presented herein would provide solutions and strategies to the various problems related to animal nutrition and production management; however, the collection of published articles shows that the presented solutions could be useful for a wider range of applications, including aspects of animal welfare and health and the safety and quality of food products of animal origin. Therefore, the review process for this Special Issue generally focused on one of the topics mentioned in the objectives above but all have in common animal nutrition as a key element of the sustainability of animal production and quality and safety for both the consumer and the environment. The Guest Editors of the Special Issue directly invited many potential authors. Out of a total of 41 full papers received, only 23 manuscripts were accepted (an acceptance rate of 56.1%) after a thorough review by two or more primary reviewers chosen for their expertise, particularly in the field of animal nutrition. All papers were improved upon as a result of the reviewers' comments, and the final decision was made by one of the Guest Editors based on the reviewers' comments and ratings.

Therefore, as part of the Special Issue "Animal Nutrition and Productions: Series II", 23 articles covering various aspects of new trends in animal nutrition and production were published through a multi- and trans-disciplinary approach. The results of research on the effect of different nutritional strategies on not only productive performance, the nutritional and sanogenic quality of products of animal origin (milk, meat, and eggs), and animal well-being and health but also on the environment are presented. In addition, various aspects are addressed related to the preservation of biodiversity and the conservation of animal genetic resources, the evaluation and monitoring of pollutants in feed and milk, and the standardization of the use of antibiotics on pig farms.

The research results clearly demonstrate that precision feeding techniques that meet the nutritional needs of animals in accordance with their genetic potential are essential for increasing productivity, ensuring animal welfare and health, and for the production of animal products that are safe for consumers, offering innovative perspectives in this field. In addition, the use of unconventional resources rich in nutrients and bioactive compounds can contribute to increasing the sustainability of animal production and obtaining products with naturally improved nutritional profiles that contribute to maintaining and promoting human health. This publication addresses researchers, postgraduates, and students studying animal sciences, veterinary science, zoology, and biochemistry.

Daniel Simeanu and Daniel Mierlita
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Editorial

Animal Nutrition and Productions: Series II

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The Food and Agriculture Organization of the United Nations estimates that by 2050, there will be a 58% increase in dairy consumption and a 73% increase in meat and egg consumption, worldwide, which would put additional pressure on the availability of natural resources. In addition, to feed almost two billion more people by 2050, it is necessary to sustainably increase the yield of agri-food products of animal origin [1]. Within such a context, raising animals and especially providing the animal feed quality and quantity required will be a challenge in line with the three pillars of sustainability (economy, social, environment), which will have a far-reaching impact on food prices, human nutrition, and the global economy. It is undeniable that solving these constraints is complex and multifactorial, in order to securely raise animals and obtain safe and affordable food of animal origin to meet the increased demand.

The key element of the sustainability of animal food production is animal nutrition, which affects almost every sector of animal production: performance and efficiency, product quality and safety, health and welfare, farm economic viability, and environmental protection.

This Special Issue "Animal Nutrition and Productions: Series II" is a collection of 23 articles that capture the latest knowledge and innovations in animal nutrition and production and cover current topics through multi- and trans-disciplinary approaches, such as additives and novel feeds, animal productivity, reproductive efficiency, animal welfare and health, and product safety and quality. This collection consists of 19 original research articles, 3 review articles, and a meta-analysis, explored by 143 researchers from many different countries (Romania, China, Mexico, Poland, Czech Republic, Italy, and Canada). Most articles were written by authors from Romania (12 articles), followed by authors from China (4 articles), Mexico (3 articles), Italy, Poland, the Czech Republic, and Saudi Arabia (1 article each).

The research topics addressed by the authors focus on the evaluation of the effect of different nutritional strategies on the productive performances, nutritional value, and safety of agro-food products of animal origin (milk; eggs: chicken and quail; meat: chicken broiler, duck, mutton, veal, pork), the welfare and health of the animals, their reproductive function, and also the effect on the environment. Other researchers had objectives such as an evaluation of the quality of rabbit meat in relation to the gender and type of muscle, an analysis of the quality of polyfloral honey, an analysis of the quality of some dairy products, an evaluation and monitoring of pollutants in feed and milk, a standardization of the use of antibiotics in pig farms, and also studying aspects related to the preservation of biodiversity and the conservation of genetic resources.

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Nutritional strategies play an essential role in determining animal productivity and the nutritional and safety properties of animal products, using some key indicators: the type and composition of the diet and the balance of dietary macronutrients, vitamins, and minerals. In addition, the use of alternative feed sources rich in nutrients and bioactive compounds contributes to increasing the sustainability of animal production, improves animal health, and leads to obtaining animal products with improved nutritional profiles which are also safe for consumers [2].

The scientific papers included in this Special Issue discuss the identification of novel feed ingredients, the optimization of nutrient availability, precision feeding techniques in meeting the nutritional requirements of animals, and different feeding strategies for ruminants, poultry, and pigs.

Ruminant nutrition research focuses on evaluating the effect of diet on milk and dairy products' quality, reproductive performance in goats and rams, and the influence of feed additives and meat yield and quality in lambs and calves.

Studies on poultry feeding strategies (laying hens, laying quails, broilers, and ducks) have focused on four main topics, namely feed additives, bioproductive performance, agro-food product quality (eggs and meat), and fowl health and welfare.

In the case of pigs, feeding strategies have focused on the opportunity of using alternative feed sources, and their effect on yield, meat quality, and environmental protection, but also aspects related to the need of regulating the use of antibiotics in pig farms in relation to maintaining and promoting public health.

The nutritional quality and content of bioactive compounds, with the potential to improve consumers' health, in milk and milk products are influenced by certain factors, with the most important being those related to animal nutrition and feeding. Thus, milk obtained from pasture-raised cows (generically called grass milk) is sought after and preferred by consumers because they consider it healthier than that obtained from cows fed indoors on stock feed [3].

In this respect, Rațu et al. [4] evaluated the effect of grazing on the chemical composition and fatty acid profile of milk and yogurt compared to that of cows on an indoor feeding system with TMRs (total mixed rations) based on corn silage, grass hay, and concentrates. Grazing cows provided better milk quality, especially in terms of fatty acid profile, i.e., polyunsaturated fatty acid (PUFA) content (CLA: conjugated linoleic acid, LA: linoleic acid, and ALA: α-linolenic acid). In addition, the fatty acid profile of yogurt was improved compared to the original profile in raw milk, due to the increase in the proportion of PUFAs resulting from fermentation processes.

At the same time, the studies carried out by Maciuc et al. [5] demonstrated that cheeses marked with the quality label "mountain product", obtained from animals that had access to pasture, had better nutritional and safety properties than the conventionally produced cheeses, i.e., a higher content of minerals (Ca, P, Fe) and PUFA, which made better values of the health lipid indices possible (atherogenic index, thrombogenic index, ratio of hypocholesterolemic/hypercholesterolemic fatty acids) compared to conventional cheeses. In conclusion, cheeses bearing the "mountain product" quality label are more nutritious, contribute to maintaining and promoting the health of consumers, and are obtained in a sustainable production system where mostly local inputs are used as feeding resources.

Milk, in addition to its nutrient and bioactive compound content, can be contaminated with a number of pollutants with adverse effects on the consumers' health [6]. The occurrence of pollutants in feedstuffs depends on the pollution profile of the geographical area, and the transfer of pollutants from feed to milk is influenced by the amount and profiles of dietary pollutants, by pollution sources, and by the interaction between some categories of pollutants [7].

The studies carried out by Rodriguez-Cordero et al. [8] and Carrillo-Muro et al. [9] focused on the use of calcium propionate (gluconeogenic compound) in the diet of growing calves and finishing lambs, respectively, with the aim of increasing dietary energy availability and thus improving both growth performance and body fat reserves, as well as

meat quality, without compromising animal health. The studies concluded that calcium propionate (CaPr) is an effective feed additive that provides additional energy to growing animals. Early diet supplementation of growing calves with CaPr (20 g/calf/day) increased average daily gain (ADG), body mass (BW), feed efficiency (FCR), and body fat reserves, while the metabolic profile also improved (blood urea nitrogen decreased and the activity of some enzymes increased) [8]. In lambs, supplementation of the finishing diet with CaPr (10 g/lamb/day) for a period of 24 to 28 days prior to slaughter can improve growth performance and carcass weight without affecting organ mass or meat quality [9].

Numerous studies have demonstrated the importance of nutritional factors in the reproductive activity of animals. Adopting correct feeding strategies positively influences puberty onset, ovulation rate, embryo survival, and semen quantity and quality. In this context, Pascal et al. [10] concluded that supplementing rams' diets with fat-soluble vitamins (A, D, E) and trace elements (Zn, Fe, Mn, Cu, Co, I, Se) significantly improved reproductive performance, as a result of increasing ejaculate volume and improving sperm quality (increased concentration of viable spermatozoa and decreased proportion of abnormal spermatozoa), and increased testosterone levels, which improved mating behavior. This topic was also addressed by Mohammed and Al-Suwaiegh [11] who supplemented the diet of pregnant goats (4 weeks prior to mating and 4 weeks post partum) with *Nigella sativa* seeds (10.0 and 20.0 g seeds of *N. sativa* per kg of diet) with the aim of improving ovarian follicle development, health status, and milk composition. Such a feeding strategy was effective in improving feed conversion, milk nutritional quality, metabolic profile, and ovarian follicle development.

In addition to improving poultry productivity, there is high interest in enhancing the nutritional and sensory qualities of poultry products (eggs and meat), thereby influencing consumers' preferences and choices, as shown in some of the papers published in this Special Issue. The meta-analysis conducted by Orzuna-Orzuna et al. [12] led to the conclusion that supplementing the diet of laying hens with essential oils improves the productive performance (increases egg yield and egg mass and decreases the feed conversion ratio) and egg quality (mineral shell thickness and strength and intensity of the yolk color). In addition, dietary inclusion of essential oils improved blood serum antioxidant status and intestinal morphology in laying hens. The best results were obtained when the primary bioactive compounds of the essential oils were menthol, cinnamaldehyde, terpinen-4-ol, or mixtures of bioactive compounds. In contrast, studies by Li et al. [13] demonstrated that replacing vitamin D3 in the diet of laying hens with 25-hydroxyvitamin D3 (125 μg/kg) did not influence the production performance, whilst it negatively impacted shell thickness and weight and improved bone strength (femur and tibia) throughout the late laying period.

The most important and suitable sources of protein for poultry are soybean products and by-products, which due to cultivation requirements have become progressively more limited and expensive. In this context, lately, research attention has focused on the identification and validation of alternative protein sources for animal feed. According to Struti et al. [2], the introduction of white lupine beans from low-alkaloid varieties into the diet of laying quails (250 g/kg), in association with specific exogenous enzymes, makes the major substitution of soybean meal in the diet (by more than 60%) possible without affecting egg yield, egg weight, physico-chemical parameters of egg quality, or FCR. Meanwhile, white lupine beans can be used as part of a strategy to improve the nutritional quality of eggs by increasing the content of omega-3 fatty acids and carotenoids in the yolk and lowering the cholesterol concentration.

Similarly, Bondar et al. [14], following the analysis of sources in the literature, state that spirulina (*Spirulina platensis*) can be used as an alternative feed source for fowl, being widely recognized as a valuable source of protein (55–70%), essential fatty acids (18–20%), various vitamins (such as thiamin, riboflavin, pyridoxine, vitamin B12, vitamin C), and numerous bioactive compounds (tocopherols, chlorophyll, carotenoids, phenols), with positive effects on productive performance, intestinal integrity, and immunity.

In recent years, there has been increased interest in improving the production performance of animals, maximizing the efficiency of feed utilization, and increasing the safety of products intended for human consumption [15]. Trace elements are essential nutrients in improving the growth performance of fast-growing broilers. In this sense, Lv et al. [16] investigated the effects of replacing inorganic trace elements with organic ones (sucrose-chelated trace elements) in broiler diets and concluded that this advanced mineral chelation technology can reduce the trace element requirements of broilers. Thus, using chelated trace elements (Cu, Fe, Zn, and Mn) at a dose of 1–1.5 g/kg instead of inorganic trace elements (2.0 g/kg diet) did not affect growth performance, dietary energy, nutrient utilization, antioxidant capacity, or trace element deposition in the liver. It turns out that organic trace elements can reduce mineral excretion in broilers, which can decrease environmental pollution.

Although global poultry meat consumption is following an upward trend, consumers blame the poor sensory properties and low nutritional value of the meat provided by fast-growing chickens. To respond to the new market preferences, the research carried out by Usturoi et al. [17] evaluated the effect of dietary protein supplementation on growth performance and meat quality in slow-growing broilers (Hubbard JA757 hybrid). The results demonstrated that the level of crude protein increase in the diet of chickens (+0.5% CP in the growth phase and +1.0% CP in the finishing phase, compared to the recommendations of the Hubbard company) led to an improvement in the production performances (LW and FCR) of meat and carcass quality (more breast and thigh %).

A limiting factor for the development of animal production is represented by the shortage of feed ingredients. In tropical and subtropical areas, rubber trees are widespread and their seeds are often discarded and not redeemed effectively. Rubber seed oil contains important amounts of n-3 PUFA (approx. 26% of total fat), which gives them the opportunity to be used in animal feed not only as a concentrated source of energy but also as a potential resource to obtain food products of animal origin enriched in n-3 PUFA (functional foods). Zhao et al. [18] demonstrated that replacing soybean oil with rubber seed oil reduced abdominal fat deposition and increased n-3 PUFA levels in meat without affecting the growth performance of Pekin ducks. In addition to nutritional factors, the housing system can influence productive performance, carcass traits, and bone composition in ducks. Krunt et al. [19] revealed that ducks reared on deep litter with access to a swimming pond had a higher live weight and better feed conversion ratio but a lower proportion of breast in the carcass structure than ducks maintained on deep litter without access to a pond. Housing conditions did not affect the fracture toughness of the fowl tibia and femur; however, ducks that had access to the pond had more Ca and Mg in the tibia and more Mg in the femur.

Classic diets for pig nutrition are based on a mixture of corn and soybean meal as the main sources of energy and protein, which correspond very well to the nutritional requirements of pigs. However, there is currently a growing discrepancy between yield, availability, and demand for feed. Thus, the identification and use of locally accessible resources are necessary to ensure animal feed requirements. Roguski et al. [20] propose the partial substitution of cereal grains and protein components in pig feed with Wet Distillers' Grains plus Solubles (WDGSs), which are by-products in the bio-ethanol industry and are characterized by a high concentration of crude protein and crude fat (% of DM—dry matter). The authors concluded that WDGSs can be used in the liquid feeding of pigs in a proportion of up to 15% of the DM, without affecting the pig performance, carcass traits, meat quality, proximate composition, or physical–chemical traits of pork. Similar topics were also addressed in the papers of Chiofalo et al. [21] and Mihaila et al. [22]. Chiofalo et al. [21] evaluated the agronomic traits, chemical composition, fatty acid profile, and in vitro fermentation characteristics of twelve varieties of *Amaranthus* spp. grown in a semi-arid Mediterranean area. Among the varieties studied, *A. hypochondriacus* proved to have the best agronomic traits and the seeds had the best nutritional value (high protein and starch content and a good fatty acid profile), being potential ingredients for pig diets.

Mihaila et al. [22] investigated the possibility of substituting soybean meal in pig feed with guar meal, which is a by-product rich in protein and carbohydrates, obtained after mechanical separation of the endosperm from the germ and coat of guar seeds. The obtained results demonstrated that guar flour can replace 100% soybean meal in a pig finishing diet, without changing animal performance or carcass traits, and can have a positive impact on the main greenhouse gas emissions resulting from enteric fermentation (E-CH_4) and manure (M-CH_4 and NO_2).

Food safety, the environment, and public health are threatened by farmers' overuse of veterinary antimicrobials. Based on data collected from 675 swine farms in China, Si et al. [23] reported that the main factors involved in this phenomenon are a lack of access to valid information or skill constraints, as the Internet has become the main source of information for young farmers. The authors consider it necessary to standardize the use of antimicrobials by farmers through the intervention of governmental bodies and the application of sustainable agricultural policies.

In response to the growing emphasis on the quality of products of animal origin, Frunză et al. [24] investigated the effect of gender and muscle type on the fatty acid profile, health lipid indices, texture, color, and sensory quality of rabbit meat. Research has shown that male meat was healthier (richer in essential fatty acids, with a better ratio of hypo-/hypercholesterolemic fatty acids and lower values of atherogenic indices (AIs) and thrombogenic indices (TIs)). The flesh of females was more tender but less pigmented than that of males, while overall sensory attributes were better in males. The authors recommend slaughtering females 3–4 weeks earlier than males in order to avoid excessive fat deposition and, consequently, the development of unfavorable lipidic indices for consumers' health. The studies carried out by Albu et al. [25] also fall under the same theme, evaluating the quality of multifloral honey from the northeast of Romania. The analyzed honey samples were distinguished by a high content of antioxidants (phenols and flavonoids), confirming its therapeutic character. The predominant minerals in the honey were K, Ca, and Na, and in a limited number of samples the Pb content was above the detection limit but within the limit recommended by the legislation. The use of the FTIR spectral method confirmed the difference between the samples, investigated through pollen analysis, and highlighted the differences in the chemical composition of the honey.

The preference and orientation of consumers towards 'natural products' have encouraged many farmers to introduce grazing in the management of dairy farms, as it has a positive impact on the content of nutrients and bioactive compounds in milk but also on the health and behavior of dairy cows. The studies carried out by Blaga Petrean et al. [26] showed that the general welfare of dairy cows improved when they had access to pasture, proving the superiority of this management system that allows cows to exhibit a wide range of natural behaviors.

The decrease in the genetic diversity of animal populations imposes the need to conserve endangered species in order to maintain the biodiversity of animal genetic resources. In this sense, the study by Davidescu et al. [27] had the main aim of highlighting and comparing the most recent articles on the origin, evolution, genetic diversity, and phylogenetic relationships of Podolian cattle, with special emphasis on the endangered Romanian Gray Steppe breed, whose importance is given by the special biological properties it possesses (adaptability and resistance against diseases and to severe climate and habitat conditions, and longevity). The breed should be well preserved for the improvement of other cattle and for the protection of biodiversity. The authors propose taking immediate measures and allocating adequate financial resources to conserve the valuable genetic diversity of Romanian Gray Steppe cattle, which still represent a valuable genetic pool.

In 2022 we proposed to produce a second volume of the *Animal Nutrition and Production* Special Issue; here, we have succeeded, let us say with more success—the second edition has 23 published articles compared to only 20 in series I. The audience created by the second Special Issue also indicates that nutrition and animal productions are of interest to researchers from all over the world. This time, the range of addressed topics was very

varied and very interesting, as indicated by the large number of views on the second series (over 27,000 at the time of publishing this Editorial). We thank the Editorial Board of the *Agriculture* journal and the Farm Animal Productions section for allowing us to collaborate and complete the second Special Issue of Animal Nutrition and Productions: Series II.

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Pasture Access Effects on the Welfare of Dairy Cows Housed in Free-Stall Barns

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Abstract: Despite considerable research regarding the benefits of natural living conditions on several aspects of the health and well-being of dairy cows, the effects of pasture access on their overall welfare are less studied. In this comparative study, the Welfare Quality® protocol was applied in 22 zero-grazing and 17 grazing access farms with an ulterior statistical exploration of the differences found. Moreover, correlations were calculated between pasture access and animal-based welfare measures. Aside from the multiple benefits of pasturing identified within the welfare measures, criteria, and principles, in the overall classification, the farms with permanent confinement ranked lower than the grazing farms. Although both systems used free-stall barns, allowing the cows' movement, the grazing animals showed improved overall welfare. Yet, the origin-related adaptation of the animals could play a role. The authors recommend research-based tailoring whenever these conditions are intended to be transposed in technology, especially in intensive systems.

Keywords: grazing; animal welfare; zero-grazing; welfare quality protocol

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1. Introduction

In terms of animal-based health parameters, cows with access to pasture proved to fare better than those housed continuously indoors [1]. Previous studies have shown less lameness in systems with grazing [2,3], a reduced number of integument lesions [4], and a decreased incidence of mastitis [5] compared to indoor housing. Additionally, mortality was found to be lower in herds with pasture access than in those without [6,7]. Other studies focused on different benefits of pasture access beyond strictly health advantages. Pasture-based systems offer increased freedom for cows to express their full behavioral repertoire [1,8]. When on pasture, they display better herd synchronization [9], spend more time lying [10], and show less agonistic behaviors [11] compared to confined animals. Concluding their study, Crump et al. [12] state that giving dairy cattle pasture access appears to induce more positive emotional states than cubicle housing.

From an economic perspective, including current market tendencies combining the preference for natural products with the ethical considerations of the consumers, the farmers could be encouraged to allow their dairy herds to graze. According to Stampa et al. [13], many consumers are willing to pay a higher price for milk from cows kept on pasture than from cows kept indoors. Yet, some farmers have concerns regarding the challenges the cows may be exposed to while grazing. The risk of encountering internal and external

parasites or contagious diseases from other animals and the vulnerability of facing suddenly changing and possibly extreme weather conditions (such as high temperature) are some possible caveats listed by several authors [1,8]. Especially in the changing environmental conditions increasingly experienced lately, the heat stress during summer months has the potential to impair the welfare of the cows both on the pastures and inside the barns [1]. Thus, grazing per se does not guarantee animal welfare; however, it must be remembered that no management system does. To obtain a standard model of such a type of cattle housing that can serve as a guide for both policymakers and consumers willing to pay a higher price for welfare-friendly products, it is necessary to assess the overall welfare of the cows on pasture.

Continuous indoor housing systems cannot be considered adequate from the animals' point of view. In the past years, the "zero-grazing" system has spread considerably in Romania because of the concerns of the farmers for their animals and often due to workers' convenience or the lack of pasture lands [14]. Especially in large farms with high numbers of cows, leading and handling the herd becomes problematic and requires hiring additional personnel, at least for the warm season. A similar tendency can be observed in most European countries [15]. For the past few years, the proportion of farms offering access to pasture has been declining, as an increasing number of these have been converted to all-year-housing systems [16]. The percentage of farms with access to pasture varies widely between countries and regions [17]. The studies performed until now focused primarily on the impact of grazing on dairy cows' health and behavior and much less on their overall welfare quality [18].

Taking into consideration all the mentioned aspects, this study aimed to compare the overall welfare status of dairy cows with access to pasture to that of animals housed continuously in farms from Transylvania, Romania. In addition, the study investigated the relationship between pasture access and animal-based welfare measures of dairy cows kept in free-stall barns.

2. Materials and Methods

2.1. The Farms

The study was performed during the grazing season (April–October) at 39 dairy farms (17 with access to grazing (GF) and 22 with no access, i.e., zero-grazing (ZGF)) from Transylvania, Romania. The farm selection criteria comprised the following: a free-stall housing system, a minimum of 30 milked dairy cows, and the farmers' agreement to take part in the study. The number of animals per farm varied from 32 to 170, with an average of 81 cows (±32.42 standard deviation). The mean number of milked cows in the ZGFs was 93.41 (±33.76 standard deviation, between 43 and 170 animals). The GFs had slightly fewer, between 32 and 105 cows, with a mean number of 65.59 (±23.16 standard deviation). All the GFs (17 farms) and half of the ZGFs (11 farms) reared Romanian spotted cattle; the rest of the ZGFs (11 farms) had Holstein cows. The daily milk production per cow in the two farming systems was similar: a mean quantity of 19.88 ± 5.13 L (between 12 and 30 L) in the GFs and 20.82 ± 7.11 L (between 10 and 33 L) in the ZGFs. The cattle barns were closed or half-opened, providing cubicles for the cows' rest. None of the farms had outside paddocks or dry lots. In the ZGFs the cows left the housing area only for milking and in the GFs the pasture represented the only outside access. At the assessment moment, the barns were found to either have sawdust (in the majority of the farms) or no bedding (i.e., bare concrete flooring). All farms performed the milking in parlors, twice a day. In the GFs, the cows were pasturing, on average, around eight hours a day, the pasture access of the cows depending on the length of the daylight during the study period.

2.2. Welfare Assessment

Because none of the standardized protocols have specifically assessed the grazing dairy cows' overall welfare to date, the Welfare Quality® protocol [19] was used, which includes four major welfare principles, 11 criteria, and 29 measures (Table 1).

Table 1. The principles, criteria, and parameters of the Welfare Quality® assessment protocol for dairy cows [19].

Welfare Principles	Welfare Criteria	Assessed Parameters
1. Good feeding	1. Absence of prolonged hunger	Body condition score
	2. Absence of prolonged thirst	Water provision; cleanliness of water points; water flow; functioning of water points
2. Good housing	3. Comfort around resting	Time needed to lay down; animals colliding with housing equipment during lying down; animals lying partly or completely outside the ling area; cleanliness of udders, flank/upper legs, lower legs
	4. Thermal comfort	As yet, no measure has been developed
	5. Ease of movement	Presence of tethering; access to outdoor loafing area or pasture
3. Good health	6. Absence of injuries	Lameness; integument alterations
	7. Absence of disease	Coughing; nasal discharge; ocular discharge; hampered respiration; diarrhea; vulvae discharge; milk somatic cell count; mortality; dystocia; downer cows
	8. Absence of induced pain	Disbudding/dehorning; tail docking
4. Appropriate behavior	9. Expression of social behaviors	Agonistic behaviors—assessed by observation of head butts; displacements; chasing; fighting; chasing-up
	10. Expression of other behaviors	Access to pasture
	11. Human-animal relationship	Avoidance distance
	12. Positive emotional state	Qualitative behavior assessment—by observation of the cows' "body language" regarding 20 behavioral terms (active, relaxed, fearful, agitated, calm, content, indifferent, frustrated, friendly, bored, playful, positively occupied, lively, inquisitive, irritable, uneasy, sociable, apathetic, happy, distressed)

The data collection was performed according to the protocol's instructions [19]. The majority of parameters were recorded directly, at animal and herd level. A few measures regarding farm resources and management were scored based on the farmers' declarations. In each farm, two trained assessors evaluated the cows. The overall sample comprised 1662 cows (740 housed in GFs and 922 in ZGFs). The number of animals assessed per farm was established in conformity with the instructions of the Welfare Quality® assessment protocol (between 30 and 62 per farm, depending on the total number of cows on each farm). After having their necessary numbers, the animals were randomly chosen at the morning milking (every nth cow in the milking parlor) and marked. Part of the measurement was immediately recorded (body condition score, integument alterations, nasal, ocular, and vulvar discharge, diarrhea, and lameness while the cows were exiting the parlor), and then the same animals were followed in order to obtain other parameter scores, where random sampling was required by the protocol. Also, the farm resources were examined, and the farmer was interviewed.

After the collection of this first data set, the rest of the cow-related parameters were recorded inside the barns in the ZGFs and at the pasture in the GFs. Also, the water supplies were verified on the pasture, if there were any. The avoidance distance assessment for the grazing cows started 30 min after the arrival of the cows on the pasturing parcel, in actively grazing animals. Even in the absence of a feed bunk and feeding rack/neck rail, the evaluation respected the instructions of the assessment protocol to record the measure. Detailed information about the scoring methodology can be found in the Welfare Quality® Assessment Protocol for Cattle [19].

For the calculation of the criteria and principle scores, and the final classification of the farms (as not classified, acceptable, enhanced, and excellent), the Welfare Quality® scoring system's software (2023) was used [20].

All the procedures involving animals were carried out under the ethical guidelines of the Romanian National Animal Protection Law [21]. Being a purely observational and fully non-invasive study, no approval from the National Ethics Committee was needed.

2.3. Statistical Analysis

All statistical analyses were performed using the SPSS for Windows version 17 software (SPSS version 17, SPSS Inc., Chicago, IL, USA). Descriptive statistical indicators were calculated (median, range) for the scores of the measures, criteria, and welfare principles. The statistical significance of the grazing effect on welfare (measures, criteria, and principles of welfare) in the studied farms was determined by the Mann–Whitney test because the data did not have a normal distribution. To study the relationship between pasture access and animal-based welfare indicators, the Spearman rank correlation was used. The p values of less than 0.05 were considered statistically significant for both comparisons and correlations.

3. Results and Discussion

3.1. The Assessment of the Welfare Measures

Table 2 shows the results obtained by assessing the indicators of "Good feeding" and "Good housing" principles in the GFs and ZGFs. The percentage of very thin cows did not vary significantly between the two management systems, although it was slightly higher in the ZGFs.

Table 2. Scores (median, range) of the animal-based measures related to the principle of good feeding and housing in the GFs (n = 17) versus the ZGFs (n = 22), and the significance of the difference between the two management systems.

Criteria and Measures	GF		ZGF		p Value
	Median	Range	Median	Range	
Very lean cows (%)	11.32	27.17	17.07	30.97	0.230
Duration of lying down movements (s)	4.32	0.75	5.31	1.13	<0.001
Lying down movements with collisions (%)	0.00	0.00	0.00	3.39	0.644
Cows which lie partly outside the lying area (%)	0.00	0.00	0.00	4.35	0.154
Cows with dirty udder (%)	14.89	6.86	19.51	23.46	0.001
Cows with dirty flank and upper legs (%)	16.22	9.18	24.39	21.32	<0.001

GF: farms with grazing access for the cows; ZGF: zero-grazing farms. If the p value is less than 0.05, the difference between the GFs and ZGFs is significant.

In the opinion of several authors [9,22,23], compared to cattle kept exclusively indoors, grazing dairy cows are more prone to the risk of inadequate nutrient intake, possibly leading to lower body condition scores. Thus, in practical farming, this translates to an increased importance of good pasture management, regarding the control of both the floristic composition (and the nutritive value of grasses) and the judicious use of pasturing techniques to avoid overgrazing. Grazing is often seen by farmers as an economical way to spare feed (and thus money), but, especially in cows with high milk production, it must cover their metabolic needs in order to keep them from losing their body condition. Controlling feed intake in grazing cows is indeed more difficult than in cows kept indoors on a mixed unique ration, for example, as in the latter scenario both the composition and the distribution of food allow close monitoring. In severe cases, as mentioned by Brügemann et al. [24], the variable nutrient content of pastures (which depend on several factors, such as meteorological conditions, plant types and their vegetation stage, soil characteristics, and management measures, among others) poses even higher malnutrition risk in cows which have not been selected for pasture-based systems. In our study, most of the assessed

cows were of the well-adapted local breed, Romanian spotted cattle, which could explain the lower percentage of very lean cows in the grazing management compared to the ZGFs. The low body score of the cows in ZGFs evaluated in this study may favor the occurrence of lesions and may be interrelated with lameness; such associations were also demonstrated by Randall et al. [25] and Oehm et al. [26].

The median values for the number and total length of the water troughs were significantly lower ($p < 0.05$) in the GFs (the median for the water troughs' number was 2, and for their length it was 280, respectively) compared to the ZGFs (the median for the troughs' number was 5, and for their length it was 700). For the cleanliness of water points and for the water flow sufficiency the difference was not significant ($p > 0.05$) between the two systems. The majority of the GFs did not have water sources at the pasture; thus, the cows could drink only at the barns (from the moment of evening arrival at the barn until the next morning's departure again to the pasture). A similar setup was reported in the paper of de Andrade Kogima et al. [18]. The main barrier for the farmers to install on-pasture waterers was cost-related, as many times the pasturing areas were at considerable distances and a water pipe system would have had considerable costs. In specific situations, the pastures were not owned by the farmers but rented in yearly terms, adding insecurity to any investment in those lands. Although Wagner et al. [27] note as another possible reason the higher effort needed to check the water cleanliness in the pasture than in the barn (leading to insufficient or soiled water points), in our study this was not mentioned by the farmers.

The lying down movements took longer for cows in the ZGFs than for those in the GFs (Table 2). These results are congruent with the findings of Gieseke et al. [28] in Germany and those of de Andrade Kogima et al. [18] in Brazil. Previous investigations also showed improved lying behavior in cows that have access to pasture [10,29,30] compared to those without it, and our study added one more piece of evidence to these.

The percentage of collisions with elements of the environment was assessed in the barn in all the studied cows and the difference was not significant between the two management systems. In the ZGFs the occurrence of these collisions was considerably lower than that observed in other studies [18,31,32]. A possible reason for our lower values could have been the generous space allowance of resting places in the studied farms. The same reason could explain the low percentage (under 5%) of the cows lying partly or completely outside the resting area in the investigated ZGFs. According to the protocol [19], this does not represent a serious welfare problem.

The ZGFs had a higher percentage of cows with dirty legs and flanks compared to the farms that allowed their animals to have outside access. As mentioned (in the Materials and Methods section), some of the farms did not have any bedding. Unfortunately, even in the barns where sawdust was used, in the ZGFs, the bedding was dirtier than in the GFs, soiling the cows' bodies when they were lying down. As a considerable body of literature signals [14,33,34], the poor body hygiene of cows is mainly caused by deficient cleaning of the barn floors and insufficient changing of the bedding. According to these studies, barn hygiene does not represent a priority for the farmers. Even if the consequences may not be straightforwardly evident, several studies [33,35] note that poor hygiene increases the risk of mastitis and worsens lameness in dairy cows. Unsurprisingly in this context, the Welfare Quality® protocol establishes a warning threshold in this regard with a cutoff of 50% dirty lower hind legs and 19% dirty udders. Exceeding these percentages indicates a serious problem in any dairy herd, regardless of their housing and/or management system. In our study, the cows were cleaner in the GFs than in the ZGFs, in line with the results reported by other researchers [18,27]. Arnott et al. [1] also conclude that the cows are generally cleaner outdoors, except from exposure to adverse meteorological conditions (rainy weather, causing mud in the outside environment).

As Table 3 shows, the medians for most of the animal-based measures indicated significantly better ($p < 0.05$) health of the cows in the GFs than in the ZGFs. The positive effects of pasture access on the overall health status of dairy cows are multiple and the

greater opportunity to exercise plays an important role in this regard. The studies prove that cattle walk more while on the pasture than inside the barn, but also warn that long trips between the barns and grazing area may induce tiredness [8,36]. The most vulnerable category in this scenario is that of the lame cows in which movement may need to be restricted. Thus, the watchperson has an important role and should be experienced enough to decide which cows can walk the distance needed without detrimental effects on their health and welfare.

Table 3. Scores (median, range) of the animal-based measures related to the principle of good health in the GFs (n = 17) versus the ZGFs (n = 22), and the significance of the difference between the two management systems.

Criteria and Measures	GF		ZGF		p Value
	Median	Range	Median	Range	
Lame cows (%)	6.38	12.89	29.78	12.54	<0.001
Cows with at least one hairless patch (%)	6.34	9.06	14.63	29.34	<0.001
Cows with at least one lesion (%)	4.25	10.63	20.97	28.04	<0.001
Cows with ocular discharge (%)	0.00	0.00	0.00	3.22	0.20
Diarrhea (%)	0.00	23.07	0.00	4.83	0.067
Vulvar discharge (%)	0.00	0.00	0.00	7.41	0.122
Mastitis (%)	4.00	6.00	8.00	16.00	<0.001
Mortality (%)	0.00	1.00	1.00	3.00	<0.001
Dystocia (%)	0.00	2.00	1.00	2.00	<0.001
Downer cows (%)	0.00	0.00	0.00	1.00	0.067
Dehorned cows (%)	100.00	50.00	70.00	100.00	0.079

GF: farms with grazing access for the cows; ZGF: zero-grazing farms. If the p value is less than 0.05, the difference between the GFs and ZGFs is significant.

The median of lame cows was lower (good health) in the GFs than in the ZGFs, similar to the findings of other studies [2,3,10]. For example, Haskell et al. [2] found that, in farms with permanent housing, the percentage of lameness was higher than in those that provided pasture access to cows (39% versus 15% lameness prevalence). Other authors suggested, as well, that reduced or no pasture access represents a risk factor for lameness [37,38]. Other elements contributing to the lameness risk in dairy cows include solid concrete flooring, slippery walking alleys [39], uncomfortable and dirty barns [33,37,40], and an increased degree of dirtiness on the cows' hind legs [33,34]. Lameness is considered one of the most important problems in the intensive dairy industry [41]. Its effects interlink and trigger a downward spiral of health and production, severely impacting the welfare of the suffering cow. The discomfort and impaired balance lead to a reduction in time spent feeding, then the lower nutrient intake pairs with a higher metabolic demand imposed by pain, causing not only poor body condition scores but also a lower milk yield. All these have consequences, such as substantial negative effects on reproductive parameters and fertility performance, and, ultimately, an increased culling risk [42]. Even if the main cause of dairy cow lameness is considered to be either infectious or non-infectious claw lesions [43], the pain can be caused by a wide array of issues of the bones, joints, or soft structures (tendons and ligaments) of the locomotor system. Pasturing, due to its context (access to free exercise), seems beneficial for all the components of dairy cows' gait health, as grazing cows are less often lame than those kept indoors [8,10]. The free exercise on a larger surface and the more flexible and shock-absorbing nature of the earth (compared to concrete) seems protective for the upper parts of the cows' legs. At the same time, the cleanliness of the pasture ground and reduced probability of standing in manure act beneficially on the claws' health [15,23], reducing the overall risk of lameness.

The proportion of cows with hairless patches and lesions was significantly ($p < 0.001$) lower in the GFs than in the ZGFs, probably due to softer lying surfaces and lack of contact with injury-producing objects, such as gates, cubicle partitions, and feeding troughs. This finding is congruent with the results of other studies [15,22,23,29].

No significant differences were found between the two studied management systems for the percentage of cows with ocular or vulvar discharge, which is similar to the reports of Corazzin et al. [29] and Matiello et al. [44].

The pasture access did not significantly influence the prevalence of diarrhea in the studied cows. In this regard, our findings are in contrast with the results of other researchers. Several authors [23,27,29] consider that grazing may increase the risk of diarrhea, and Burow et al. [22] note a higher occurrence of this syndrome in cows with pasture access compared to the all-year-round confined ones. The causes of adult dairy cattle diarrhea are very diverse, even when only the nutritional risks are considered, and some may be indeed related to fresh grass consumption. As Armbrecht et al. [23] and Wagner et al. [27] very accurately mention, the high water and/or low protein and crude fiber content of the green fodder has the potential to soften the cows' feces. Especially when sudden diet changes occur, and/or when high quantities of grass are ingested, the manure can become considerably loose in the pasturing cows. The important element of this situation is not always possible to capture during the snapshot of a welfare assessment, but the assessor should always try to avoid confusing these temporarily loose feces with pathological diarrhea. Interviewing the farm personnel may aid this process.

In our research, the percentage of cows with mastitis was significantly ($p < 0.001$) lower in the GFs than in the ZGFs, which may be another advantage of the cleaner lying surfaces, simply limiting the exposure to pathogens, as other authors have also suggested [1,15,23]. Alongside lameness and infertility, mastitis is one of the major issues affecting dairy cattle globally [43]. It is a multifactorial disease recognized, a quarter of a century ago, as being one of the most frequent and costly health problems of the dairy industry [45]. Unfortunately, the past decades have not reduced the challenge of controlling dairy cow mastitis, especially because of the rising threat of antimicrobial resistance, with new and emerging mechanisms of resistance appearing and spreading globally. Not only does treating the cows with mastitis become more difficult, but large quantities of milk become improper for human consumption because of pathogenic bacteria, antimicrobial residues, and/or non-zoonotic micro-organisms, the latter being a source of transferable genetic resistance [46]. In this context, all the means of mastitis prevention or incidence reduction are extremely valuable, and the cows' access to pasture seems beneficial in this regard. White et al. [47] state that grazing lactating cows have a lower prevalence of mastitis. Washburn et al. [5] establish that these are 1.8 times less prone to clinical signs, and eight times less likely to be culled because of mastitis compared to cows with no pasture access. In cases like we found in the ZGFs (mastitis prevalence exceeding 4.5% of the assessed cows), the Welfare Quality® protocol [19] recommends the implementation of a farm-level action plan to remediate this problem as quickly as possible.

Mortality was significantly higher ($p < 0.001$) in the ZGFs than in the GFs. This result is not different from that of other studies reporting the decrease in mortality in dairy herds with pasture access [1,6,8] by up to 75% [48]. In a study [48] grouping Swedish dairy herds according to their mortality percentage, the authors found that the likelihood of being in the high mortality group increased when the cows' pasture access was restricted for the summer season. Additionally, the same authors [48] observed that a herd size ranging from 50 to more than 100 cows per group presented a higher mortality risk compared to smaller herds (from 30 to 50 cows).

In line with other publications [1], in our study, the percentage of cows with dystocia at parturition was higher in the ZGFs than in the GFs.

Regarding the behavioral measures studied in the cows, all of these differed significantly ($p < 0.05$) between the two management systems (Table 4). All the measures were better in GFs.

Table 4. Scores (median, range) of the animal-based measures related to the principle of appropriate behavior in the GFs (n = 17) versus the ZGFs (n = 22), and the significance of the difference between the two management systems.

Criteria and Measures	GF		ZGF		p Value
	Median	Range	Median	Range	
Frequency of butts (per cow per hour)	0.33	0.43	1.78	2.29	<0.001
Frequency of displacements (per cow per hour)	0.20	0.30	1.34	1.87	0.01
Cows that can be touched (%)	43.73	35.42	24.19	17.02	<0.001
Cows that can be approached at 50 cm but not touched (%)	52.54	25.67	23.80	51.05	0.001
Cows that can be approached between 50 cm and 1 m (%)	38.30	42.01	12.76	46.50	<0.001
Cows that cannot be approached (%)	4.25	4.25	8.47	25.77	<0.001
Qualitative Behavior Assessment (QBA)	4.15	1.57	0.61	3.65	<0.001

GF: farms with grazing access for the cows; ZGF: zero-grazing farms. If the p value is less than 0.05, the difference between the GFs and the ZGFs is significant.

The frequency of head butts and displacements was higher in the ZGFs than in the GFs. In the same way, cattle tend to display less agonistic behaviors (e.g., pushing, avoiding, and threatening) while on the pasture or in larger spaces [11] where their movement possibilities are not limited; thus, the submissive individuals can avoid confrontation with the dominant ones. The duration of maintaining established cattle groups is also important because during each regrouping the cows experience the stress of breaking former social bonds and adapting to a new social environment, leading to increased agonistic behaviors, decreased feeding and rumination times, and lower milk production [49].

The parameters measuring the percentage of cows that can be touched or approached explore the human–animal relationship, indicating the quality of previous experiences of the cows with their human handlers. Our results for these measures are similar to those of de Andrade Kogima [18]. In the comparative evaluation of confined versus pasturing cattle, many authors found that grazing cows are often more avoidant when approached than those in the barn. Batini [50], for example, concludes that the cows become less approachable after being re-confined following a grazing season of four months. The possible explanation provided is the lesser extent of human manipulation on the pasture (as frequency), which leads to the need to re-habituate to human handling. In similar terms, Armbrecht et al. [23] emphasize the importance of improved animal handling on pasture for the added cow welfare benefits of grazing. Not only the avoidance of aversive stimuli (e.g., the use of electric prodders, yelling, hitting the cows while leading them between the pasture and farm) but the addition of positive interactions (e.g., gentle scratching, food rewards) would be needed in the very well-justified opinion of these authors. Yet, the mentioned studies do not fully describe the practical daily management of the pasturing and confined cows. Even if grazed daily, the cows in our study were still handled for milking twice a day, and thus their habituation to human handling could not be "forgotten". In our viewpoint, the human-related response of the cows at each farm (approachability, avoidance distance, willingness to be touched) depends very much on the quality of interactions with the personnel of that specific farm, reinforced continuously by daily experiences. Some of the farms used electric fences only, but others had a watchperson guarding the cows on the pasture. In the latter scenario, the interactions with that person could substantially shape the human-related on-pasture behavior of the cows, because even if cows can discriminate between humans, they also generalize their experience with one person to other people, especially in similar contexts [51].

The Qualitative Behavior Assessment (QBA) was scored by the assessor after observing the behavior and body language of the cows, taking into consideration the descriptors pre-established in the Welfare Quality® protocol [19] (Table 5).

Table 5. Descriptive statistics (median, range) for descriptors (expressed in millimeters) of the Qualitative Behavior Assessment (QBA) in the GFs (n = 17) versus the ZGFs (n = 22), and the significance of the difference between the two management systems.

Descriptor	GF		ZGF		p Value
	Median	Range	Median	Range	
Active	100.10	17.0	80.30	43.00	<0.001
Relaxed	68.30	74.00	45.40	66.90	0.005
Fearful	30.20	25.00	50.20	45.00	0.003
Agitated	0.00	0.00	25.20	37.10	<0.001
Calm	110.20	26.50	95.30	34.90	<0.001
Content	120.20	14.90	95.00	55.10	<0.001
Indifferent	20.00	10.00	30.00	28.00	0.001
Frustrated	0.00	0.00	10.00	15.40	<0.001
Friendly	60.00	25.30	25.00	29.80	<0.001
Bored	15.20	9.90	20.00	30.30	0.219
Playful	45.40	40.40	30.00	35.20	<0.001
Positively occupied	100.00	15.20	90.00	35.00	<0.001
Lively	60.00	73.20	40.00	30.20	0.004
Inquisitive	0.00	0.00	5.00	15.00	<0.001
Irritable	0.00	0.00	5.00	15.00	<0.001
Uneasy	0.00	15.30	10.00	25.20	<0.001
Sociable	119.70	7.10	100.20	35.30	0.001
Apathetic	0.00	0.00	5.00	10.00	<0.001
Happy	120.00	14.50	90.00	40.00	<0.001
Distressed	0.00	15.00	20.00	29.80	<0.001

GF: farms with grazing access for the cows; ZGF: zero-grazing farms. If the p value is less than 0.05, the difference between the GFs and the ZGFs is significant.

The QBA was meant to bring the positive emotional state indicators of the evaluated animals to the welfare assessment protocol [19]. Although it is not generally accepted as an ideal method and its limitations are extensively discussed in the literature [52–54], QBA continues to be used until the development of more appropriate indicators.

The emotional state of cows on pasture assessed by the QBA method by other researchers seems to be at least similar, or even more positive, than that of cows kept indoors [23,27]. Emotional states that refer to positive feelings (such as active, relaxed, calm, content, friendly, playful, positively occupied, and happy) were shown at a higher frequency by the cows in the GFs than in ZGFs (Table 5). On the farms without pasture access, the cows displaying negative emotional states (such as frustration, irritableness, apathy, and distress) were significantly more prevalent ($p < 0.05$). The inquisitive behavior was recorded at a higher rate in the ZGFs than in the GFs. Although it is less researched in cattle, curiosity and the tendency to investigate are considered parts of a mindset related to play behavior, which is at the foundation of complex object-related and social abilities in cows, but play behavior can indicate both positive and negative emotional states [55]. Another perspective on curiosity is its relationship with learning (motivation to explore, to acquire novel information), but this topic has been scarcely researched in cattle [56]. Thus, neither the valence of this behavior displayed more frequently in the ZGFs, nor the significance of its underlying emotional state, is conclusive.

The explanation for the higher scores of positive emotional state indicators in the grazing cows may be the possibility for them to exercise relatively unrestricted movement, the natural environmental enrichment, and their access to comfortable resting surfaces, which would explain their preference to spend more time on the pasture than in confined areas [57]. Although animal preferences do not always directly relate to their better welfare [58], they indicate the pursuit of more positive emotional states. For example, feeding occupies considerably less of the confined cows' daily time budget (a third) compared to those kept on the pasture [9], well below what is considered usual in the natural bovine

behavioral repertoire [59]. Yet, this time "saving" brings no benefit for the confined cows, it only favors temporal leisure and negative emotional states [12].

Several studies show that access to grazing enables natural social behaviors in dairy cows [1,8,12,44], which is an important welfare aspect. When the competition for resources is low (as it happens on adequate pastures) the feeding and lying (ruminating) behaviors synchronize much better in the grazing herds than in the confined groups. Several preference studies [57,60,61] demonstrate that spending time on grasslands is valuable for these animals. The reported results prove that if they are given free choice, the cows favor the simple grass over the more nutritious (and assumably more palatable) rations, preferring to spend time on the pasture. Even if compared to free-stall housed herds (similar to our study) which are free to move inside the barns, the species-specific behaviors of pasturing animals are superior both as duration and as frequency and display range [62]. Therefore, the better positive emotional state of the cows on pasture found in our research is not surprising.

3.2. Wefare Criteria and Principles Assessment

The comparison in terms of criteria showed no significant difference between the two management systems, except for the "Absence of prolonged hunger" and the "Absence of pain induced by management procedures". Significant differences ($p < 0.05$) were found for all the welfare principles between the two management systems, three of these being in favor of the GFs (Table 6).

Table 6. Descriptive statistics (median, range) for welfare criteria and principle scores in dairy farms with or without access to pasture and the significance of the difference between the two management systems (GF, ZGF).

Principles and Criteria *	GF		ZGF		*p* Value
	Median	Range	Median	Range	
Good feeding	47.90	56.60	64.80	11.20	<0.001
APH *	83.50	48.70	76.19	40.00	0.070
APT *	3.00	57.00	60.00	40.00	<0.001
Good housing	74.00	10.00	70.90	18.10	0.01
CAR *	58.70	16.00	31.15	13.50	<0.001
Good health	48.40	18.00	39.60	34.30	<0.001
AI *	85.40	18.30	46.00	43.50	<0.001
AD *	100.00	13.90	86.10	59.50	0.006
APIMP *	14.00	28.00	13.00	87.00	0.147
Appropriate behavior	69.60	16.60	27.50	39.40	<0.001
ESB *	96.00	4.90	57.00	37.90	0.05
GHAR *	60.00	26.50	38.10	22.70	<0.001
PES *	88.90	13.10	56.90	39.60	<0.001

* marks the criteria (the entries without are the principles). GF: farms with grazing access for the cows; ZGF: zero-grazing farms. If the *p* value is less than 0.05, the difference between the GFs and the ZGFs is significant. APH: Absence of prolonged hunger; APT: Absence of prolonged thirst; CAR: Comfort around resting; AI: Absence of injuries; AD: Absence of diseases; APIMP: Absence of pain induced by management procedures; ESB: Expression of social behaviors; GHAR: Good human–animal relationship; PES: Positive emotional state.

When the welfare criteria and principles scores are compared in the two types of farms (GF vs. ZGF) in Table 6, the significantly higher scores ($p < 0.05$) of those farms providing their cows with access to pasture compared with the farms that housed their animals permanently became evident for the majority of the assessed criteria and three of the four welfare principles.

Although no significant differences were found for the "Good feeding" principle between the two farm types, slightly higher scores were obtained in the ZGFs than in the GFs for the criterion "Absence of prolonged hunger", and higher for the criterion "Absence of prolonged thirst". The lower scores for the criterion "Absence of prolonged thirst" in the GFs are related to the insufficient water supplies on the pastures. Similar results are given

by other research papers [18,27], as well. Prolonged thirst has a stronger negative impact on the animals' welfare than prolonged hunger. This effect can be more detrimental in the case of milking cows, whose metabolism uses considerable water quantities (in addition to the animals' maintenance needs) to produce milk. The higher their milk production is, the more important it is that they have uninterrupted free access to water. The Romanian spotted is a mixed breed, not primarily specialized for milk production. This feature could aid the adaptation of these cows to periodic water restrictions (eight hours per day, on average, while grazing). Although all the GFs (and half of the ZGFs) had exclusively cows of this breed, continuous access to water is paramount for dairy cows. Unfortunately, the studied GFs did not fulfill this requirement. Some of the farmers insisted that their cows were used to this drinking schedule (only when at the barns, during the night), their intake was not restricted quantitatively when they had access to water, and the water content of grass (higher than that of hay or other "dry" forage) should contribute to the animals' overall daily fluid requirement. In more specific studies, the veracity of these assertions could be verified by testing the dehydration degree of the cows, or by simply offering them water at different moments of the day, to assess their thirst level.

The scores for the principle of "Good housing", and also for the criterion "Comfort around resting" were significantly higher ($p < 0.05$) on the farms with pasture access, meaning that the cows felt more comfortable on the pasture than inside the barn. Recently, de Andrade Kogima et al. [18] reported similar results in a study performed in Brazil. Previous research also showed an improved lying behavior in dairy cows on pasture [27,29].

The "Good health" welfare principle and the criteria "Absence of injuries" and "Absence of diseases" were significantly influenced by grazing ($p < 0.05$). These results are in line with those in the scientific research proving that cows with access to pasture have a better health status. The access of dairy cows to pasture prevents and reduces the incidence of lameness [2], improves the behavioral parameters in the sense of displaying natural behaviors [63], increases the resistance of their immune system, stimulates the reproductive function, and thus enhances their overall degree of welfare [64].

Significant differences ($p < 0.05$) were found between the median scores for the "Appropriate behavior" welfare principle and the criteria included when the two farming types were compared. The pasture-based systems are perceived to offer greater behavioral freedom than those with continuous housing [1].

The evaluation of the "Expression of social behaviors" criterion is performed by observing the agonistic behaviors of the cows. The results obtained for this criterion are in agreement with previous studies, namely less agonistic behaviors when on pasture compared to during the housed period [1].

The significantly higher scores for the criterion "Good human-animal relationship" in GFs are rooted most probably in the positive experiences of the cows during interactions with their handlers. The overall positive effects of pasturing on the emotional balance of the cows would be very difficult to prove, because of the multitude of interlinked factors, but de Andrade Kogima et al. [18] reported similar results to those in the present research.

In this study, the "Positive emotional state" was significantly higher in the GFs than in the ZGFs ($p < 0.001$). These results are congruous with those obtained by other researchers [23,27].

3.3. The Overall Welfare Assessment

Based on the scores obtained for the four welfare principles, each farm was classified into a welfare category.

The GFs were in the "enhanced" (15 farms) and in the "acceptable" (two farms) categories, while the ZGFs were categorized as "enhanced" (five farms), "acceptable" (14 farms), and "not classified" (three farms). None of the assessed farms reached the "excellent" level (Figure 1). A better overall welfare quality in grazing dairy cows was reported recently by de Andrade Kogima et al. [18].

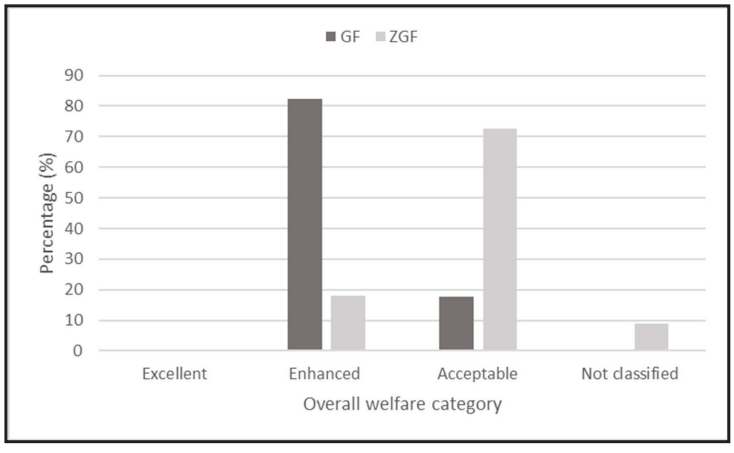

Figure 1. Classification of the assessed farms in the overall welfare categories comparatively, according to their management system. GF: farms with grazing access for the cows; ZGF: farms without pasture access (zero-grazing).

3.4. Correlations between Pasture Access and Animal-Based Welfare Parameters

Table 7 presents the correlations found between pasture access and certain animal-based welfare measures. Pasture access correlated positively with certain indicators and negatively with others (Table 7).

Table 7. Significant correlations between pasture access and animal-based welfare measures in the dairy cows in the assessed farms.

Pasture Access	r_s	Pasture Access	r_s
Very lean cows	−0.62	Mortality	−0.63
Duration of lying down movements	−0.76	Dystocia	−0.59
Cows with dirty lower legs	−0.67	Frequency of head butts	−0.74
Cows with dirty udder	−0.42	Frequency of displacements	−0.66
Cows with dirty flank and upper legs	−0.52	Cows that can be touched	0.51
Lame cows	−0.72	Cows that can be approached by 50 cm but not touched	0.41
Cows with at least one lesion	−0.68	Cows that cannot be approached	−0.47
Vulvar discharge	−0.44	QBA	0.88
Mastitis	−0.58		

The negative correlations between pasture access and comfort and health-related indicators sustain the conclusions of several studies stating that grazing cows need less time to rise and lay down [27]. Their increased comfort around resting while on the pasture may be due to more space available, as well as softer and more comfortable lying surfaces [1], and also because they can freely select and choose the spot where they want to rest. Cows are generally cleaner outdoors [1], are less lame and injured (i.e., having fewer lesions, swellings, and hairless patches) [23], have a better general health status, and decreased mortality [8]. The negative correlations found between pasture access and behavioral indicators underline the observations that cows on pasture engage in less agonistic interactions [1]. The positive correlations with the indicators comprised in the "Good human-animal relationship" criterion suggest that pasturing cows are less reactive

and avoidant to the approach of people, as noted in our study too. Similar results have been reported in working horses [65]. The strong correlations with QBA highlight the importance of pasture access for the well-being of dairy cows. The association between positive emotions and pasturing was also demonstrated by Motupalli et al. [57].

4. Conclusions

This study showed that the overall welfare of dairy cows was better when they had access to pasture than in permanent confinement. Even if grazing was limited to the daylight period only in the warm season and both farm types provided free-stall management, the positive effects of freedom on grasslands were evident. Due to the high number of significant differences in the assessed welfare measures, criteria, and principles, the GFs ranked higher in the final farm classification, proving the superiority of a management system that allows the cows to display a wide range of natural behaviors. Because most assessed animals were of a local breed (Romanian spotted cattle), thus well adapted to the local conditions and a grass-based diet, the study conditions do not represent a guideline for managing highly productive ultra-ameliorated dairy cows that may have different needs. Yet, the authors consider that, regardless of breed, all dairy cows would benefit from pasture access, tailored to their characteristics, to achieve better health and a higher welfare quality.

5. Study Limitations

The main limitation of the present study was the need to adapt the Welfare Quality® assessment protocol for dairy cows to on-pasture evaluation because of the lack of a specifically developed protocol. This implied a slight modification for the measurement of the avoidance distance, for example. Other authors also noted the shortcomings of the protocol while assessing dairy cows on the pasture [66]. One aspect which needs special attention is the lack of a measure for the thermal comfort of the dairy cows. Although several measures are equally feasible for indoor and outdoor assessments, a specific and standardized welfare assessment protocol for dairy cows on pasture is needed that is representative of the overall welfare status of the cows [66].

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Comparative Assessment of the Nutritional and Sanogenic Features of Certain Cheese Sorts Originating in Conventional Dairy Farms and in "Mountainous" Quality System Farms

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Abstract: In order to highlight the influence of cattle farming systems on dairy products, assessments were carried out on certain varieties of cheese—marked with the "Mountain product" quality label in comparison with those conventionally produced ones not bearing the quality label. The study was carried out using products obtained from raw milk issued from seven farms and transformed into cheese in four small dairy factories from the mountainous area of Dornelor Basin, Suceava County, Northeastern Romania. The analyzed cheese issued from the "mountain" production system were "Călimani" Schweizer, "Călimani" Cașcaval, "Călimani" smoked Cașcaval, and "Călimani" Telemea—salty brined cheese. Both the "Mountain cheese" and conventional cheese samples produced throughout the same shift were collected and kept under refrigeration conditions until laboratory analysis in order to compare the production systems. The physico-chemical analysis revealed higher amounts of minerals (2.8 to 10.7% Ca; 2.8 to 9.5% P; 12.3% to double the amount of Fe, $p < 0.001$) and polyunsaturated fatty acids (+5.6 to +13.7%), in mountain cheeses versus the conventionally processed ones. Also, the sanogenic indices had higher values in the "Mountain cheese", such as the polyunsaturation index (+4.3 to 7.8%) and hypocholesterolic/hypercholesterolic fatty acid ratio (+1.8 to 3.7%), while the atherogenic index and the thrombogenic index had lower values (−1.9 to −4.3%) compared to the conventionally produced cheese, thus revealing healthier properties for consumers. The *Enterobacteriaceae* family species were identified in "Mountain cheese", while they were absent from conventionally processed cheese, knowing the raw matter milk is thermally treated at ultra-high temperatures in the latter ones. In the "Mountain cheese", such microorganisms were found within the safety regulation limits and contributed to providing flavor, taste, color, and specific texture, making it superior in terms of sensorial quality compared to the conventionally produced cheese.

Keywords: mountain cheese; conventional cheese; sanogenity; quality; microorganisms

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1. Introduction

The present research was carried out in the Dornelor Basin, a mountainous region with high tourism and agro-tourism potential due to its own varied gastronomy, traditions, culture, and hospitality of the inhabitants, and also due to the herds of cattle raised in the area [1,2]. In the studied area, there are generally cattle breeds with innate disease resistance and suitable adaptability, namely the breeds Pinzgau of Transilvania, Brown, Romanian Spotted, Romanian Spotted with Black, and crossbreeds of these breeds [3–6].

Studies on consumer perception of dairy cow welfare in traditional mountain farms and also the integrated system approach have shown that mountain products are health-

ier because of their geographical origin, farm locations, and small-scale production systems [7–12].

In the mountainous areas of Europe, with a humid and cold climate [13–15], as in the mountainous area of the Dorna Basin, rearing dairy cattle is an important agricultural activity, and the milk obtained here is often processed into cheeses, according to the specifications of origin protected (PDO) or with the mention of quality "mountain product" in Romania, thus bringing added value to the farmer's household. The registration of products under the "mountain product" quality label scheme is fully carried out by the National Agency of the Mountain Zone, is free of charge, and represents support granted by the Romanian state to producers in the mountain area. The conditions for obtaining the optional quality mention "mountain product" are the raw materials, but the animal feed must come mainly from mountain areas, and in the case of processed products, their processing must also be performed in mountain areas [16–18]. Dairy products obtained in mountain areas are healthier for human consumers due to better fatty acids profile and sanogenic indices than those obtained in lowlands, related to many influential factors: altitude and geographical area [19] maintenance system (grazing free roaming on alpine pastures vs. confined cattle) [20], predominance of pasture or of haystack and concentrates in diet [21], different floral composition of pastures [22], and metabolism of cattle breeds adapted to highlands [23]. The products that can be certified as "Mountain product" are processed or unprocessed animal products (milk; fermented milk beverages; cheese; meat; meat products; eggs; bee products) and processed or unprocessed vegetable products [24–26]. The benefits of capitalizing on dairy products with the quality label "mountain product" include a reduction of value-added tax to 5% from 9%; superior utilization of products; recognition of the quality of products obtained from less-polluted areas; promoting producers on the "mountain product" platform; and joining the "Produs Montan" Association [27–30].

In the National Register of Mountain Products (RNPM), 1081 dairy products are registered, which represent 30.88% of all mountain products registered under the logo "mountain product" (animal, vegetable, beekeeping products). Out of these, 214 are also registered as traditional milk products, and 3 products are fully registered under the Protected Geographical Indication system. The mountain products recorded in the period 2018–2021 indicated an increased trend. Considering the small number of studies in the mountainous area on milk production and the quality of dairy products, the aim of the research was to follow the influence of conventional and "mountain" production systems on the microbiology and quality of some dairy products. The utility of our findings resides in the fact that results can be taken as a reference for future research and can serve as a suitable recommendation for consumers regarding the quality and sanogenity of products obtained in the mountain area compared to the conventionally produced ones.

2. Materials and Methods

The study area is located within the Local Action Group, Suceava County, Northeastern Romania (geographical area comprised between the coordinates 47°08′–47°47′ Northern latitude and 24°94′–25°83′ Eastern longitude). To carry out this study, 7 local producers were taken into account (farms and small-size family dairy processing workshops). The farms hosted lactating cows from multiple breeds adapted to the mountainous area: Pinzgau of Transilvania, Brown, Romanian spotted, and less Romanian black spotted [31].

European Union legislation has allowed the member states considerable flexibility in the implementation of the disadvantaged areas helping scheme. These proposals were included in Regulation (EC) no. 1698 of 2005 with reference to support for rural development [17]. In Romania, the disadvantaged mountain area mostly overlaps the Carpathian area and comprises administrative-territorial units (UAT) located at average altitudes higher than or equal to 600 m (Regulation (EC) no. 1257 of 1999) [17].

The mountain area of the Dornelor Basin looks like a flat relief with an average altitude of 800 m. It presents a higher step of hilly type with altitudes between 800 and 1000 m

located at the contact with the forested mountain area and a lower step consisting of terraces and meadows along the river basins.

The farms that provided the raw milk for developing the studied "Mountain" cheese are located at an average altitude of 800 m and have a minimum herd of 21 heads, 19 ha of permanent grassland, and a maximum herd of 66 heads and 100 ha of permanent grassland. The cattle are maintained indoors throughout the winter, while in the summer, the maintenance is free-moving in summer camps at altitudes above 1000 m. In the farms studied, milking is performed mechanically, using mobile individual milking devices. Cows are fed on a seasonal basis, in winter from stock: mountain hay, clover hay, timothy semi-hay, hay, fodder pumpkins, fodder beet, potatoes, corn fodder flour, wheat bran, dicalcium phosphate, and semi-dehulled sunflower meal, and in the summer, feeding is performed ad libitum on pastures composed of mountain meadows gramineas, "ottava" (2nd scythe hay), corn feed flour, wheat bran, rye bran, dicalcium phosphate, and salt.

No animals were used for experimental purposes, but the dairy products developed from their milk bear the quality label "Mountain product" in comparison with the conventionally manufactured products. Dairy products labelled "Mountain product" are obtained from raw materials originating from the mountain area and processed in the same area. These products offer consumers access to healthy food originating from a territory with low pollution levels and surrounded by natural reserves. It is relevant to notice that the milk used in manufactured conventional cheese products is collected from multiple small-scale family-size producers, and the heterogeneity of the raw matter is quite high, while the feeding of dairy cows is not provided in a homogeneous manner, hence the hypothesis that the nutritional and sanogenic quality of the cheese is expected to be different between the "Mountain"-labeled products and the conventional ones. The commercial names of the analyzed products are "Călimani" Schweizer, "Călimani" Cascaval, "Călimani" smoked Cascaval, and "Călimani" Telemea—brine-salted cheese (Figure 1). The "Călimani" particle in all these cheeses' commercial names is given by the mountain massif patronizing the area and from the national park bearing the same name, an emblematic symbol for the area. Cascaval is the Romanian type of stretched-curd cheese, with no fermentation eyes and hard edible crust, light to intense yellow colored, close in sensorial and nutritional traits with the Italian cheese type Caciocavallo (hence the name Cascaval, pronounced *cashkahvahl* in Romanian, bore by this sort of cheese across the whole Mediterranean and Balkan countries). Schweizer is a locally prepared version of the Emmentaler Swiss-type cheese (hence the name Schweizer), a light-yellow semi-hard cheese with fermentation eyes (holes) the size of a grape berry or of a cherry. Telemea is a sort of semi-soft salt-brined cheese produced traditionally in Romania and belonging to the Feta cheese family.

In accordance with the County Sanitary-Veterinary Authority analysis, the raw milk used to manufacture the two categories of cheese ("mountain product" and conventional) presented the commodity quality, freshness, and sanogenity traits according to compositional quality safety regulations (total germs count—TGC and somatic cells count—SCC):

- Raw milk for "mountain product": density = 1.298 g/cm^3, pH = 6.53; fat = 4.1%, protein = 3.42%, out of which casein = 33.7%; lactose = 5.82%; TGC = 87,000 CFU/cm^3 (vs. max. admitted limit of 100,000 CFU/cm^3), SCC = 112,300 cells/cm^3 (vs. max. admitted limit of 400,000 cells/cm^3);
- Raw milk for conventional cheese: density = 1.282 g/cm^3, pH = 6.61; fat = 3.9%, protein = 3.27%, out of which casein = 28.3%; lactose = 4.92%; TGC = 67,000 CFU/cm^3 (vs. max. admitted limit of 100,000 CFU/cm^3), SCC = 83,700 cells/cm^3 (vs. max. admitted limit of 400,000 cells/cm^3).

In brief, the technological flow of cheese manufacturing has several stages: (a) raw milk reception; (b) milk filtering; (c) temporary refrigerating storage; (d) thermization—low pasteurization ("mountain product") or high pasteurization—UHT treatment (conventional); (e) normalizing for fat content; (f) rennet enzymes and coagulating salts inoculating; (g) heating for coagulating; (h) clotting and clot cutting; (i) whey removal; (j) pressing; (k) cutting of crude curd; (l) boiling and salting of curd; (m) filtering; (n) putting into molds;

(o) inoculating with probiotic lactic cultures; (p) maturation (ripening); (q) packaging; (r) storage prior to marketing.

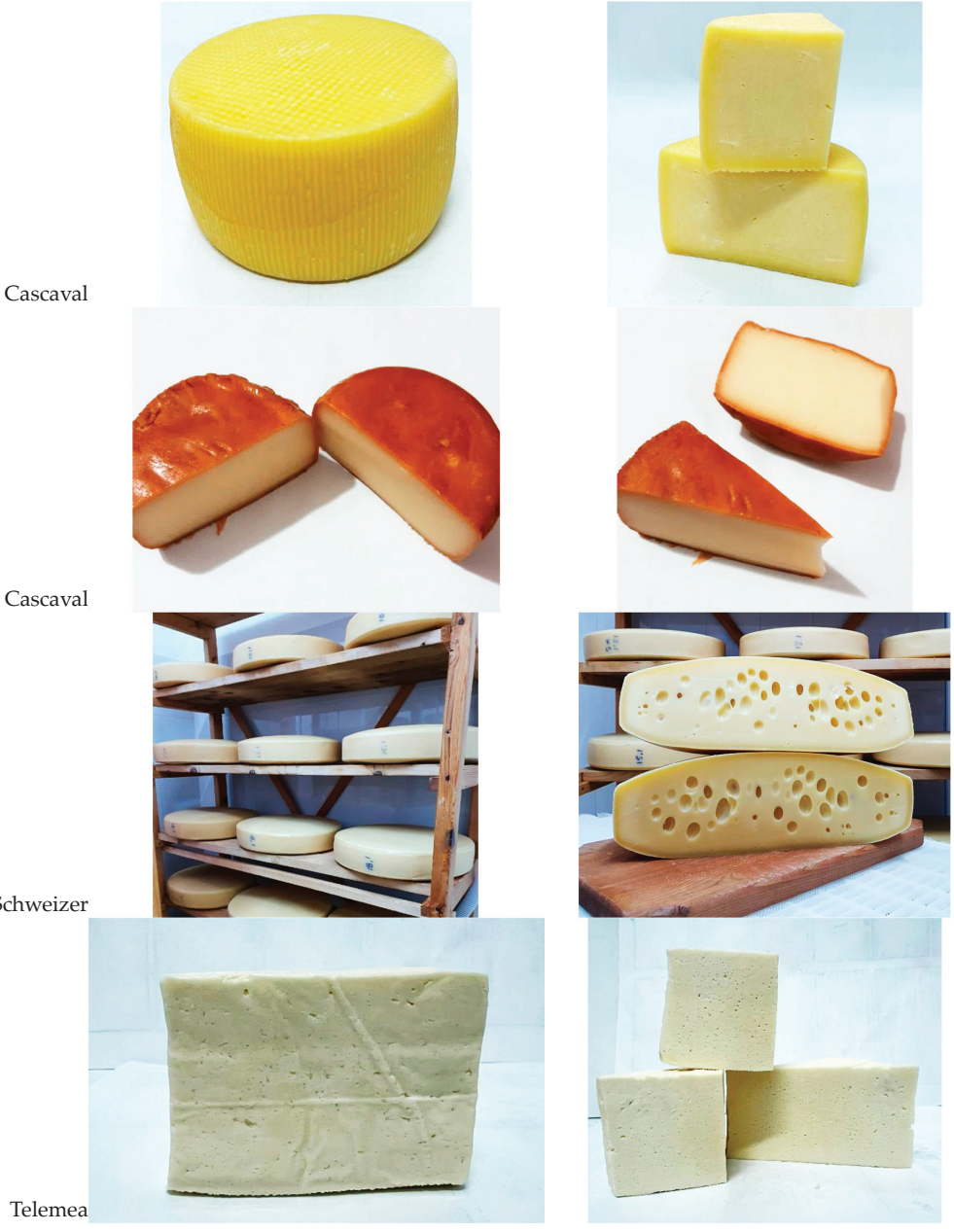

Figure 1. Photographs of analyzed Mountain cheese types (original captures).

In the rennet enzymes inoculating stage, naturally harvested rennet (abomasum content of youth calves, dried or lyophilized) is used for the "Mountain product", while the conventional cheese types are inoculated with biotechnologically originated commercial products enzymes for clotting. Temperature ranges between 30 and 33 °C in the clotting

recipient. Coagulating salts usually consist of calcium chloride solution, 25%, introduced at 50–70 mL/100 L milk. Calcium chloride is used in conventional cheese manufacturing only.

In smoked Cascaval, stage (p) is followed by smoking, then the flow is resumed. In Telemea cheese, the flow modifies from (j) pressing, and the rectangular pressed curd is transferred into barrels with saline (brine) solution 18–20%, which is stored upon marketing.

The maturation (ripening) stage lasts 12–24 h in Cascaval-type cheese and at least 60 days in Schweizer, at room temperature (15–20 °C) and in relative humidity conditions ranging between 70 and 80% in Cascaval and 80–90% in Schweizer.

Samples of these cheese types, both "Mountain product" labeled and conventionally produced, were collected at 7 days after the ripening period ended from 4 small dairy factories in Dornelor Basin (500 g/type/processor). Each processor produces both mountain-labeled products and conventional products, using milk from different sources:

- Raw milk for "Mountain product" produced in the study area, collected from 7 dairy farms in Calimani National Park, with cows grazing on the mountain meadows and fed mostly haystack harvested from the same area. Cows were not provided herbs or maize silage throughout the cold season;
- Raw milk for conventional products, collected from many small-size producers in the whole of Suceava County, both from mountain areas and hilly-plain regions, whose feeding was not traced, but, usually, the smallest farm holders use local pastures, complete with corn silage and alfalfa hay, bought from all Northeast Romania, upon availability.

2.1. Cheese Sampling

Four local producers (small local dairy workshops) were used to sample the cheese for multiple reasons:

- They use, on a daily basis, the same raw matter procured from the farmers raising dairy cows in the specific study area;
- Only one set of samples for analysis, from only one producer (local dairy workshop) would have been very subjective and narrow as data collected, considering there could be differences due to workshop influence;
- The workshops use the same recipe to prepare each type of cheese and the same raw matter.

The four types of cheese were chosen to be studied due to the high demand existing in conventional distribution chains (retailers, small shops) and farmers' markets. They are one of the most consumed cheeses in Romania (Cascaval types, both regular and smoked; Schweizer-type cheese and white cheese preserved in salted brine—Telemea cheese).

Samples were issued from the cheese batches that were produced on the very same day, using the same batch of raw matter milk. Milk typical analysis prior to transformation into cheese was unique, both for the locally produced milk transformed into mountain products and for the county-collected milk transformed into conventional products.

Collection of cheese samples was carried out on the matured finished product in accordance with the AOAC 920.122-1920 Cheese—Collection of Samples Procedure [32], i.e., 4 × 500 g in sealed plastic bags from each cheese type from each producer.

The labelled sealed bags were introduced into a refrigerating container for 8 h while they were transported from the study area to the university, then transferred into a laboratory refrigerator and kept between 1 and 4 °C and 30–50% relative humidity for 12 days until they were submitted to analysis to investigate composition, fatty acids profiling, and microbiological features.

Prior to analysis, for each type, the cheese was minced in small shards of approximately 1–2 mm each, and the samples from the 4 different producers were mixed together (4 × 500 g/cheese type/production system) in order to obtain a homogeneous bulk sample that could depict, on average, the mean value of the products.

The analytical samples were randomly collected from the bulk homogenized sample (2000 g/cheese type/production system) and weighed:

- 3–5 g/repetition in proximate composition investigations;
- 5 g/repetition in fatty acids profiling;
- 3 g/repetition in mineral micronutrients analysis;
- 50 g/repetition in the microbiological investigations.

Considering these sampling conditions and the analytical sample preparation, the external factors that could interfere with cheeses' stability and sanogenity were reduced to the greatest extent.

2.2. Chemical Proximate Composition and Gross Energy Content

Sample analyses were carried out at the Milk and Dairy Products Inspection Laboratory, part of the Centre for Quantitative and Qualitative Monitoring of Animal Productions and Food Research Infrastructure, Faculty of Food and Animal Sciences, Iasi University of Life Sciences, Romania (https://eeris.eu/ERIF-2000-000P-1926, accessed on 15 November 2023).

The chemical composition of dairy products refers to the water (moisture) content (g/100 g product) as well as to the dry matter compounds (g/100 g product). Several standardized methods proposed by AOAC International (www.aoac.org, accessed on 15 November 2023) were used as analytical protocols. For each element of chemical proximate composition and for gross energy content as well, 25 analytical repetitions/calculations have been carried out.

Water and dry matter (total solids) contents were assessed through sample drying (AOAC Method 926.08-1927) [33] in a MEMMERT UF110 + air convection oven (manufacturer: Memmert GmbH, Germany) throughout two successive stages: (1) 24 h at 60 °C; (2) minimum 6 h at 103 °C or until reaching the constant mass.

Crude ash (total minerals) was assessed through calcination in NABERTHERM B180 furnace (manufacturer: Nabertherm GmbH, Lilienthal, Germany), where the sample was burned at 550 °C, throughout 24 h, in accordance with AOAC Method 935.42-1935, ash of cheese, gravimetric method [34].

Total nitrogen and Total proteins were assessed through the AOAC Method 920.123-1920, nitrogen in cheese [35], applied on devices BEHR K8 digester, BEHROSOG 3 fume evacuator (digestion stage), BEHR S5 module (distillation—titration stage) (manufacturer Behr GmbH, Germany). The values achieved via the titrimetric procedure were input into calculation using the initial sample mass and other volumes of reagents, and the total protein content in edible products was obtained.

Total lipids in cheese products were assessed by AOAC Method 920.125-1920 [36], using a Behrotest 6er Probe extractor (manufacturer Behr GmbH, Stuttgart, Germany).

Nitrogen-free extract (NFE-total carbohydrates) content was calculated by difference, using the relation (1) [37].

$$\text{NFE (g/100 g)} = 100\ \text{g} - [\text{W (g/100 g)} + \text{TM (g/100 g)} + \text{TP (g/100 g)} + \text{TL (g/100 g)}] \quad (1)$$

in which NFE = nitrogen-free extract; W = water content; TM = total minerals; TP = total proteins; TL = total lipids.

Gross energy was calculated using the Atwater relation (2) proposed by FAO [38]:

$$\text{GE (Kcal/100 g)} = 4.36\ \text{Kcal} \times \text{g TP} + 8.79\ \text{Kcal} \times \text{g TL} + 3.87\ \text{Kcal} \times \text{g NFE} \quad (2)$$

in which GE = gross energy; TP = total proteins; TL = total lipids; NFE = nitrogen-free extract.

2.3. Lipid Profile (Fatty Acids, Cholesterol)

Fatty acid profile and cholesterol content were investigated within the laboratories of the National Institute of Research and Development for Biology and Animal Nutrition Balotesti, Ilfov County, in 6 analytical repetitions per sample.

Fatty acids profiling was realized using a method comprising two consecutive stages: (1) preparation of methyl esters (ISO/TS 17764-1:2002 method) [39] and (2) gas chromatographic method (ISO/TS 17764-1:2002 method) [40].

In order to better depict the sanogenity of the compared products, based on the fatty acids analysis, certain nutritional-sanogenic indices were calculated using the equations compiled from the literature by Simeanu et al. [41]: polyinsaturation index (PI)—Equation (3); atherogenic index (AI) (Equation (4)); thrombogenic index (TI) (Equation (5)); and hypocholesterolic/hypercholesterolic ratio (h/H) (Equation (6)):

$$PI = C18{:}2\ n-6 + (C18{:}3\ n-3 \times 2) \quad (3)$$

$$AI = (C12{:}0 + C16{:}0 + 4 \times C14{:}0)/[\Sigma MUFA + \Sigma(n-6) + \Sigma(n-3)] \quad (4)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/[0.5 \times \Sigma MUFA + 0.5 \times \Sigma(n{-}6) + 3 \times \Sigma(n-3) + \Sigma(n-3)/\Sigma(n-6)] \quad (5)$$

$$h/H = (C18{:}1 + PUFA)/(C12{:}0 + C14{:}0 + C16{:}0) \quad (6)$$

Cholesterol content was assessed via the gas chromatograph—direct saponification method in accordance with the AOAC 994.10 protocol [42].

2.4. Mineral Micronutrients Analysis

The concentrations of Ca, P, and Fe were investigated within the laboratories of the National Institute of Research and Development for Biology and Animal Nutrition Balotesti, Ilfov County, in 6 analytical repetitions per sample. Calcium content was assessed using a complexometric–titrimetric method with EDTA derived from AOAC Method no. 968.31-1969 [43]. Iron content was measured through flame atomic absorption spectrophotometry using a method indicated by Singh et al., 2015 [44]. Phosphorus was assessed in accordance with the methodology specified by the EU Regulation 152/2009 (European Commission, 2009) [45].

2.5. Microbiological Analysis

Microbiological assessments have been carried out in the Microbiology and Biosecurity Laboratory, Sanitary-Veterinary and Food Safety Directorate, Iasi County, Romania, in 6 analytical repetitions per sample. Certain microorganisms' detection was pursued, and quantification proceeded: *Enterobacteriaceae*—most probable number method, according to SR EN ISO 21528-1/2017 [46]; *Staphylococcus aureus* and other species—DIN-EN ISO 6888-2/A1:2021 method [47]; *Escherichia coli* betaglunoridase positive—ISO 16649-2:2001, 2007 method [48]; *Listeria monocytogenes*-ISO 11290-1/2017 method [49].

2.6. Statistical Analysis

The analytical data were statistically processed for main descriptors (mean, standard deviation, coefficient of variation) via GraphPad Prism 9.4.1 (673) software; then, the data were compared for the significance of differences between the analytical means of conventional and "Mountain"-labeled dairy products, using the unpaired *t*-test followed by Welch's correction. Also, relative (%) differentiations have been calculated and presented within the discussion chapter [50].

3. Results

3.1. Proximate Chemical Composition and Gross Energy

Table 1 shows the statistical descriptors resulting from the 25 replications of each analytical investigation related to proximate chemical composition, table salt content, and calculation of the gross energy content, carried out on the "Călimani" Cascaval samples of both origins ("Mountain" labeled and conventional).

Table 1. Gross composition and gross energy content of "Călimani" Cascaval and of the corresponding conventional product (n = 25).

Cascaval Type	Statistics	Water (g/100 g)	Dry Matter (g/100 g)	Total Minerals (g/100 g)	NaCl (g/100 g)	Total Proteins (g/100 g)	Total Lipids (g/100 g)	Nitrogen Free Extract (g/100 g)	Energy (Kcal/100 g)
"Mountain"	Mean ± StDev	$56.54^{a} \pm 0.40$	$43.46^{a} \pm 0.40$	$1.62^{a} \pm 0.60$	$0.77^{a} \pm 0.11$	$22.55^{a} \pm 0.32$	$19.04^{a} \pm 0.73$	$0.25^{a} \pm 0.16$	$264.58^{a} \pm 5.96$
Conventional	Mean ± StDev	$45.26^{d} \pm 0.62$	$54.74^{d} \pm 0.62$	$4.38^{d} \pm 0.91$	$2.88^{d} \pm 0.18$	$22.95^{d} \pm 0.37$	$25.84^{d} \pm 0.85$	$1.57^{d} \pm 0.18$	$331.22^{d} \pm 6.37$

Student test results: analytical means with different superscripts per column differ significantly for $p < 0.001$ when ^{a} vs. ^{d} superscripts were used.

Data issued from the chemical analysis of "Călimani" smoked Cascaval were processed statistically and presented in Table 2.

Table 2. Gross composition and gross energy content of "Călimani" Smoked Cascaval and of the corresponding conventional product (n = 25).

Smoked Cascaval Type	Statistics	Water (g/100 g)	Dry Matter (g/100 g)	Total Minerals (g/100 g)	NaCl (g/100 g)	Total Proteins (g/100 g)	Total Lipids (g/100 g)	Nitrogen Free Extract (g/100 g)	Energy (Kcal/100 g)
"Mountain"	Mean ± StDev	$56.39^{a} \pm 0.50$	$43.61^{a} \pm 0.50$	$1.52^{a} \pm 0.65$	$0.78^{a} \pm 0.13$	$22.50^{a} \pm 0.30$	$19.16^{a} \pm 0.80$	$0.43^{a} \pm 0.23$	$266.11^{a} \pm 6.33$
Conventional	Mean ± StDev	$47.19^{d} \pm 0.61$	$52.81^{d} \pm 0.61$	$2.80^{d} \pm 1.18$	$1.60^{d} \pm 0.27$	$23.86^{d} \pm 0.36$	$25.62^{d} \pm 1.65$	$0.54^{b} \pm 0.08$	$329.18^{d} \pm 14.31$

Student test results: analytical means with different superscripts per column differ significantly for $p < 0.05$ when ^{a} vs. ^{b} superscripts were used; $p < 0.001$ when ^{a} vs. ^{d} superscripts were used.

Table 3 reveals the statistically processed data derived from the analyses and computations run on the "Călimani" Schweizer samples of both origins.

Table 3. Gross composition and gross energy content of "Călimani" Schweizer and of the corresponding conventional product (n = 25).

Schweizer Type	Statistics	Water (g/100 g)	Dry Matter (g/100 g)	Total Minerals (g/100 g)	NaCl (g/100 g)	Total Proteins (g/100 g)	Total Lipids (g/100 g)	Nitrogen Free Extract (g/100 g)	Energy (Kcal/100 g)
"Mountain"	Mean ± StDev	$36.38^{a} \pm 0.50$	$63.62^{a} \pm 0.50$	$4.03^{a} \pm 0.45$	$1.95^{a} \pm 0.11$	$26.52^{a} \pm 0.27$	$27.46^{a} \pm 0.27$	$5.61^{a} \pm 0.51$	$376.33^{a} \pm 2.99$
Conventional	Mean ± StDev	$43.69^{d} \pm 0.68$	$56.31^{d} \pm 0.68$	$2.29^{d} \pm 0.26$	$3.76^{d} \pm 1.65$	$22.86^{d} \pm 0.49$	$26.09^{d} \pm 0.32$	$5.00^{d} \pm 0.44$	$346.28^{d} \pm 3.03$

Student test results: analytical means with different superscripts per column differs significantly for $p < 0.001$ when ^{a} vs. ^{d} superscripts were used.

Another studied mountain product was the "Călimani" Telemea—brine-salted cheese. Table 4 presents the data with reference to this "Mountain product", analyzed from a chemical and energetic point of view, along with the values measured for a similar conventionally processed product.

Table 4. Gross composition and gross energy content of "Călimani" Telemea and of the corresponding conventional product (n = 25).

Telemea Type	Statistics	Water (g/100 g)	Dry Matter (g/100 g)	Total Minerals (g/100 g)	NaCl (g/100 g)	Total Proteins (g/100 g)	Total Lipids (g/100 g)	Nitrogen Free Extract (g/100 g)	Energy (Kcal/100 g)
"Mountain"	Mean ± StDev	$61.80^{a} \pm 0.51$	$38.20^{a} \pm 0.51$	$5.39^{a} \pm 0.17$	$3.57^{a} \pm 0.25$	$16.09^{a} \pm 0.36$	$15.45^{a} \pm 0.36$	$1.27^{a} \pm 0.70$	$209.34^{a} \pm 2.53$
Conventional	Mean ± StDev	$63.66^{d} \pm 0.50$	$36.34^{d} \pm 0.50$	$4.91^{d} \pm 0.17$	$4.22^{d} \pm 0.28$	$17.09^{d} \pm 0.39$	$12.96^{d} \pm 0.52$	$1.39^{d} \pm 0.67$	$192.25^{d} \pm 3.23$

Student test results: analytical means with different superscripts per column differ significantly for $p < 0.001$ when ^{a} vs. ^{d} superscripts were used.

3.2. Fatty Acids Profile

Table 5 shows the results issued from the gas chromatographic analysis of lipids profile in the cheese, expressed as fatty acids methyl esters (FAMEs) (g FAME/100 g total FAME), the ratios between various groups of fatty acids, as well the cholesterol content and the sanogenic indices calculated on the basis of fatty acids profile (polyunsaturation atherogenic index, thrombogenic index, and hypocholesteromic/hypercholesterolemic ratio).

Table 5. Fatty acids profile and sanogenic indices in the "Călimani" Mountain products investigated in comparison with the conventional ones (n = 6).

Fatty Acid	Cheese Type (Fatty Acids Methyl Esters Content, Expressed as g FAME/100 g Total FAME)							
	Cascaval		Smoked Cascaval		Schweizer		Telemea	
	M *	C **	M	C	M	C	M	C
Butyric acid	0.12	0.14	0.18	0.22	0.14	0.19	0.25	0.28
Caproic acid	1.43	1.49	1.61	1.69	1.45	1.47	0.18	0.19
Caprylic acid	1.36	1.38	1.36	1.42	1.32	1.41	1.32	1.38
Nonanoic acid	0.07	0.10	0.21	0.12	0.02	0.04	0.02	0.03
Capric acid	2.92	2.95	2.83	2.89	2.90	2.95	2.78	2.85
Undecanoic acid	0.35	0.34	0.34	0.42	0.35	0.42	0.33	0.39
Tridecanoic acid	3.43	3.48	3.28	3.31	3.48	3.54	3.31	3.41
Lauric acid	0.10	0.12	0.10	0.11	0.10	0.11	0.11	0.12
Myristic acid	12.78	12.81	12.68	12.74	13.18	13.25	12.75	13.01
Myristoleic acid	1.49	1.51	1.52	1.48	1.61	1.58	1.55	1.42
Pentadecanoic acid	0.73	0.76	0.80	0.85	0.82	0.84	0.91	0.95
Pentadecenoic acid	1.90	2.10	1.88	1.74	2.02	1.98	2.15	2.08
Palmitic acid	33.93	34.22	33.73	33.92	33.81	33.85	33.93	34.03
Palmitoleic acid	1.95	1.97	2.13	2.08	1.96	1.92	2.25	2.14
Heptadecanoic acid	0.55	0.62	0.57	0.62	0.56	0.62	0.62	0.69
Heptadecenoic acid	0.98	0.97	0.99	0.87	1.00	0.97	0.99	0.95
Stearic acid	8.71	8.82	8.34	8.41	8.12	8.24	8.05	8.06
Oleic cis acid	21.19	21.08	21.83	21.79	20.89	20.74	22.50	22.30
Linoleic trans Ω-6 acid	0.32	0.26	0.29	0.25	0.35	0.31	0.25	0.21
Linoleic cis Ω-6 acid	1.79	1.68	1.71	1.68	1.73	1.68	1.79	1.73
Arachidic acid	0.05	0.06	0.05	0.04	0.10	0.14	0.09	0.12
Gamma linolenic Ω-3 acid	0.15	0.11	0.15	0.13	0.17	0.15	0.16	0.12
Alpha linolenic Ω-3 acid	1.51	1.41	1.32	1.28	1.46	1.38	1.35	1.29
Conjugated linolenic acid	0.60	0.51	0.65	0.61	0.67	0.61	0.63	0.58
Eicosadienoic Ω-6 acid	0.10	0.08	0.13	0.11	0.11	0.09	0.12	0.09
Eicosatrienoic Ω-3 acid	0.11	0.05	0.13	0.12	0.15	0.11	0.11	0.08
Eicosatetraenoic Ω-3acid	0.26	0.17	0.19	0.15	0.14	0.10	0.21	0.19
Arachidonic Ω-6 acid	0.11	0.10	0.13	0.12	0.10	0.08	0.09	0.06
Other fatty acids	1.01	0.71	0.87	0.83	1.29	1.33	1.20	1.25
Total SFAs	66.53	67.29	66.08	66.76	66.35	67.07	64.65	65.51
Total UFAs	32.46	32	33.05	32.41	32.36	31.7	34.15	33.24
Total MUFAs	27.51	27.63	28.35	27.96	27.48	27.19	29.44	28.89
Total PUFAs	4.95	4.37	4.70	4.45	4.88	4.51	4.71	4.35
SFAs/UFAs	2.05	2.10	2.00	2.06	2.05	2.12	1.89	1.97
PUFAs/MUFAs	0.18	0.16	0.17	0.16	0.18	0.17	0.16	0.15
Omega 3 FAs	2.03	1.74	1.79	1.68	1.92	1.74	1.83	1.68
Omega 6 FAs	2.92	2.63	2.91	2.77	2.96	2.77	2.88	2.67
Omega 6/Omega 3	1.44	1.51	1.63	1.65	1.54	1.59	1.57	1.59
Polyunsaturation index	5.13	4.76	4.64	4.49	5.00	4.75	4.74	4.52
Atherogenic index	2.62	2.67	2.56	2.62	2.68	2.74	2.49	2.59
Thrombohenic index	0.55	0.59	0.55	0.57	0.54	0.56	0.53	0.54
Hypocholesterolemic/hypercholesterolemic ratio	0.56	0.54	0.57	0.56	0.55	0.53	0.58	0.57
Cholesterol content (mg/100 g sample)	28.99	31.24	35.76	38.25	43.54	45.19	16.37	17.84

* M = "Mountain product"-labeled cheese samples; ** C = conventional cheese samples.

3.3. Content of Calcium, Phosphorus, Iron

The certain macro- (calcium, phosphorus) and trace- (iron) minerals in the analyzed samples are presented in Table 6.

Table 6. Calcium, phosphorus, and iron in the analyzed "Mountain" and conventional cheeses (n = 6).

Cheese Type		Statistics	Ca (g/100 g)	P (g/100 g)	Fe (mg/100 g)
Cascaval	"Mountain"	Mean ± StDev	0.65 0.04	0.63 0.04	2.46 [a] 0.16
	Conventional	Mean ± StDev	0.62 0.04	0.64 0.03	2.19 [c] 0.18
Smoked Cascaval	"Mountain"	Mean ± StDev	0.74 0.09	0.72 0.06	2.49 0.16
	Conventional	Mean ± StDev	0.72 0.07	0.71 0.07	2.31 0.20
Schweizer	"Mountain"	Mean ± StDev	0.73 0.05	0.74 0.05	5.87 [a] 0.25
	Conventional	Mean ± StDev	0.74 0.06	0.72 0.05	2.27 [d] 0.20
Telemea	"Mountain"	Mean ± StDev	0.72 [a] 0.07	0.69 [a] 0.05	2.19 [a] 0.18
	Conventional	Mean ± StDev	0.65 [b] 0.04	0.63 [b] 0.04	2.46 [c] 0.16

Student test results: analytical means with different superscripts per column within each cheese type differ significantly for $p < 0.05$ when [a] vs. [b] superscripts were used; $p < 0.01$ when [a] vs. [c] superscripts were used; $p < 0.001$ when [a] vs. [d] superscripts were used.

3.4. Microbiological Assessment

Table 7 reveals the results on the microorganisms' identification and/or quantification in the analyzed "Mountain products" in comparison with the conventionally produced ones.

Table 7. Microorganisms in the analyzed dairy "Mountain" versus conventional cheese (n = 6).

Cheese Type		*Enterobacteriaceae* MPN/g	*Staphylococci* CFU/g	*Escherichia coli* CFU/g	*Listeria monocytogenes* CFU/25 g
Cascaval	"Mountain"	1.6	<10	-	-
	Conventional	0	<10	-	-
Smoked Cascaval	"Mountain"	4.3	<10	-	-
	Conventional	0	<10	-	-
Schweizer	"Mountain"	4.3	<10	-	-
	Conventional	0	<10	-	-
Telemea	"Mountain"	9.3	<10	-	-
	Conventional	0	<10	-	-

MPN—most probable number; CFU—colony-forming units.

4. Discussion

4.1. Chemical Composition and Gross Energy

Water content in the "Călimani" Cascaval was 56.54 g/100 g, while dry matter reached 43.46 g/100 g. Total proteins per raw sample reached 22.55 g/100 g, total minerals was 1.62 g/100 g, salt (NaCl) content was 0.77 g/100 g, and lipids were quantified at 19.04 g/100 g. The gross energy content per 100 g of product was calculated at 264.58 kcal (Table 1). The conventionally processed product of the same type was much richer in salt

(up to the maximum admitted inclusion level, 3 g/100 g) and lipids (26.00 g/100 g, +46.47% compared to the "Mountain product"). It also had slightly more proteins (22.95 g/100 g, +1.99%, compared to the "Mountain product") while the gross energy content was 25.2% higher (331.22 kcal/100 g, vs. 264.58 kcal/100 g in "Mountain product" samples). Therefore, the mountain product was less caloric and lighter in terms of fat content, so healthier and dietetic, especially for consumers facing cardiovascular risks and metabolic syndrome [51,52]. In all run comparisons of proximate composition compounds, the differences between "Mountain product" and conventional samples were found to be statistically significant for $p < 0.001$.

Samples of "Călimani" smoked Cascaval (Table 2) had a water mean proportion of 56.39 g/100 g, while total solids reached 43.61 g/100 g. Total minerals accounted for 1.52 g/100 g, salt (NaCl) 0.78 g/100 g, total proteins 22.50 g/100 g, total lipids 19.16 g/100 g, and the nitrogen-free extract reached 0.43 g/100 g. The gross energy content per 100 g edible portion was 266.11 kcal. The conventionally obtained equivalent product had more than double salt content (1.60 g NaCl/100 g), 33% more fat (25.62 g/100 g), and 6.7% more proteins (23.80 g/100 g). Due to the higher concentration of organic matter, caloric content was higher as well (329.18 kcal/100 g). In most of the comparisons, the "Mountain product"-labeled samples differed significantly from the conventional ones ($p < 0.001$).

Analyzing the results obtained for the two Cascaval-type "Mountain products" ("Călimani" Cascaval and "Călimani" smoked Cascaval), no significant differences could be observed between them. In fact, it is about the same product that is marketed as it is primarily obtained or in its smoked form. Smoking is an effective and relatively simple preservation method practiced in the mountain area in order to maintain dairy and meat products' quality and stability throughout storage, therefore prolonging shelf life or improving the sensorial appeal of such products.

In Italy, a study was carried out on hard cheeses bearing the Protected Designation of Origin (PDO) label. The chemical composition revealed a crude fat of 27.89% in Grana Padano cheese, 29.90% in Montasio cheese, 30.21% in Asiago cheese, 31.37% in Parmigiano Reggiano cheese, and the highest crude fat of 36.16 for Cheddar cheese. Correspondingly, for the same types of cheese, crude protein was 34.21% for Grana Padano cheese, 27.67% for Montasio cheese, 30.46% for Asiago cheese, 34.01% for Parmigiano Reggiano cheese, and 26.59% for Cheddar cheese [53–56]. In comparison, the hard cheese labeled with the "Mountain product" quality logo originating in the mountainous area of the Dornelor Basin in Romania presented a crude fat of 20.17% and a crude protein of 22.55%, values that are lower than the cheese produced in Italy.

In "Călimani" Schweizer samples (Table 3), water content reached 36.38 g/100 g, while dry matter was calculated as 63.62 g/100 g. Total minerals reached 4.03 g/100 g, salt (NaCl) 1.95 g/100 g, total proteins 26.52 g/100 g, total lipids 27.46 g/100 g, and the nitrogen-free extract 5.61 g/100 g. Due to the highest proportion of fat and lactose (nitrogen-free extract), the gross energy content of this cheese type reached 376.45 Kcal/100 g edible portion (about 39% in excess of the caloric content, in comparison with the Cascaval-type analyzed products, that have less lipids and lactose). The conventional equivalent product presented less salt (1.70 g/100 g), less proteins (23 g/100 g), and also less lipids (26 g/100 g), a situation that led to a lower caloric content (328 kcal/100 g), in accordance with the producer statement on the label. In all comparisons, the results of gross chemical composition elements differed significantly ($p < 0.001$) in relation to the production system ("Mountain product" or conventional).

Research on Emmentaler cheese, the equivalent of Schweizer cheese from Romania, produced in six regions of Europe: Allgau in Germany, Brittany in France, Switzerland, Finland, Savoie in France, and Vorarlberg in Austria highlighted Emmentaler cheese from Vorarlberg Austria with the highest crude fat content of 34.2% and the lowest crude fat content of 30.0% in Emmentaler cheese produced in Bretagne, France. If we compare with the Schweizer, a "Mountain product" from Romania, the value for crude fat was 27.46%, lower than the types of Emmentaler cheese from Western Europe. Regarding protein,

the highest value of 28.87% was identified in Emmentaler cheese from the Savoie region of France, and the lowest value of 26.81% was recorded in Emmentaler cheese from the Vorarlberg region of Austria. Mountain-produced Schweizer cheese from Romania had a protein value of 26.52%, close to that of the Austrian Emmentaler-type cheese. It should be noted that the types of surveyed Emmentaler cheese have protected origin (PDO) [53–56].

The originality of cheeses depends on several factors, such as milk- and cheese-making processes (including microbiology and technology), which are both dependent on geographical origin. Climate, geology, feed, and the cattle breed itself influence the quality of the milk, while local, regional, or national traditions influence the making of the cheeses.

In "Călimani" Telemea (Table 4), total solids content reached 38.20 g/100 g, while water content was measured at 61.80 g/100 g. Total minerals were found in 5.39 g/100 g quantity and salt at the level of 3.57 g/100 g. Total proteins reached 16.09 g/100 g, lipids 15.45 g/100 g, and nitrogen-free extract 1.27 g/100 g. The average gross energy content reached 209.34 kcal/100 g. The conventionally processed Telemea had higher salt content, was slightly richer in proteins (17.09 g/100 g), and had lower total fat content (12.96 g/100 g), hence the lower gross energy content (192.2 kcal/100 g).

Comparing the "Călimani" Telemea, which is a soft cheese with 16.09% protein, 15.44% fat, and nitrogen-free extract of 1.22%, with other types of soft cheese such as Mozzarella with crude fat 16.11% and crude protein 18.23%, Casatella with 24.69% crude fat and 15.66% crude protein, Gorgonzola with 27.65% crude fat and 18.69% crude protein, and Taleggio with 26.73% crude fat and crude protein of 19.73%, we note that in terms of protein, Telemea cheese has a close value to Casatella cheese, and as crude fat, it is close to Mozzarella cheese. The total minerals in Telemea cheese were, on average, 5.39%, and in the rest of the literature-surveyed cheeses, the limits were between 4.77 and 5.34% [53–55].

If we analyze the chemical composition of dairy products, we can see that the highest protein content was found in "Călimani" Schweizer (26.52 g/100 g), followed by "Călimani" Cascaval (22.55 g/100 g), "Călimani" smoked Cascaval (22.50 g/100 g), and "Călimani" Telemea (16.09 g/100 g). The highest energy content occurred in "Călimani" Schweizer (376.33 kcal/100 g), while the lowest one was in "Călimani" Telemea (209.34 g/100 g). Concerning total minerals content, higher values were measured in the "Mountain product"-labeled cheese, in comparison with other data reported in the literature for conventional equivalent sorts of cheese [57–62].

In "Călimani" Schweizer, total proteins reached 26.52 g/100 g, slightly lower than the same organic compound reported for the Emmental cheese, a similar type produced in the Switzerland mountain area [63]. On the contrary, a similar type of cheese (Emmental family) produced conventionally in Ireland had different total protein values, varying within the limits of 22.78 and 23.48 g/100 g, thus underlying the higher quality of the "Mountain products" [64].

4.2. Fatty Acids Profile and Sanogenic Indices

The fatty acid content of the "Mountain product"-labeled and conventional cheese is presented in Table 5. Thus, palmitic acid varied between 33.93 g FAME/100 g total FAME in the "Mountain" Cascaval and 34.22 g FAME/100 g total FAME in the conventional samples (+0.8%). In "Mountain" smoked Cascaval, it was measured at 33.73 g FAME/100 g total FAME versus 33.92 g FAME/100 g total FAME in the conventional product. In both Schweizer samples, the values were pretty similar, between 33.81 and 33.85 g FAME/100, while in Telemea samples, they varied between 33.93 and 34.03 g FAME/100 g total FAME.

Myristic acid was found in amounts between 12.78 and 12.81 g FAME/100 g total FAME in the Cascaval samples, with lower values in the "Mountain"-labeled ones. The highest value (13.25 g/100 g FAME) was found in the conventionally produced Schweizer.

Oleic acid was found in variable proportions, between 20.74 g FAME/100 g total FAME in the "Mountain product"-labeled Schweizer and 22.50 g FAME/100 g total FAME in the "Mountain product" Telemea. Stearic acid was found with close values in all dairy products, between 8.05 and 8.82 g FAME/100 g total FAME.

Table 5 shows that saturated fatty acids (SFAs) were predominant in all samples (64.65–67.29%), while monounsaturated fatty acids (MUFAs) reached 27.19–29.44%, and the polyunsaturated fatty acids (PUFAs) were found in a proportion of 4.35–4.95%. In all comparisons, the "Mountain product"-labeled samples were richer in MUFAs and PUFAs and lower in SFAs than their conventionally corresponding samples, suggesting the influence of animals feeding on the quality of the lipidic profile. Also, the ratio of saturated fatty acids to total unsaturated fatty acids (SFAs/UFAs) varied between 1.89 and 2.11 in the analyzed products. The proportion of unsaturated fatty acids was higher [65,66] in other equivalent cheese types produced locally in the mountains. Moreover, if a comparison is made between the analytical findings and the nutritional statements on the conventionally produced equivalent sorts of cheese, we found the SFAs/UFAs ratios lower in "Mountain products", meaning that the level of lipids unsaturation is better and healthier in the "Mountain product" samples (values between 1.89/1 and 2.05/1 in analyzed products, compared to the conventionally produced cheese, bearing SFAs/UFAs ratios between 1.97/1 and 2.12/1).

In Italian hard cheese (Asiago, Grana Padano, Montasio, Parmigiano Reggiano, and Cheddar), SFAs had values, between 17.72 and 22.80%, UFAs had values between 8.63 and 11.37%, MUFAs had values between 7.36 and 9.87%, and PUFAs had values between 1.25 and 1.70% [53–56].

In the analyzed cheese products, the content of Ω-6 fatty acids was 1.5-fold richer than the content of Ω-3 fatty acids (between 2.63% and 2.96% for Ω-6 FA and between 1.68% and 2.03% for Ω-3 fatty acids).

The polyunsaturation index was in all situations higher in the "Mountain product"-originating sample (5.13 in Cascaval, 4.64 in smoked Cascaval, 5.00 in Schweizer, and 4.74 in Telemea) compared to the conventionally produced equivalent samples (4.76 in Cascaval, 4.49 in smoked Cascaval, 4.75 in Schweizer, and 4.52 in Telemea), revealing a 3.3%-7.7% better polyunsaturation in "Mountain product"-labeled cheese. The same trend was observed in the hypocholesterolemic/hypercholesterolemic fatty acid ratios, which varied between 0.55 and 0.58 in the "Mountain product" cheese and between 0.53 and 0.57 in the conventionally produced cheeses. Therefore, consumption of "Mountain product"-type cheese with such an h/H fatty acids ratio will most likely reduce the cholesterol neosynthesis in consumers' hepatocytes [67,68].

Both vascular sanogenic indices were found with 1.9–4.0% lower values (atherogenic index) and with 1.8–7.2% lower values (thrombogenic index) in the "Mountain product" samples than in the conventionally produced ones, suggesting that consumers may have lower probability in developing atherosclerosis and thrombogenesis if they choose to consume "Mountain product"-labeled cheese against opting out for the conventionally produced one.

On the cholesterol content, the found levels varied between the upper limit of 43.54 mg/100 g (Schweizer) and the lowest one of 16.37 g/100 g (Telemea) in the "Mountain product"-labeled cheese (Table 5) compared with the 17.84 g/100 g (Telemea)–45.19 g/100 g (Schweizer) interval found in the conventionally produced cheese. The data revealed that conventionally produced cheese was 3.7–8.9% richer in dietary cholesterol than the "Mountain product"-labeled one, probably due to dairy cattle dietary intake of more saturated fatty acids or with a lipid profile richer in hypercholesterolic fatty acids [69,70].

4.3. Content of Calcium, Phosphorus, Iron

Among the "Mountain product" samples, the smoked Cascaval had the highest calcium content (0.74 g/100 g), while the lowest one occurred in Cascaval (0.65 g/100 g). In conventionally produced equivalent sorts of cheese, calcium content varied between 0.62 and 0.64 g/100 g product, suggesting slightly better levels of such macro minerals in the "Mountain product"-labeled samples. Telemea cheese was the only product whose samples differed significantly ($p < 0.01$) for calcium content, with the "Mountain product" having a

higher content of this macroelement (0.72 g/100 g) vs. the conventionally produced cheese (0.65 g/100 g).

The phosphorus content in the analyzed samples varied within the 0.63–0.74 g/100 g range within the "Mountain product" category and between 0.63 and 0.71 g/100 g limits in the conventionally produced cheeses. The highest values were found in both Schweizer samples, while the only significant difference ($p < 0.05$) occurred, again, in Telemea cheese, which had 7.8% more phosphorus in the "Mountain product" sample than in the conventional one.

The iron content presented the larger variations, in relation to the analyzed product category, between 2.19 mg/100 g (conventional) and 2.46 mg/100 g ("Mountain") ($p < 0.01$) in the Cascaval sort; 2.39 mg/100 g (conventional) and 2.49 mg/100 g ("Mountain") in the smoked Cascaval sort; 2.27 mg/100 g (conventional) and 5.87 mg/100 g ("Mountain") ($p < 0.001$) in the Schweizer sort; 2.46 mg/100 g (conventional) and 2.19 mg/100 g ("Mountain") ($p < 0.05$) in the Telemea sort.

Usually, such dairy products have higher contents of calcium (1.48% of dry matter) and phosphorus (1.17% in dry matter) and the lowest content of iron, 2.69 mg/100 g dry matter [71,72]. Our findings were close to those in the literature for Fe and lowest for Ca and P, probably due to different salt mixtures used in the coagulation process, along with enzymatic products.

4.4. Microbiological Assessment

The microorganism content of the analyzed "Mountain product"-labeled cheeses (Table 7) varied, in the case of *Enterobacteriaceae*, between 1.6 MPN/g (Cascaval) and 9.3 MPN/g (Telemea). A study on six types of Emmentaler cheese, the equivalent of Schweizer cheese from Romania, produced in six regions of Europe: Allgau from Germany, Brittany from France, Switzerland, Finland, Savoie from France, and Vorarlberg from Austria highlighted the microorganism content of the analyzed products: 5.18 MPN/g was found in Emmentaler cheese produced in Allgau, Germany, 3.3 MPN/g was found in cheese produced in Bretagne, France, 2.1 MPN/g was found in cheese produced in Switzerland, 3.7 MPN/g was found in cheese produced in Finland, 6.3 MPN/g was found in cheese produced in Savoie, France, and 5.51 MPN/g was found in cheese produced in Vorarlberg, Austria. In the hard cheese produced in the Dornelor Basin, Romania, we found values of 1.6–4.3 MPN/g in the "Mountain product"-labeled smoked Cascaval and Schweizer of 4.3 MPN/g, a value close to that of cheese produced in Finland and cheese produced in Allgau, Germany [53–56].

A possible explanation for the microorganism content and color of the cheese can be found in the geology of the production region, in the feed ingredients composition and diet formulations involved in the production of Protected Designation of Origin (PDO) or of the "Mountain cheese". Most locally produced feedstuffs are allowed in cattle diets, and they are usually richer in antioxidants and pigment molecules and in a specific beneficial microflora [72–74]. Also, the usage of predominant roughages and green pasture biomass in diet structure over concentrated feedstuffs affects the above-mentioned traits. The microorganisms belonging to the *Enterobacteriaceae* group were lacking from the conventional products due to the ultra-thermal treatment applied to the raw milk. In the "Mountain product", such bacteria, not totally destroyed through a low-temperature thermization will develop specific flavor, color, and taste, thus improving the sensorial quality in comparison with the conventionally produced cheese [75,76].

The European Union and national regulations on the microbiological quality and sanitation of milk and dairy products [76] specify as compulsory for cheese the assessment of *Staphylococcus aureus*, with a legal threshold of up to 10 CFU/1 g product (samples were negative for both "Mountain product" and conventional cheese). Also, all tested samples issued from both production systems tested negative against the occurrence of *Escherichia coli* and *Listeria monocytogenes*, proving the observance of hygienic and good practices of production in all situations.

5. Conclusions

Cheese types bearing the quality label "Mountain product" provide consumers with healthier food originating from a territory with low pollution and have better nutritional quality than conventionally produced similar cheese types while complying with the microbiological norms of sanitation. The sanogenic indices, such as the polyunsaturation index and hypocholesterolic/hypercholesterolic fatty acid ratio, had higher values in the "Mountain cheese", while the atherogenic index and the thrombogenic index had lower values compared to their corresponding conventionally produced cheese.

The studied products proved they met all the quality conditions to be cataloged and marketed, both on the national and the European market, under the title of "Mountain product". The opportunity to register such products under this quality and origin label system will strongly influence dairy farmers to orientate toward local, sustainable businesses, to use mostly local inputs as feed resources, and to process their raw milk within short-chain, locally transforming dairy plants. Also, consumers have become more and more aware of the quality and sanogenity of dairy products issued from mountainous areas because they tend to be safer, healthier, and even bear better sensory properties than the conventionally produced cheeses issued from big players in the dairy industry.

As research follow-up, "Mountain" and conventional cheese types should be investigated for amino acid contents and protein quality to better depict the nutritional value and sanogenity and also to identify the factor of variation affecting their nutritional and dietetic quality.

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Article

Selected Characteristics of Multifloral Honeys from North-Eastern Romania

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Abstract: The aim of this research was to evaluate some characteristics (moisture, total solid substances, specific gravity, pH, FA, ash, electrical conductivity, TPC and TFC, potassium, calcium, magnesium, sodium, phosphorus, zinc, copper, manganese, nickel, cobalt, and lead) of fifteen multifloral honey samples. The quality of the investigated honey was confirmed by the obtained results: moisture, FA, and EC values were below the limit value regulated by the legislation. The average content of total polyphenols and total flavonoids of 29.91 mg GAE/100 g and 2.13 mg QE/100 g confirm the antioxidant properties of honey. Determination of minerals showed that potassium (101.4–1212.6 mg kg^{-1}) was the most abundant mineral in honey, followed by sodium (40.7–302.3 mg kg^{-1}) and calcium (41.8–230.9 mg kg^{-1}). Lead was found in two samples, with a content under the limit stipulation by legislation; nickel was found in one sample of 0.10 mg kg^{-1}, and the content of cobalt was below the detection limit. Significant correlations ($p < 0.001$) were observed between mm Pfund and electrical conductivity, TPC, TFC, P, Ca, and Zn; strong correlations ($p < 0.001$) were between electrical conductivity with Ash, TPC, TFC, K, and P. FTIR analysis confirmed the differences obtained by analyzing multifloral honey samples.

Keywords: honey; phenols; minerals; FTIR

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1. Introduction

Bees have a special ability to transform the melliferous flower nectar, as well as other sweet secretions present in plants or excretions of various insects, into a sweet product—honey [1–3]. The predominant components in honey are carbohydrates (mainly monosaccharides, such as fructose and glucose) and water. Also, there are many other components in small amounts: enzymes; vitamins (vitamin B6, riboflavin, pantothenic acid); phenolic acids; flavonoids; amino acids; and minerals, all contributing to a unique composition impossible to reproduce [4,5]. Due to its components, honey is known as a complete food, as well as having outstanding therapeutic qualities (antioxidant, antifungal, antibacterial, and antiviral effects) [6,7].

The physico-chemical properties, as well as the organoleptic ones (smell, taste), are greatly influenced by several factors: bee species; seasonal and environmental factors; geographical region. Various activities of the beekeepers can influence the quality of honey. However, the main influence on the overall quality of honey is the floral source [6,8–10]. Due to favorable conditions in terms of climate, as well as the diversity of the melliferous

flora, Romania is an important honey producer [11]. The well-known types of honey produced and marketed in Romania are the common monofloral honeys: acacia; lime; rape; and sunflower. There are regulations established by some European countries regarding the minimum percentage of pollen required for the characterization of monofloral honey [3,12]. Under the limits provided by the legislation, honey is called poly(multi)flora, having as dominant two or more types of different plants' pollen. The properties of the flowers that are mixed together give multifloral honey a special composition (extremely variable), which makes it unique. The melissopalynological analysis is usually used to identify the botanical origin and the pollen spectrum to complete the information on the studied honey samples [13,14]. The studies carried out on this food showed that all these characteristics are closely related [15–17]. European Union Council Directive 2001/110/EC indicates the maximum allowed levels for some parameters, such as 0.1% for the content of water-insoluble solids, 0.5% for pressed honey, 20% moisture content, 50 meq kg^{-1} free acidity, and 0.8 mS cm^{-1} for electrical conductivity for nectar honey (no less than 0.8 mS cm^{-1} for chestnut honey). The acidity of honeys is considered a freshness indicator, considering that at low pH, the growth of microorganisms is inhibited [18–20].

The therapeutic properties of honey are attributed to its antioxidant capacity. The phenolic compounds (flavonoids, phenolic acids) present in honey are responsible for the antioxidant activity, and this sweet food is sometimes used as an ingredient. Flavonoids are floral markers for the geographical and botanical origin of honey and are correlated with some parameters, mainly with total phenol content and color [4,21].

The mineral elements in honey come from the soil and are absorbed by the plants. The uptake from the soil is not selective, and both the essential and toxic to human health minerals (K, Ca, Mg, Na, P, Cu, Mn, Fe) are absorbed (Pb, Cd, Hg). The amount of each element may be an indicator of the environmental quality [22].

The method that is used more and more often due to some advantages (rapidity, nondestructive analytical method) is the Fourier transform infrared spectroscopy (FTIR) technique. This is used to scan and identify substances or chemical groups present in honey, and at the same time, information is received related to quality, the authenticity of the honey, and whether it has been adulterated or not [23,24].

The aim of this research was to characterize multifloral honey samples from North-Eastern Romania from a botanical, physicochemical, and mineral perspective and to find the similarities or differences by using various methods of analysis, including FTIR spectroscopy as a nondestructive method.

2. Materials and Methods

2.1. Honey Samples

Fifteen multifloral honey samples produced by *Apis mellifera* species were collected in October 2017 in Romania. Samples collected from the beekeepers came from two different areas: nine multifloral honey samples (S1, S2, S3, S4, S5, S10, S11, S13, S14) were collected from I-Iasi county (47°15′ N 27°19′ E) and six multifloral honey samples (S6, S7, S8, S9, S12, S15) were collected from II-Vaslui county (46°35′ N 27°46′ E) (Figure 1). Three jars of 400 g for each sample were kept in the dark in a laboratory. Before performing the analyses, the crystallized samples were liquefied at a maximum temperature of 45 °C, and all samples were homogenized.

2.2. Physicochemical Determinations

The botanical origin of the fifteen multifloral honey samples was established using the melissopalynological method of Louvreaux et al. (1978) [25], with some modifications to the centrifugation process (time, speed). Ten grams of sample, dissolved in 20 mL of 5‰ sulphuric acid (Merck KGaA, Darmstadt, Germany), were centrifuged twice (UNIVERSAL 320 HETTICH centrifuge, Hettich GMBH—Tuttlingen, Germany) at 3500 rpm for 30 min. After removing the liquid, 20 mL of distilled water was added and again centrifuged twice at 3500 rpm for 30 min. After removing the liquid, the entire amount of sediment

was placed on a glass slide in two separate drops. After complete drying, the two spots were included in the gelatin–glycerin mixture and covered with lamella. The samples were examined by counting at least 800 pollen grains [26] with an optical microscope (Optika Microscopes Italy, Ponteranica, Italy) under a light microscope with 40× and 100× objective lenses.

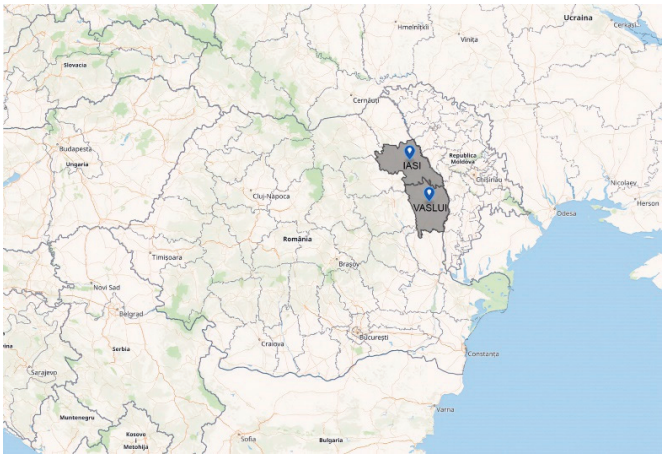


Figure 1. Map showing the areas where the multifloral honey samples were collected.

Fifteen multifloral honey samples were palynological analysed. Relative frequency classes were determined according to the international melissopalyno-logical nomenclature PP—"predominant pollen" (more than 45% of pollen grains counted), SP—"secondary pollen" (representing 15–45% of the total pollen), IMP—"important minor pollen" (3–15%), MP—"minor pollen" (less than 3%) [7].

The Pfund value was determined using the method described by Rațiu et al. [27]. The honey aqueous solutions (50% (w/v)) were centrifuged (UNIVERSAL 320 HETTICH centrifuge, Hettich GMBH—Tuttlingen, Germany), and the absorbance at 635 nm was measured using a Shimadzu UV-1700 Pharma Spec spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The absorbance units were converted in mm Pfund using a mathematical relation. The shades of honey color are related to mm Pfund from water white (0 mm Pfund) to dark amber (140 mm Pfund).

By applying the temperature correction, the refractive index was read on an ABBÉ Kruss AR 2008 refractometer (Kruss Scientific GMBH, Hamburg, Germany), and the moisture content (M, %) was determined based on the correspondence between the water content and the refractive index at 20 °C [28].

The total soluble solids (total soluble sugars, Brix degrees) were determined from the correspondence between the refractive index and degrees Brix [29].

Specific gravity was determined by the gravimetric method using a pycnometer device. The results were expressed in g/cm^3 [30].

The pH of the honey solution (10 g of honey in 75 mL of distilled water) was measured using the MULTI 3320 multiparameter (WTW GMBH, Weilheim, Germany) [30].

Free acidity was determined by the titration method: a honey solution (10 g of honey in 75 mL of distilled water) was titrated with 0.1 N NaOH (Chemical Company, Romania), and the result was expressed in meq kg^{-1} [28].

The ash content (g/100 g) was determined by sample calcination in a furnace (Nabertherm B180, Nabertherm GMBH, Lilienthal, Germany) [28].

Electrical conductivity was measured with the MULTI 3320 multiparameter (WTW GMBH, Weilheim, Germany) in a 20% solution (at dry matter) with ultrapure water (Barn-

stead EASY PURE II, Thermo Fisher Scientific Co., Ltd., Des Moines, IA, USA); the electrical conductivity was expressed in mS cm^{-1} [30].

2.3. Total Phenol Content and Total Flavonoid Content

The total phenols and total flavonoids were extracted with an alcoholic solution (1:1 equal parts of methanol (Merck KGaA, Darmstadt, Germany) and acidified water with pH = 2 (adjusted with HCl, Merck KGaA, Darmstadt, Germany), and the extractive honey solution (10%) was homogenized and filtered through filter paper. An aliquot of the filtered honey solution was mixed with 0.2 mL of Folin–Ciocalteu's phenol reagent (Merck KGaA, Darmstadt, Germany) for 5 min, and 75 g/L Na_2CO_3 (Merck KGaA, Darmstadt, Germany) was added to a total volume of 10 mL. The sample was then incubated for 30 min in the dark at room temperature and spectrophotometrically analyzed at 742 nm. The linear range ($y = 0.0993x + 0.0741$; $R^2 = 0.9991$) for gallic acid (Merck KGaA, Darmstadt, Germany) was 2–12 mg L^{-1}. The total phenol content was expressed as mg of gallic acid equivalents (GAE)/100 g [31,32].

For total flavonoids, equal volumes of 2% $AlCl_3$ (Merck KGaA, Darmstadt, Germany) and the same honey solution used at total phenols determination were mixed, and after 10 min, the absorbance was measured at 430 nm. A standard solution of quercetin (Sigma-Aldrich, St. Louis, MO, USA) was prepared and used to obtain the calibration curve (concentration range 0.5–5 mg L^{-1}; $y = 0.01330x + 0.0111$; $R^2 = 0.9998$). The total flavonoid content was expressed as mg of quercetin equivalents (QE)/100 g [31,33].

2.4. Mineral Elements (K, Ca, Mg, Na, P, Zn, Cu, Mn, Ni, Co, and Pb)

The ash resulting from the sample calcination was moistened with ultrapure water and subsequently evaporated, calcinated, treated with 6 M HCl (Merck KGaA, Darmstadt, Germany), heated, and dissolved in 0.1 M nitric acid (Merck KGaA, Darmstadt, Germany). The extract was filtered and diluted with ultrapure water to 25 mL. The phosphorus concentration was spectrophotometrically determined with molybdovanadate reagent (Merck KGaA, Darmstadt, Germany) at 430 nm (Shimadzu UV-1700 Pharma Spec spectrophotometer, Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The calibration curve was linear in the concentration range of 5–50 mg L^{-1} ($y = 0.0209x + 0.0150$; $R^2 = 1.000$) [34]. Ca, Mg, Zn, Cu, Mn, Ni, Co, and Pb were determined by flame atomic absorption spectrometry. For Ca ($\lambda = 422.7$ nm), the linear range was 1–10 mg L^{-1}, and for Mg Mg ($\lambda = 285.2$ nm) 0.1–0.5 mg L^{-1} (Ca: $y = 0.0264x + 0.0140$, $R^2 = 0.9995$; Mg: $y = 0.3997x + 0.0253$, $R^2 = 0.9991$); the calibration curve for Zn ($\lambda = 213.9$ nm) and Mn ($\lambda = 279.5$ nm) was linear in the concentration range of 0.05–0.6 mg L^{-1} (Zn: $y = 0.4929x + 0.0101$; $R^2 = 0.996$; Mn: $y = 0.0959x + 0.0005$; $R^2 = 0.999$) and for Cu ($\lambda = 324.7$ nm), Ni ($\lambda = 232$ nm), Co ($\lambda = 240.7$ nm), and Pb ($\lambda = 283.3$), the calibration curve was linear in the concentration range of 0.1–1.0 mg L^{-1} (Cu: $y = 0.0097x + 0.2310$; $R^2 = 0.999$; Ni: $y = 0.1036x + 0.0035$; $R^2 = 0.996$ Co: $y = 0.1562x + 0.0054$; $R^2 = 0.997$; Pb: $y = 0.0643x + 0.0031$; $R^2 = 0.997$). Na ($\lambda = 589$ nm; $y = 0.0970x + 0.0017$, $R^2 = 0.996$, 1–10 mg L^{-1}) and K ($\lambda = 766.5$ nm, $y = 0.1010x + 0.0128$, $R^2 = 0.998$, 1–10 mg L^{-1}) were determined by flame atomic emission spectrometry (Analytik Jena novAA 350, Analytik Jena GmbH, Jena, Germany).

2.5. FTIR Spectra

Infrared spectra were obtained using a Jasco FT/IR-660 Plus Fourier Transform Infrared Spectrometer (Tokyo, Japan). A small quantity of liquefied and homogenized samples was incorporated into a KBr (Sigma-Aldrich, Darmstadt, Germany) pellet. Spectral measurements were recorded in the wavenumber range from 4000 cm^{-1} to 400 cm^{-1} (32 scans, resolution 4 cm^{-1}) [23].

2.6. Statistical Analyses

For all the samples, three replicates were analyzed. The results were statistically analyzed (STATISTICA 12.0, StatSoft Inc., Tulsa, OK, USA) to obtain an overview of physicochemical parameter contributions by testing via Pearson's correlation coefficient, principal component analysis, and hierarchical cluster analysis.

3. Results

3.1. Physicochemical Determinations

The plant families of pollen grains identified in the studied honey samples are summarized in Table 1.

Table 1. Plant families of pollen grains in the investigated honey samples.

Family	Sample														
	Area 1										Area 2				
	S1	**S2**	**S3**	**S4**	**S5**	**S10**	**S11**	**S13**	**S14**	**S6**	**S7**	**S8**	**S9**	**S12**	**S15**
Apiaceae	IMP	MP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP
Asteraceae	IMP	IMP	SP	SP	SP	IMP	IMP	SP	IMP	SP	SP	IMP	SP	IMP	SP
Boraginaceae	-	-	-	-	-	-	-	-	-	-	MP	-	MP	-	IMP
Brassicaceae	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
Cyperaceae	-	IMP	IMP	-	-	-	-	-	-	MP	-	MP	-	MP	-
Fabaceae	IMP	SP	IMP	SP	SP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	IMP	SP
Fagaceae	-	MP	MP	-	-	-	-	MP	-	-	-	-	-	-	-
Lamiaceae	MP	-	MP	MP	-	-	MP	-	-	-	MP	-	MP	MP	-
Malvaceae	SP	IMP	IMP	IMP	IMP	MP	IMP	SP	IMP	IMP	IMP	IMP	SP	IMP	IMP
Plantagigaceae	MP	MP	-	-	-	-	MP	MP	-	MP	-	-	-	MP	-
Poaceae	MP	MP	MP	MP	IMP	IMP	IMP	MP	IMP	MP	IMP	IMP	IMP	IMP	MP
Rosaceae	IMP	MP	MP	MP	IMP	SP	SP	-	SP	IMP	-	SP	-	SP	MP
Salicaceae	IMP	IMP	IMP	IMP	IMP	IMP	MP	MP	MP	IMP	SP	IMP	IMP	IMP	IMP

SP—secondary pollen (15–45%); IMP—important minor pollen (3–15%); MP—minor pollen (less than 3%).

Tables 2 and 3 show the results for honey samples from area I and area II, respectively.

Table 2. Physicochemical parameters for multifloral honeys from area I.

Parameter	Descriptive Statistics	Sample								
		S1	**S2**	**S3**	**S4**	**S5**	**S10**	**S11**	**S13**	**S14**
mm Pfund	Min-Max Mean ± SD CV	59.0–60.1 59.4 ± 0.4 0.7	24.1–25.6 24.8 ± 0.5 1.9	55.3–57.1 56.2 ± 0.6 1.0	48.2–50.4 48.9 ± 0.7 1.4	69.0–70.5 69.7 ± 0.5 0.7	40.4–41.9 41.2 ± 0.5 1.3	30.4–31.4 30.9 ± 0.3 1.1	24.4–25.9 25.2 ± 0.5 2.0	44.0–45.6 44.9 ± 0.5 1.1
RI	Min-Max Mean ± SD CV	1.495–1.496 1.496 ± 0.00 0.01	1.493–1.494 1.493 ± 0.00 0.01	1.491–1.492 1.492 ± 0.00 0.01	1.488–1.489 1.488 ± 0.00 0.01	1.488–1.489 1.490 ± 0.00 0.01	1.488–1.489 1.490 ± 0.00 0.01	1.492–1.493 1.492 ± 0.00 0.02	1.491–1.490 1.491 ± 0.00 0.01	1.493–1.494 1.494 ± 0.00 0.01
M %	Min-Max Mean ± SD CV	16.3–16.5 16.4 ± 0.07 0.43	17.4–17.5 17.4 ± 0.04 0.21	17.9–18.0 18.0 ± 0.04 0.22	19.2–19.4 19.3 ± 0.06 0.29	19.1–19.2 19.1 ± 0.05 0.28	19.0–19.2 19.2 ± 0.06 0.33	17.5–17.8 17.6 ± 0.09 0.51	18.0–18.2 18.1 ± 0.05 0.28	17.0–17.2 17.1 ± 0.05 0.29
TSS °Brix	Min-Max Mean ± SD CV	82.0–82.2 82.1 ± 0.07 0.08	81.0–81.1 81.0 ± 0.03 0.04	80.5–80.6 80.6 ± 0.04 0.05	79.1–79.3 79.2 ± 0.06 0.07	79.4–79.5 79.4 ± 0.05 0.07	79.3–79.5 79.4 ± 0.06 0.08	80.7–81.0 80.9 ± 0.09 0.11	80.2–80.5 80.4 ± 0.05 0.06	81.3–81.4 81.4 ± 0.06 0.07
SG g/cm3	Min-Max Mean ± SD CV	1.440–1.442 1.441 ± 0.00 0.03	1.434–1.435 1.434 ± 0.00 0.01	1.431–1.432 1.431 ± 0.00 0.02	1.421–1.422 1.421 ± 0.01 0.03	1.423–1.424 1.423 ± 0.00 0.03	1.422–1.424 1.423 ± 0.00 0.03	1.432–1.434 1.433 ± 0.00 0.04	1.429–1.430 1.430 ± 0.00 0.02	1.436–1.437 1.436 ± 0.00 0.03

RI—refractive index; M—moisture; TSS—total soluble solids; SG—specific gravity; SD—standard deviation; CV—coefficient of variation.

Table 3. Physicochemical parameters for multifloral honeys from area II.

Parameter	Descriptive Statistics	Sample					
		S6	**S7**	**S8**	**S9**	**S12**	**S15**
mm Pfund	Min-Max	46.3–48.2	25.2–26.3	63.4–65.3	42.3–43.4	47.6–48.6	13.8–15.0
	Mean ± SD	47.5 ± 0.64	25.8 ± 0.42	64.5 ± 0.53	42.9 ± 0.33	48.0 ± 0.34	14.5 ± 0.41
	CV	1.36	1.63	0.81	0.77	0.71	2.87
RI	Min-Max	1.494–1.495	1.494–1.495	1.491–1.492	1.487–1.488	1.494–1.495	1.493–1.494
	Mean ± SD	1.495 ± 0.00	1.495 ± 0.00	1.492 ± 0.00	1.488 ± 0.00	1.495 ± 0.00	1.494 ± 0.00
	CV	0.01	0.01	0.01	0.02	0.02	0.01
M %	Min-Max	16.6–16.8	16.8–16.9	17.9–18.0	19.4–19.6	16.6–16.9	17.0–17.1
	Mean ± SD	16.7 ± 0.06	16.8 ± 0.05	18.0 ± 0.06	19.5 ± 0.09	16.7 ± 0.09	17.1 ± 0.03
	CV	0.38	0.29	0.33	0.46	0.56	0.20
TSS °Brix	Min-Max	81.7–81.9	81.6–81.7	80.5–80.7	79.0–79.2	81.6–81.9	81.4–81.5
	Mean ± SD	81.8 ± 0.06	81.7 ± 0.04	80.6 ± 0.06	79.0 ± 0.09	81.8 ± 0.09	81.4 ± 0.03
	CV	0.07	0.05	0.07	0.11	0.11	0.04
SG g/cm^3	Min-Max	1.438–1.440	1.438–1.439	1.431–1.432	1.420–1.421	1.438–1.440	1.436–1.437
	Mean ± SD	1.439 ± 0.00	1.438 ± 0.00	1.431 ± 0.00	1.420 ± 0.00	1.423 ± 0.00	1.436 ± 0.00
	CV	0.03	0.02	0.03	0.04	0.05	0.02

RI—refractive index; M—moisture; TSS—total soluble solids; SG—specific gravity, SD—standard deviation; CV—coefficient of variation.

Table 4 summarizes the analysis results for pH, free acidity, ash, electrical conductivity, total phenol content, and total flavonoid content of honey samples from area I.

Table 4. pH, free acidity, ash, electrical conductivity, total phenol content, and total flavonoid content of multifloral honeys from area I.

Parameter	Descriptive Statistics	Sample								
		S1	**S2**	**S3**	**S4**	**S5**	**S10**	**S11**	**S13**	**S14**
pH	Min-Max	4.26–4.27	5.01–5.02	3.73–3.74	4.24–4.25	3.85–3.86	3.78–3.79	3.58–3.59	4.07–4.08	3.75–3.76
	Mean ± SD	4.26 ± 0.0	5.02 ± 0.00	3.74 ± 0.0	4.25 ± 0.0	3.86 ± 0.0	3.79 ± 0.0	3.58 ± 0.0	4.08 ± 0.0	3.76 ± 0.0
	CV	0.03	0.02	0.02	0.02	0.03	0.08	0.09	0.07	0.07
FA meq kg^{-1}	Min-Max	47.9–48.3	28.5–28.8	39.9–40.5	38.5–39.0	49.4–50.6	34.3–34.9	43.2–43.9	42.4–42.9	23.7–24.4
	Mean ± SD	48.1 ± 0.14	28.7 ± 0.10	40.3 ± 0.19	38.8 ± 0.16	49.9 ± 0.40	34.7 ± 0.18	43.6 ± 0.22	42.7 ± 0.17	24.1 ± 0.21
	CV	0.29	0.35	0.46	0.42	0.80	0.52	0.50	0.41	0.89
Ash %	Min-Max	0.211–0.300	0.436–0.515	0.201–0.281	0.125–0.191	0.258–0.384	0.128–0.144	0.092–0.105	0.224–0.314	0.160–0.185
	Mean ± SD	0.233 ± 0.03	0.484 ± 0.03	0.241 ± 0.03	0.155 ± 0.02	0.325 ± 0.05	0.133 ± 0.01	0.099 ± 0.00	0.280 ± 0.03	0.176 ± 0.01
	CV	11.60	5.36	11.49	13.99	13.97	6.08	4.20	9.59	4.48
EC mS cm^{-1}	Min-Max	0.537–0.539	0.735–0.736	0.295–0.297	0.399–0.401	0.503–0.504	0.302–0.305	0.318–0.319	0.498–0.499	0.496–0.497
	Mean ± SD	0.538 ± 0.00	0.736 ± 0.00	0.296 ± 0.00	0.400 ± 0.00	0.504 ± 0.00	0.304 ± 0.00	0.318 ± 0.00	0.499 ± 0.00	0.496 ± 0.00
	CV	0.16	0.10	0.24	0.21	0.10	0.29	0.17	0.11	0.11
TPC mg GAE/100g	Min-Max	34.43–35.44	28.06–29.26	30.91–32.56	23.66–24.37	38.83–40.34	30.86–31.67	32.58–33.41	28.40–29.84	32.89–34.87
	Mean ± SD	34.86 ± 0.35	28.63 ± 0.38	31.73 ± 0.71	24.05 ± 0.26	39.54 ± 0.47	31.23 ± 0.27	33.03 ± 0.31	29.02 ± 0.47	33.93 ± 0.70
	CV	1.00	1.33	2.22	1.08	1.20	0.86	0.95	1.62	2.05
TFC mg QE/100g	Min-Max	2.38–2.85	1.69–2.04	2.26–2.74	1.99–2.35	2.60–3.01	1.67–2.17	1.75–2.10	1.64–1.97	2.18–2.78
	Mean ± SD	2.63 ± 0.16	1.92 ± 0.11	2.51 ± 0.16	2.18 ± 0.13	2.75 ± 0.13	1.97 ± 0.16	1.97 ± 0.11	1.77 ± 0.10	2.41 ± 0.18
	CV	5.98	5.86	6.29	6.06	4.56	7.92	5.75	5.74	7.47

FA—free acidity; EC—electrical conductivity; TPC—total phenol content; TFC—total flavonoid content; SD—standard deviation; CV—coefficient of variation.

Table 5 shows the results of pH, free acidity, ash, electrical conductivity, total phenol content, and total flavonoid content of honey samples from area II.

3.2. Mineral Elements (K, Ca, Mg, Na, P, Zn, Cu, Mn, Ni, Co, and Pb)

Table 6 shows the content of macroelements and microelements determined in multifloral honey from area I.

Table 7 shows the content of macroelements and microelements determined in multifloral honeys from area II.

Table 5. pH, free acidity, ash, electrical conductivity, total phenol content, and total flavonoid content of multifloral honeys from area II.

Parameter	Descriptive Statistics	Sample					
		S6	**S7**	**S8**	**S9**	**S12**	**S15**
pH	Min-Max	3.84–3.85	3.94–3.95	4.04–4.05	3.77–3.78	3.88–3.89	3.92–3.93
	Mean ± SD	3.84 ± 0.00	3.95 ± 0.00	4.05 ± 0.00	3.78 ± 0.00	3.88 ± 0.00	3.92 ± 0.00
	CV	0.03	0.02	0.02	0.07	0.09	0.03
FA meq kg^{-1}	Min-Max	49.4–50.0	47.6–48.0	41.5–41.7	20.7–21.4	46.3–47.1	18.9–19.4
	Mean ± SD	49.7 ± 0.21	47.8 ± 0.14	41.6 ± 0.09	21.0 ± 0.22	46.8 ± 0.23	19.1 ± 0.19
	CV	0.42	0.29	0.21	1.07	0.50	0.10
Ash %	Min-Max	0.177–0.261	0.062–0.089	0.190–0.214	0.111–0.132	0.146–0.158	0.061–0.078
	Mean ± SD	0.216 ± 0.03	0.079 ± 0.01	0.201 ± 0.01	0.121 ± 0.01	0.152 ± 0.00	0.070 ± 0.01
	CV	12.98	11.74	4.02	5.09	2.76	7.46
EC mS cm^{-1}	Min-Max	0.485–0.486	0.208–0.209	0.510–0.511	0.300–0.302	0.418–0.419	0.168–0.170
	Mean ± SD	0.486 ± 0.00	0.209 ± 0.00	0.511 ± 0.00	0.301 ± 0.00	0.419 ± 0.00	0.169 ± 0.00
	CV	0.10	0.21	0.10	0.24	0.13	0.42
TPC mg GAE/100g	Min-Max	26.37–27.78	25.73–26.73	27.96–29.07	29.88–31.41	26.67–27.84	22.78–23.65
	Mean ± SD	26.88 ± 0.0.48	26.22 ± 0.34	28.65 ± 0.35	30.43 ± 0.73	27.24 ± 0.42	23.18 ± 0.31
	CV	1.77	1.31	1.21	2.38	1.53	1.33
TFC mg QE/100g	Min-Max	2.27–2.84	1.08–1.61	1.87–2.11	1.94–2.34	2.05–2.44	1.36–1.85
	Mean ± SD	2.57 ± 0.19	1.28 ± 0.15	2.00 ± 0.08	2.11 ± 0.12	2.28 ± 0.13	1.60 ± 0.16
	CV	7.26	11.72	4.00	5.68	5.60	10.08

FA—free acidity; EC—electrical conductivity; TPC—total phenol content; TFC—total flavonoid content; SD—standard deviation; CV—coefficient of variation.

Table 6. Macroelement and microelement content (mg kg^{-1}) of multifloral honeys from area I.

Parameter	Descriptive Statistics	Sample								
		S1	**S2**	**S3**	**S4**	**S5**	**S10**	**S11**	**S13**	**S14**
K	Mean ± SD	818.6 ± 4.06	1212.6 ± 18.68	110.6 ± 1.63	112.3 ± 1.76	403.9 ± 2.07	210.1 ± 2.09	354.1 ± 1.50	486.9 ± 2.99	415.7 ± 2.58
	CV	0.50	1.54	1.48	1.56	0.51	1.00	0.42	0.61	0.62
Ca	Mean ± SD	116.1 ± 2.70	88.9 ± 3.52	141.2 ± 1.27	199.9 ± 1.39	46.5 ± 1.93	99.4 ± 1.97	102.5 ± 1.39	164.4 ± 2.14	195.3 ± 1.92
	CV	2.33	3.96	0.90	0.70	4.15	1.98	1.36	1.30	0.99
Mg	Mean ± SD	64.1 ± 2.85	43.6 ± 1.73	40.3 ± 1.83	44.9 ± 1.55	61.2 ± 1.55	40.0 ± 1.54	35.8 ± 1.83	56.9 ± 1.89	58.7 ± 1.31
	CV	4.44	3.97	4.56	3.44	2.53	3.86	5.11	3.33	2.22
Na	Mean ± SD	144.5 ± 3.61	279.8 ± 5.39	113.2 ± 1.46	302.3 ± 1.71	139.6 ± 1.73	221.7 ± 1.59	112.1 ± 1.71	75.3 ± 1.78	100.7 ± 1.98
	CV	2.50	1.93	1.29	0.57	1.24	0.72	1.53	2.36	1.97
P	Mean ± SD	61.9 ± 1.39	54.6 ± 1.28	46.9 ± 0.87	31.8 ± 1.45	85.5 ± 1.83	51.3 ± 2.06	42.8 ± 1.17	44.3 ± 1.94	58.3 ± 1.83
	CV	2.25	2.34	1.86	4.55	2.14	4.02	2.72	4.39	3.13
Zn	Mean ± SD	1.33 ± 0.03	2.87 ± 0.03	4.85 ± 0.03	6.19 ± 0.18	5.11 ± 0.10	1.58 ± 0.08	0.76 ± 0.07	5.69 ± 0.09	4.66 ± 0.07
	CV	2.10	1.22	0.71	2.83	2.02	5.01	9.23	1.58	1.53
Cu	Mean ± SD	1.01 ± 0.01	0.96 ± 0.02	1.08 ± 0.04	1.30 ± 0.09	1.12 ± 0.11	1.82 ± 0.07	1.49 ± 0.08	1.99 ± 0.08	1.61 ± 0.08
	CV	1.42	2.33	3.97	6.61	9.58	4.09	5.66	4.18	5.23
Mn	Mean ± SD	0.43 ± 0.01	0.77 ± 0.02	0.27 ± 0.02	0.53 ± 0.04	4.31 ± 0.15	1.17 ± 0.09	0.43 ± 0.03	1.62 ± 0.0	0.51 ± 0.04
	CV	3.17	3.16	7.52	7.48	3.51	7.67	8.09	3.39	7.83
Ni	Mean ± SD									
	CV	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Co	Mean ± SD									
	CV	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Pb	Mean ± SD									
	CV	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

SD—standard deviation; CV—coefficient of variation; LOD—limit of detection.

Table 7. Macroelement and microelement content (mg kg^{-1}) of multifloral honeys from area II.

Parameter	Descriptive Statistics	Sample					
		S6	**S7**	**S8**	**S9**	**S12**	**S15**
K	Mean ± SD CV	471.5 ± 1.89 0.40	150.0 ± 1.76 1.17	639.8 ± 1.64 0.26	224.5 ± 2.12 0.95	424.4 ± 4.74 1.12	101.4 ± 1.83 1.81
Ca	Mean ± SD CV	225.5 ± 1.58 0.70	92.5 ± 0.89 0.96	230.9 ± 1.93 0.84	78.7 ± 1.96 2.49	120.6 ± 2.68 2.22	41.8 ± 1.37 3.29
Mg	Mean ± SD CV	65.5 ± 1.91 2.92	60.5 ± 1.77 2.92	56.6 ± 1.23 2.18	37.7 ± 1.43 3.79	48.4 ± 1.54 3.18	32.7 ± 1.45 4.43
Na	Mean ± SD CV	94.8 ± 2.12 2.24	40.7 ± 1.91 4.70	165.2 ± 1.44 0.87	241.1 ± 2.12 0.88	178.6 ± 1.12 0.63	84.6 ± 1.86 2.20
P	Mean ± SD CV	63.9 ± 2.40 3.75	36.5 ± 1.13 3.10	67.3 ± 1.66 2.47	39.4 ± 1.25 3.16	60.8 ± 1.88 3.10	28.6 ± 1.62 5.66
Zn	Mean ± SD CV	7.23 ± 0.16 2.21	6.18 ± 0.14 2.30	13.66 ± 0.18 1.32	4.44 ± 0.10 2.23	1.54 ± 0.08 5.49	4.97 ± 0.10 1.96
Cu	Mean ± SD CV	2.18 ± 0.13 5.99	0.77 ± 0.07 8.95	1.99 ± 0.08 4.05	2.12 ± 0.09 4.38	0.76 ± 0.07 8.80	1.49 ± 0.04 2.93
Zn	Mean ± SD CV	7.23 ± 0.16 2.21	6.18 ± 0.14 2.30	13.66 ± 0.18 1.32	4.44 ± 0.10 2.23	1.54 ± 0.08 5.49	4.97 ± 0.10 1.96
Cu	Mean ± SD CV	2.18 ± 0.13 5.99	0.77 ± 0.07 8.95	1.99 ± 0.08 4.05	2.12 ± 0.09 4.38	0.76 ± 0.07 8.80	1.49 ± 0.04 2.93
Ni	Mean ± SD CV	<LOD	0.10 ± 0.01 12.04	<LOD	<LOD	<LOD	<LOD
Co	Mean ± SD CV	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Pb	Mean ± SD CV	<LOD	0.05 ± 0.01 14.29	<LOD	0.01 ± 0.00 5.17	<LOD	<LOD

SD—standard deviation; CV—coefficient of variation; LOD—limit of detection.

3.3. FTIR Spectra

In Table 8, the main maximum absorption wavelengths of honey sample spectra are presented. Figures 2 and 3 show the matrix plot of multifloral honey in the 4000–400 cm^{-1} range and the scores of the first two principal components computed by Principal Component Analysis, respectively.

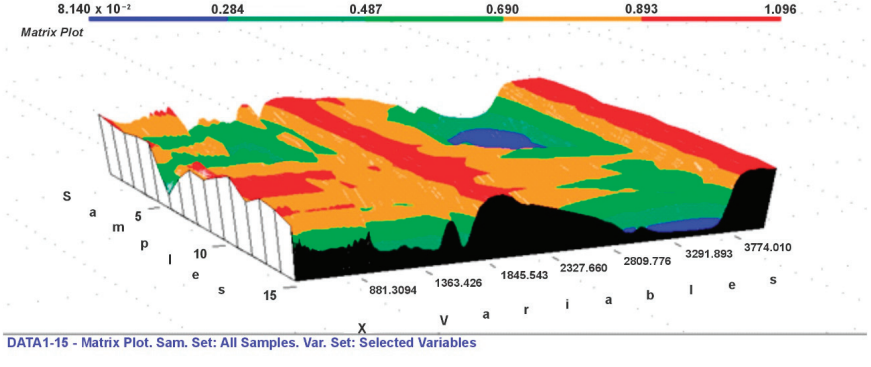


Figure 2. Matrix plot of multifloral honey in 4000–400 cm^{-1} domain.

Table 8. The position of the main bands obtained from FTIR multifloral honey samples analysis.

Sample	Spectral domains				
	D1 3500–3100 cm^{-1}	D2 3000–2800 cm^{-1}	D3 1700–1600 cm^{-1}	D4 1540–1175 cm^{-1}	D5 1175–940 cm^{-1}
	assigned to O–H stretching (carboxylic acids) and NH3 stretching (free amino acids).	assigned to: C–H stretching (carbohydrates).	C=O stretching (mainly from carbohydrates) and N–H bending of amide I (mainly proteins). O–H stretching/bending (water) and N–H bending of amide I (mainly proteins), C=C	assigned to O–H stretching/bending, C–O stretching (carbohydrates), C–H stretching (carbohydrates), and C=O stretching of ketones	assigned to C–O, C–C stretching (carbohydrates), and ring vibrations (mainly from carbohydrates).
1	3414	2931; 2882	1643	1538; 1416; 1384, 1257	1055; 919
2	3392	2932; 2878	1644	1538, 1415; 1384, 1257	1054; 920
3	3396	2932, 2878	1644	1416; 1257; 1143	1056; 918
4	3409	2933; 2884	1644;	1418; 1257; 1142	1057; 918
5	3413	2934; 2865	1644	1416; 1359, 1256, 1149,	1057; 918
6	3375	2935; 2878	1641	1413; 1346; 1253; 1140	1056; 920
7	3440	2939; 2868	1645	1553; 1452; 1251;1148	1011; 918
8	3417	2936; 2876	1644	1451; 1414; 1250, 1149	1014; 920
9	3440	2921; 2853	1645	1555; 1453; 1239; 1143	1007; 926
10	3444	2920; 2870	1645	1531; 1453; 1227; 1146	1012; 925
11	3442	2923; 2865	1645	1554; 1453; 1234; 1165	1012; 924
12	3440	2925; 2854	1645	1554; 1452; 1385, 1140	1032; 919
13	3417	2925; 2859	1645	1555; 1454; 1241; 1136	1031; 978; 916
14	3415	2930; 2872	1645	1538; 1453, 1245, 1146	1051; 922
15	3504	2934; 2878	1647	1415; 1259; 1146	1083; 919

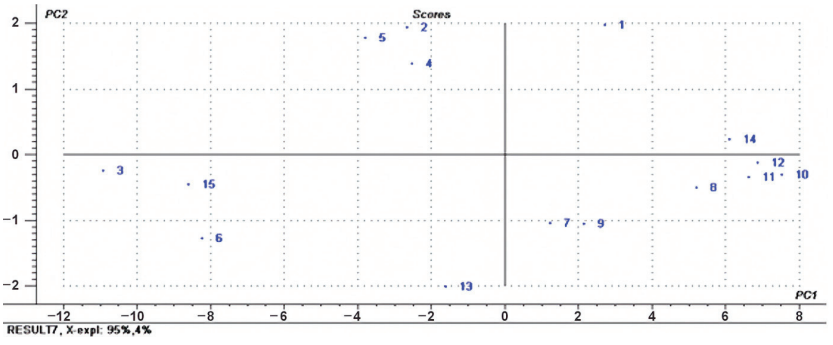


Figure 3. Principal Component Analysis.

3.4. Correlation and Multivariate Statistical Analysis

Table 9 summarizes the correlations between all the determined characteristic parameters of the analyzed honey samples.

Table 9. Pearson's correlation coefficients between the investigated honey parameters (significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).

	mm Pfund	RI	M	TSS	EC	SG	pH	FA	Ash	TPC	TFC	K	Ca	Mg	Na	P	Zn	Cu	Mn
mm Pfund	1.00																		
RI	**−0.21** *	1.00																	
M	**0.23** **	**−1.00** ***	1.00																
TSS	**−0.21** *	**1.00** ***	**−1.00** ***	1.00															
EC	**0.30** ***	0.11	−0.12	0.12	1.00														
SG	**−0.21** *	**1.00** ***	**−1.00** ***	**1.00** ***	0.13	1.00													
pH	**−0.17** *	0.11	−0.12	0.11	**0.67**	0.11	1.00												
FA	**0.45** ***	**0.23** **	**−0.22** *	**0.23** **	**0.18** *	**0.23** **	−0.09	1.00											
Ash	**0.20** *	−0.00	−0.01	0.01	**0.85** ***	0.02	**0.70** ***	0.14	1.00										

Table 9. *Cont.*

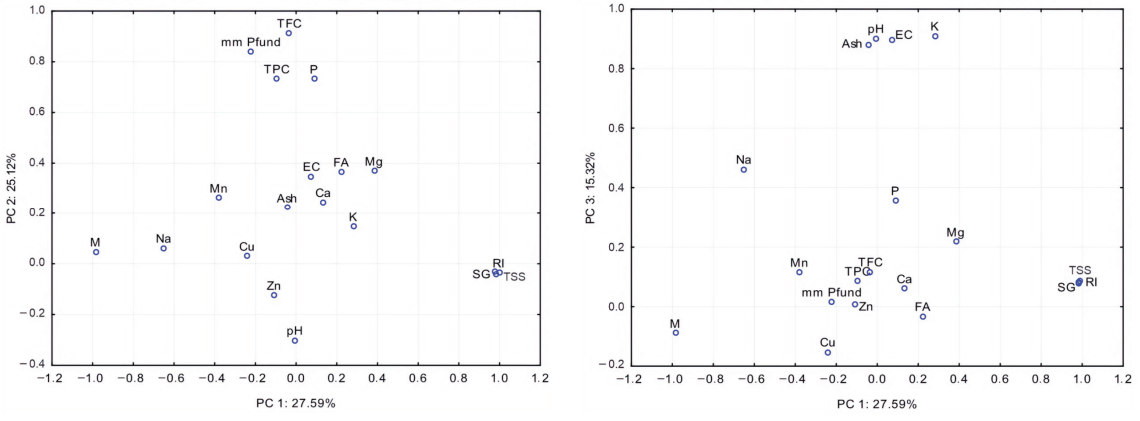
	mm Pfund	RI	M	TSS	EC	SG	pH	FA	Ash	TPC	TFC	K	Ca	Mg	Na	P	Zn	Cu	Mn
TPC	**0.55** ***	**−0.18** *	**0.17** *	**−0.17** *	**0.30** ***	−0.17	**−0.22** **	**0.23** **	**0.34** ***	1.00									
TFC	**0.75** ***	−0.05	0.05	−0.05	**0.40** ***	−0.04	−0.13	**0.27** ***	**0.35** ***	**0.57** ***	1.00								
K	0.07	**0.33** ***	**−0.34** ***	**0.34** ***	**0.89** ***	**0.34** ***	**0.74** ***	0.10	**0.78** ***	**0.22** **	0.17	1.00							
Ca	**0.33** ***	0.12	−0.12	0.12	**0.33** ***	0.13	−0.01	**0.23** **	**0.03**	**−0.21** *	**0.22** *	0.09	1.0000						
Mg	**0.43** ***	**0.37** ***	**−0.36** ***	**0.36** ***	**0.46** ***	**0.36** ***	0.09	**0.60** ***	**0.29** ***	**0.28** ***	**0.33** ***	**0.32** ***	**0.42** ***	1.00					
Na	**0.18** *	**−0.55** ***	**0.55** ***	**−0.55** ***	**0.33** ***	**−0.56** ***	**0.50** ***	**−0.28** ***	**0.28** ***	−0.09	0.14	**0.25** **	0.00	**−0.36** ***	1.00				
P	**0.71** ***	0.08	−0.07	0.08	**0.61** ***	0.09	0.02	**0.46** ***	**0.51** ***	**0.65** ***	**0.64** ***	**0.48** ***	**0.14** *	**0.61** ***	−0.03	1.00			
Zn	**0.27** **	−0.10	0.11	−0.10	0.07	−0.09	0.02	0.06	−0.01	**−0.28**	−0.10	−0.05	**0.56** ***	**0.34** ***	−0.13	0.14	1.00		
Cu	−0.01	**−0.33** ***	**0.33** ***	**−0.33** ***	−0.03	**−0.33** ***	**−0.31** ***	**−0.27** ***	**−0.18** *	−0.07	0.01	−0.12	**0.41** ***	−0.01	−0.02	−0.02	**0.40** ***	1.00	
Mn	**0.33** ***	**−0.40** ***	**0.41** ***	**−0.40** ***	0.21 *	−0.41 ***	−0.08	**0.28** ***	**0.34** ***	**0.51** ***	**0.30** ***	0.02	**−0.30** ***	**0.35** ***	−0.07	**0.55** ***	0.12	0.10	1.00

The values of significant correlation coefficients are marked in bold.

The extracted principal components and the corresponding eigenvalue are shown in Table 10 and Figure 4. Figure 5 shows the hierarchical dendrogram obtained by cluster analysis.

Table 10. Loadings and corresponding variance (%) for the extracted principal components for the analyzed honey samples.

Variable	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
mm Pfund	−0.22	0.84	0.02	0.29	0.03	0.27
RI	0.99	−0.04	0.08	0.00	−0.06	0.10
M	−0.99	0.05	−0.09	0.01	0.07	−0.09
TSS	0.99	−0.04	0.08	0.00	−0.07	0.09
EC	0.08	0.35	0.89	0.17	0.06	−0.04
SG	0.99	−0.04	0.09	0.00	−0.06	0.09
pH	−0.01	−0.30	0.90	0.00	−0.16	0.19
FA	0.22	0.37	−0.03	0.25	0.29	0.68
Ash	−0.04	0.22	0.88	−0.05	0.20	0.05
TPC	−0.10	0.73	0.09	−0.34	0.43	−0.08
TFC	−0.03	0.91	0.12	0.00	−0.05	0.01
K	0.28	0.15	0.91	−0.02	0.03	−0.07
Ca	0.13	0.25	0.06	0.85	−0.31	−0.08
Mg	0.38	0.37	0.22	0.49	0.44	0.26
Na	−0.65	0.06	0.46	−0.09	−0.52	0.05
P	0.09	0.73	0.36	0.13	0.44	0.09
Zn	−0.11	−0.12	0.01	0.87	0.18	−0.01
Cu	−0.24	0.03	−0.15	0.50	0.11	−0.74
Mn	−0.38	0.26	0.12	−0.03	0.83	0.09
Eigenvalue	5.24	4.77	2.91	2.17	1.22	1.01
% Total variance	27.59	25.12	15.32	11.40	6.41	5.30
Cumulative %	27.59	52.71	68.03	79.43	85.84	91.14



(**a**) (**b**)

Figure 4. Graphical representation of scores for (**a**) PC2 versus PC1 and (**b**) PC3 versus PC1 for the analyzed honey samples.

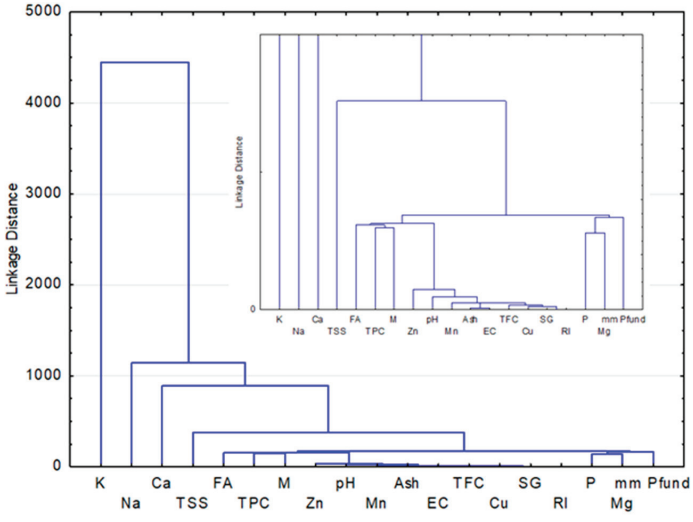


Figure 5. Hierarchical dendrogram obtained by cluster analysis.

4. Discussion

4.1. Physicochemical Determinations

Melissopalynological analysis showed that all honey samples are multifloral. The pollen grain types identified in the honey samples were part of families with various species: *Apiaceae* (*Angelica* sp., *Eryngium* sp.); *Asteraceae* (*Achillea* sp., *Taraxacum* sp., *Helianthus* sp., *Marticaria chamomilla*, *Centaureae* sp.); *Boraginaceae* (*Symphytum* sp.); *Brassicaceae* (*Brassica* sp.); *Cyperaceae* (*Carex* sp.); *Fabaceae* (*Robinia* sp., *Trifolium* sp., *Vicia* sp.m, *Medicago* sp.); *Fagaceae* (*Quercus* sp., *Fagus* sp.); *Lamiaceae* (*Mentha* sp., *Salvia* sp.); *Malvaceae* (*Tillia* sp.); *Plantagigaceae* (*Plantago* sp.); *Poaceae* (*Festuca* sp., *Sorghum* sp., *Zea mays*); *Rosaceae* (*Crataegus* sp., *Pyrus* sp., *Prunus* sp.); *Salicaceae* (*Populus* sp., *Salix* sp.) (Table 1). Secondary pollen (16–45%) was from the following families: *Asteraceae* (S3, S4, S5, S13 from Area 1 and S6, S7, S9, S15 from Area 2); *Brassicaceae* (in all samples); *Fabaceae* (S2, S4, S5 from Area 1 and S15 from Area 2); *Malvaceae* (S1, S13 from Area 1 and S9 from Area 2); *Rosaceae* (S10, S11, S14 from Area 1 and S8, S12 from Area 2). The main pollen in the analyzed samples was from

the *Brassicaceae* family (19.8–35.8%); the pollen grains from other families varied: *Apiaceae* (2.3–14.3%); *Asteraceae* (5.5–33.7%); *Boraginaceae* (2.0–3.1%); *Cyperaceae* (2.3–3.0%); *Fabaceae* (3.7–32.3%); *Fagaceae* (1.0–2.1%); *Laminaceae* (0.5–2%); *Malvaceae* (2.8–29.7%); *Plantagigaceae* (0.5–2.2%); *Poaceae* (0.5–7.2%); *Rosaceae* (1.0–31.9%); *Salicaceae* (1.0–16.8%). There are no criteria for the definition of monofloral honey. Some countries have established national conditions for the amount of pollen in one flower to classify it as monofloral. Minimum percent of pollen for the characterization of monofloral honey is excepted in *Robinia pseudoacacia* (Italy—15%; Germany—20%; Croatia—20%; Serbia—20%; Romania—25%), *Tilia* spp. (Germany—20%; Croatia—25%; Serbia—25%; Romania—30%), and *Helianthus annuus* (Romania—40%) [3,12]. The differences can be seen in the FTIR spectral analysis, where, after examining sample S1, linden honey (*Tillia* sp. = 29.7%) could also be considered monofloral, according to the regulations of other countries. In Table 1, the similarity of plant species in both areas of origin of the analyzed honey samples can be observed.

The color of honey is influenced by various factors, such as water content, HMF content, phenolic compounds, carotenoids, the amount or type of mineral elements, pollen floral types, geographical origin, time, and technological conditions (temperature, processing/handling/storage), etc. [5,33,35–37]. Honey color ranges from nearly colorless to dark brown; thus, the color of honey was classified into seven categories: water white, extra white, white, extra light amber, light amber, amber, and dark amber [32]. The color of multifloral honey samples varied from 14.5 mm Pfund (white) to 69.7 mm Pfund (light amber) in both areas I and II (Tables 2 and 3). Much research found different colors of the same type of honey: color varied from 0.1 mm Pfund in Romanian acacia honey [38] to 20.0 mm Pfund in Serbian acacia honey [35]; Bodor et al. 2021 [36] noted large differences in the color of the Hungarian samples within the same botanical group (linden), from 38.27 mm Pfund to 139.48 mm Pfund. For monofloral honey, in general, it is known that acacia honey is light-colored and chestnut honey is dark-colored; for polyflora honey, the color is given by a large number of pollen types.

Like other food, honey has water in its composition. Shelf life for foods is important because consumers should know when food is safe to be consumed. Increasing the water content can generate the fermentation process and spoilage in honey, raising its susceptibility to microbes; the physico-chemical properties, taste, texture, and aspects of a product change negatively. [22,32,39,40]. The moisture content of multifloral honey samples from area I ranged between 16.4% and 19.3%, and for samples from area II ranged between 16.7% and 19.5% (Tables 2 and 3). These values are below 20%, the maximum limit recommended for honey stipulated in Romanian standards and international regulations [28,41]. Moisture content values under this limit were found in many multifloral honey samples: from 13.91% to 15.80% in honey from Portugal [22]; from 17.11% to 17.93% in multifloral honey from Poland [42]; from 17.4% to 18.4% in multifloral honey from Chile [10]; and from 15.9 to 19.6% [13,37,43] in Romanian multifloral honey. It is known that honey is hygroscopic, and its water content is influenced by factors such as floral origin, geographical location, climatic conditions, level of maturity, the harvest season, and the water from the honey must be constantly checked [16].

The total soluble solids in honey are mainly sugars. Most honey samples had values higher than 80 Brix° of total soluble solids. When the percent of total soluble solids increases, the percent of moisture decreases, and honey has better stability during storage. According to the grading system of the United States Department of Agriculture, when results exceed 80 Brix° (<20% water), honey is qualitative [29]. Four multifloral honey samples (S4, S5, S9, S10) have lower values, from 79.0 Brix° to 79.4 Brix° (Tables 2 and 3).

Honey is heavy, with a mean value of specific gravity of 1.4 g/cm^3. Specific gravity has a practical significance in keeping track of the amount of honey safely stored because it is correlated with moisture content. The mean values of the specific gravity of multifloral honey samples ranged between 1.420 g/cm^3 and 1.441 g/cm^3 (Tables 2 and 3).

Honey has an acidic pH due to the presence of different organic acids (acetic, butyric, citric, formic, gluconic, lactic, malic, pyroglutamic, and succinic). Organic acids are respon-

sible for the flavor and aroma and are important for honey preservation. The lower value of pH in honey inhibits the growth of microorganisms. The majority of honey samples have pH values between 3.5 and 5.5 [13,42,44]. In our study, the mean values of pH were in the 3.58–5.02 range. The values of free acidity obtained in both studied areas ranged between 19.1 meq kg^{-1} and 49.9 meq kg^{-1} (Tables 4 and 5). Similar values were obtained for Romanian honey [13,37,44] and honey from Poland [42,45]. Two samples (S5-area I and S6-area II) with free acidity of 49.9 meq kg^{-1} and 49.7 meq kg^{-1}, respectively, had values close to 50 milliequivalents acid per 1000 g, the maximum allowed value specified by the legislation [Council Directive 2001/110/CE 2002], and honey must be periodically checked if is stored for a longer period.

The average values of ash content were found between 0.070% for the S15 multifloral honey sample and 0.48% for the S2 multifloral honey sample. The amount of all minerals in blossom honey is lower than 0.6; the ash content is variable due to factors such as atmospheric conditions, mineral content in the soil, and the physiology of the plant [16,17]. Due to its strong correlation with ash, the results were easily obtained, and electrical conductivity was included in new standards. The 0.8 mS cm^{-1} is the maximum value established by legislation for blossom honey [41]. Several investigations on honey samples showed similar values to the ones obtained in the present study [42,44–46]. The electrical conductivity showed variable values from 169 mS cm^{-1} (S15) to 736 mS cm^{-1} (S2) (Tables 4 and 5). Analyzing the maximum and minimum values of the ash content with the electrical conductivity values of the multifloral honey samples S2 and S15, a positive correlation can be observed between the two parameters. Ash content and electrical conductivity are parameters that can indicate the botanical origin of bee honey, whether it is blossom honey or not.

From ancient times, honey was known to have therapeutic properties (antioxidant, antibacterial, bacteriostatic). The antioxidants that occur in honey are phenolic acids and flavonoids; quantitatively, the amount of these compounds can largely vary, with a close relation with the type of plant (phenolic compounds are secondary metabolites of plants) and the environment quality traceability [8,18]. The total phenol values of multifloral honey analyzed were found to be between 23.18 mg GAE/100 g and 39.54 mg GAE/100 g. The minimum flavonoid content obtained was 1.28 mg QE/100 g for the S7 sample, and the maximum value for the S5 sample was 39.54 mg QE/100 g (Tables 4 and 5). The average content of total polyphenols and total flavonoids of 29.91 mg GAE/100 g and 2.13 mg QE/100 g confirm the antioxidant properties of multifloral honey samples. High values of total phenol content in the multifloral honey from the Czech Republic were obtained by Halouzka et al., 2016, from 36.3 mg GAE/100 g to 72.3 mg GAE/100 g, and total flavonoid content was 3.54 mg QE/100 g [18]. Multifloral analyzed honey samples from Azerbaijan honey also had a high content of polyphenols, between 18.824 GAE/100 g and 87.350 GAE/100 g [47].

A study on multifloral honey from Northern Romania showed lower values of TPC than the results in this study, between 6.28 mg GAE/100 g and 12.94 mg GAE/100 g [43]. Studies carried out on multifloral honey highlight the influence of the type of plant, region, and quality of the environment on total phenol content: values ranging between 23.69–102.16 mg GAE/100 g were found on multifloral honey from Poland [45]; lower results were obtained by Bertoncelj et al., 2007, of 12.68–19.46 mgGAE/100 g on Slovenian multifloral honey [8]. The results of TPC and TFC in our research are lower compared to the results of total phenol content (350.80–565.90 mg GAE/100 g) and of total flavonoid content (29.01–29.48 mg QE/100 g) of multifloral honey from Banat Region of Romania found by Pătruică et al., 2022 [2]; also, increased values were obtained in the study conducted by Giosanu et al., 2022, on multifloral honey samples from the south of Romania (80.19–170.79 mg GAE/100 g and 3.13–19.64 mg QE/100 g) [15].

4.2. Mineral Elements (K, Ca, Mg, Na, P, Zn, Cu, Mn, Ni, Co, and Pb)

The amount of ash in honey depends on the quality of the environment, the quality of the soil, the physiology of the plants, the climate, etc. Following the well-known soil–plant–nectar–pollen–honey path, it is not only the amount of minerals that is important but also the type of elements with which honey is enriched. There are many minerals in honey, such as macroelements (calcium, potassium, magnesium, sodium) and microelements (iron, manganese, copper, zinc, nickel, lead, and cadmium). Some elements have an important role in the human organism; some other elements are toxic. Potassium, calcium, sodium, magnesium, and phosphorus are the main honey macroelements; microelements such as zinc, copper, manganese, and nickel are present in honey in small amounts and are essential for the normal function of the human body and regulate many biological functions [10,48–50]. The content of potassium ranges from 101.4 mg kg^{-1} in the S15 honey sample to 1212.6 mg kg^{-1} in the S2 honey sample. Similar values were obtained for Romanian honey by Tudoreanu et al., 2012, and Barbeș et al., 2021 [48,50]. The elements obtained in this study ranged between 41.8 mg kg^{-1} and 230.9 mg kg^{-1} for Ca, 32.7 mg kg^{-1} and 65.5 mg kg^{-1} for Mg, 40.7 mg kg^{-1} and 302.3 mg kg^{-1} for Na, 28.6 mg kg^{-1} and 85.5 mg kg^{-1} for P, 0.76 mg kg^{-1} and 13.66 mg kg^{-1} for Zn, 0.76 mg kg^{-1} and 2.18 mg kg^{-1} for Cu, and between 0.27 mg kg^{-1} and 4.31 mg kg^{-1} for Mn (Tables 6 and 7). The higher concentrations of potassium and sodium found in the multifloral honey samples from area I could be explained by the presence of plants on soils rich in potassium, sodium, and salts in Iasi County [51].

In this study, the concentration of some elements was determined: cobalt and nickel are elements that occur naturally in small quantities in the environment but can cause negative health effects (allergenic potential, lung inflammation). Lead is a toxic heavy metal with no physiological role in the human body [46,52]. In all multifloral honey samples, cobalt was below the limit of detection. The maximum limit for Pb in honey is 0.1 mg kg^{-1}, which was established by the European Commission [EU 2023/915]. Values below the legal limits of 0.01 mg kg^{-1} and 0.05 mg kg^{-1} were recorded for Pb in the S9 and S7 multifloral samples, and in one sample (S7), Ni of 0.1 mg kg^{-1} was found. The absence or very low content of toxic elements below the limit of detection indicates that the sources of honey were not contaminated.

Many studies have been performed related to the presence of mineral elements in honey samples collected from polluted and intensively industrialized areas, as well as honey collected from unpolluted areas (Zn values were between 0.004 mg kg^{-1} and 36.40 mg kg^{-1}; Cu values were between LOD and 33.00 mg kg^{-1}, and Pb content ranged was between LOD and 3.41 mg kg^{-1}). The results lead to the same conclusion: all elements of the environment (water, air, soil) positively or negatively influence the quality of the product [2,10,13,42,46,48–50,53–59].

4.3. FTIR Spectra

Much research on honey has shown that the obtained spectra can be studied by dividing them into band domains, depending on the vibration of the functional groups [60,61]. The FTIR spectra for the analyzed honey samples show a number of common characteristics but also a number of differences. In the 4000–3500 cm^{-1} range, a series of sharp bands specific to O–H valence vibrations corresponds to O–H of carbohydrates, and O–H stretching (carboxylic acids) appears. In the D1 range, 3500–3100 cm^{-1}, a broad band appears, specific to water molecules (O–H stretching from water) in the samples, but also some small shoulders/inflections that can be attributed to N–H stretching vibration (amide A band) of the peptides and proteins and polyphenols. D2 domain range, 3000–2800 cm^{-1}, is assigned to C–H stretching (carbohydrates), symmetric and antisymmetric. The asymmetric band appears around 2930 cm^{-1}, while the symmetric band is much weaker and appears around 2870 cm^{-1} (the presence of bands between 2940 and 2850 cm^{-1} corresponds to asymmetric and symmetric stretching vibrations of the C–H bonds of the chemical structure of the carbohydrates). Bands between 2200 cm^{-1} and 2100 cm^{-1} can be assigned to C=C conjugated

and C≡C. In the D3 domain, 1700–1600 cm^{-1}, an intense band appears, centered around 1645 cm^{-1}, specific to C=O stretching (mainly from carbohydrates) valence vibrations. In the same interval, a series of weaker bands specific to O–H stretching/bending vibrations (water), N–H bending of amide I (mainly proteins), and C=C related to phenolic molecules can appear. D4, 1540–1175 cm^{-1}, is assigned to O–H stretching/bending, C–O stretching (carbohydrates), C–H stretching (carbohydrates), and C=O stretching of ketones. The bands at 1450 cm^{-1} and 1454 cm^{-1} correspond to the bending vibration of the O–CH and C–C–H bonds of the carbohydrates. The peak in the spectral range of 1340–1350 cm^{-1} and 1255–1259 cm^{-1} is characteristic of the O–H bending vibration of the C–OH group. The peaks corresponding to N–H deformation and C–N stretching vibrations from amide II and C–N amide III bands overlap. The peaks within the range 1165 cm^{-1}–1136 cm^{-1} correspond to C–H in carbohydrates and/or C–O and C–C in carbohydrates. D5, 1100–900 cm^{-1} is assigned to C–O, C–C stretching (carbohydrates), and ring vibrations (mainly from carbohydrates). The range between 900 cm^{-1} and 600 cm^{-1} is assigned to the anomeric part of carbohydrates, C–H bending (from carbohydrates), and ring vibrations (from carbohydrates) specific to honey. The main bands for the analyzed samples are presented in Table 8. The principal component analysis, PC1, PC2, presented in Figure 3 in the range 4000–400 cm^{-1}, indicates that the samples are different from each other, but similarities may appear between some samples. The distribution on the dials shows the changes that occur in the fingerprint characteristic field. In addition, it can be observed that sample S1 is totally different from the other samples. Samples S2, S4, and S5 are similar as are samples S3, S6, S15 and S7, S8, S9, S10, S11, S12, and S14. Sample S13 shows characteristics between the last two groups of samples. The spectral domains that contribute to the differentiation of honey samples can also be seen very well from the Matrix plot shown in Figure 2 (domain 4000–400 cm^{-1}). Results from other studies showed the variability of honey compounds: the characteristic peaks obtained by Mail et al., 2019 were as follows: 3272 cm^{-1}; 2934 cm^{-1}; 1643 cm^{-1}; 1416 cm^{-1}; 1345 cm^{-1}; 1256 cm^{-1}; and 1026 cm^{-1}; the characteristic peaks obtained by Aykas, 2023 were as follows: 3285 cm^{-1}; 2930 cm^{-1}; 1637 cm^{-1}; 1411 cm^{-1}; 1321 cm^{-1}; 1254 cm^{-1}; 1110 cm^{-1}; 1043 cm^{-1}; and 918 cm^{-1}; the characteristic peaks obtained by Giosanu et al., 2022 were as follows: 3233 cm^{-1}; 2935 cm^{-1}; 1646.9 cm^{-1}; 1418 cm^{-1}; 1338 cm^{-1}; 1247 cm^{-1}; 1151 cm^{-1}; 1043 cm^{-1}; and 918 cm^{-1} [15,24,62].

4.4. Correlation and Multivariate Statistical Analysis

Pearson correlation coefficients between honey parameters and the extracted PC are shown in Tables 9 and 10. A moderate correlation was observed between TPC and TFC (0.57) and between mmPfund and FA, TPC, and Mg (0.45, 0.55, 0.43), and a strong correlation (r = 0.75) was found for mmPfund with TFC and for mmPfund with P (r = 0.71). Strong correlations ($p < 0.001$) are between EC with Ash (r = 0.85), TPC, and K (r = 0.89) with P (r = 0.61). There are positive moderate correlations between the following minerals: P with Mg (r = 0.61); P with K (r = 0.48); Ca with Zn (r = 0.56); and P with Mn (r = 0.55). The research on the correlations between the quality parameters of honey has reported similar correlations both between different types of honey and within the same type of honey. Lanjwani et al., 2019, reported a good correlation between macrominerals Na, K, Mg, and Ca for honey samples from Pakistan [49]; the correlation between the color of honey and the content of mineral salts was reported by Karabagias et al., 2014 [63]; the correlation between the color of honey and the antioxidant compounds was found by Bertoncelj et al., 2007 [8]. Similar low correlations between TPC and TFC were found by Uçar et al., 2023 (−0.3418) in multifloral honey samples from Northern Cyprus, Sant'Ana et al., 2014 (0.5) in Brazilian honey samples from Northern Cyprus, and Cabrera et al., 2017 (0.45) in Argentinian honey samples [64–66]. Low values of Pearson coefficients of correlation between mmPfund and antioxidant compounds (TPC) were also reported for Algerian honey (0.693), for Irish honey (0.6), for honey from Brazil (0.4), and a correlation of 0.53 for Argentinian honey samples [65–68]. A strong correlation of 0.711 was found between color and TPC by Daci-Ajvazi et al., 2017 for multifloral honey from Kosovo [69]. Lower values of Pearson

coefficients of correlation between mmPfund and antioxidant compounds (TFC) of 0.6 and 0.685 were observed for Brazilian and Australian honeys, respectively [65,70]. Similar values of Pearson coefficients to those found in this research (0.78) were also obtained by Cabrera et al., 2017, from Argentinian honey samples and Živković et al., 2019 (0.771) from honey samples from Serbia [35,66].

The hierarchical dendrogram obtained for physicochemical parameters determined in multifloral honey samples collected in 2017 is shown in Figure 5 and indicates that K is clearly differentiated from the other parameters, similar to Na and Ca. One cluster contains the mineral elements P and Mg. Cu and SG are grouped in a cluster, and electrical conductivity and ash are grouped in a distinct cluster.

5. Conclusions

Melissopalynological analysis confirms the multifloral characteristics of the studied samples of honey.

All the quality indicators determined in this study were within the limits stipulated by the legislation. The multifloral honey samples have antioxidant potential through the amount of phenols and flavonoids, and the presence of these antioxidants confirms its therapeutic character.

Determination of minerals showed that potassium is the most abundant mineral in honey, followed by calcium and sodium. The high values of K and Na reflect the amount of these minerals in the soil. The presence of macro and microminerals ensures honey has a place in the food list.

The limited number of samples with Pb content over the detection limit indicates the existence of few possible pollution sources. The lead content value was below the limit recommended by legislation.

The use of the FTIR spectral method confirms the difference between the investigated samples by analyzing the pollen and could also highlight the differences in the chemical composition of honey.

This study confirms the close connection between the composition of honey and the quality of the environmental elements.

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Effect of Gender and Muscle Type on Fatty Acid Profile, Sanogenic Indices, and Instrumental and Sensory Analysis of Flemish Giant Rabbit Meat

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Abstract: The aim of this study was to represent quality characterization, by gender and muscle type, of rabbit meat from the Flemish Giant (FG) breed, following the fatty acid profile, sanogenic indices, and instrumental (color and texture) and sensory analysis. The biological material comprised 40 rabbits (20 females and 20 males) whose *Longissimus dorsi* (LD) and *Semimembranosus* (SM) muscles were sampled. Compared to female samples, the meat from males was more qualitative in terms of higher ratios of polyunsaturated vs. saturated fatty acids and proportions (+42%) of Essential and Desirable Fatty Acids (+21.6% EFA; +6.7% DFA). Also, the Atherogenic Index (AI) and Thrombogenic Index (TI) were better in males (−37.1% AI; −34.3% TI), as were the ratio of hypocholesterolemic/Hypercholesterolemic fatty acids (+27.8%) and the Nutritive Value Index (NVI, +11.6%). The Polyunsaturation Index (PI) was higher for females (+57.5%), with the widest differences in hind leg muscles (SM muscles), while the omega-6/omega-3 fatty acid ratio was also better (+11.3%). Female meat was more tender due to lower shear force (−6.2%... 9.3%) in both muscles. Female meat was less pigmented than that of males, while the overall sensory attributes were better scored in male samples (+3.1%... +7.1%) ($p < 0.01$). The meat of males proved to be more sanogenic (richer in EFA and DFA, with a better h/H ratio and NVI, while AI and TI were lower). We would recommend slaughtering 3–4 weeks earlier in females vs. males to avoid excessive fat deposition and, consequently, the development of unfavorable sanogenic indices for consumer health.

Keywords: Flemish Giant breed rabbit; meat; fatty acids; instrumental and sensory analysis

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1. Introduction

The worldwide food crisis, the shortage of natural resources and climate change, the desertification of agricultural land, and the population increase—both in number and life expectancy—have led to the urgent need to find new sources of proteins with high biological value. The diseases associated with an abundant and unhealthy diet lead to metabolic syndromes, mostly in developed or developing countries, characterized by the following: hypercholesterolemia, type 2 diabetes, hyperuricemia/gout, and obesity. Rabbit meat can be a handy solution for solving food security problems (access to qualitative, healthy, and sufficient food). Rabbit meat meets the needs of consumers as a functional food [1] (rich in proteins balanced in essential amino acids, polyunsaturated fatty acids (PUFA), n-3 and n-6 fatty acids, easily bioavailable vitamins, and minerals) [1–6] for people who are increasingly concerned about health and nutrition.

Rabbit meat provides multiple advantages for consumer health: absence of uric acid; small amounts of saturated fatty acids (SFA) and cholesterol [7–14]; high content of monounsaturated fatty acids (MUFA) and PUFA, with a protective role against cardiovascular diseases; and low cholesterol values [15,16]. Rabbit meat is highly appreciated for its excellent nutritional features, with lower fat, fewer saturated fats, and lower cholesterol levels compared to other commonly consumed meats [1–14,17]. Rabbit meat is lower in fat (9.2 g/100 g) and cholesterol (56.4 mg/100 g) compared to chicken, beef, and pork [7,17]. Rabbits show a great variation in breed size, from dwarf (1 kg adult weight) to giant lines (7–8 kg adult weight). Out of the wide variety of rabbit breeds, commercial production uses average-sized breeds for reproduction due to their high prolificacy and large breeds as terminal sires due to their high growth rate. This lowers the maintenance cost, allowing the production of commercial rabbits with a high growth rate [18]. At the international level, the quality of rabbit meat from medium breeds is well documented [6,8,10–13,19,20]. Dietary supplementation of feed with various non-conventional ingredients seems to influence the final quality of the meat [21–29]. As far as we know, there are very few studies on large meat breeds. The quality (including chemical composition) of meat from Flemish Giant (FG) rabbits remains insufficiently investigated [30]. Although FG is the largest breed of rabbit (10–12 kg live weight as an adult), its farming for meat production is not a tradition. Usually, in the northeast of Romania, this breed is bred for participation in exhibitions, with a spectacular appearance and size. Unfortunately, raising rabbits is no longer a habit in traditional Romanian households, as it was during the communist period, and the consumption of their meat has severely declined throughout the last 15–20 years. The use of agroindustry by-products in rabbit diets allows the use of residues that are not fit for human consumption [3]. Also, rabbits are one of the most efficient cellulose-converter animal species, ensuring the high production of low-cost meat [31]. The fact that rabbits can be raised with grass, alfalfa, clover, and food scraps (such as cabbage spines, carrot peels, pieces of dry bread, etc.) could ease/facilitate meat production in a sustainable, circular, and inexpensive way in small households. Some studies also show the advantages of raising rabbits for meat in maintaining food security, especially in armed conflicts [32]. Within such a context, our study aimed to highlight the quality of meat (characterization by gender and muscle type) in the largest rabbit breed—Giant Flemish—as a potentially sustainable, valuable food resource in terms of dietary quality (palatable, suitable for diverse culinary uses, nutritious, healthy, and sanogenic) by approaching several measurable/scalable traits: the fatty acid profile, sanogenic indices, and the instrumental (color and texture of meat) and sensory analysis.

2. Materials and Methods

2.1. Meat

The biological material was composed of 40 rabbits from the Flemish Giant breed (20 females and 20 males), slaughtered at 10 months old (via electrical stunning, respecting animal welfare and the specific technological stages), with average carcass weights of 9.45 kg. They were reared outdoors for farming purposes in a roofed pavilion with two individuals in wire mesh cages with a wood-slatted floor (length 140 cm × width 90 cm × height 70 cm), resulting in 0.63 sqm of floor surface per individual. Rabbits were fed, ad libitum, a complete commercial pelleted diet (crude protein, 16.90%; fat, 2.89%; crude fiber, 17.00%; ash, 7.57%; methionine, 0.30%; lysine, 0.65%; sodium, 0.16%; phosphorus, 0.77%; calcium, 1.00%; and Vitamin E, 45 mg/kg). The *Longissimus dorsi* (LD) and *Semimembranosus* (SM) muscles were used as actual biological material. The sampling was carried out right after slaughter, and the samples were kept at a temperature of 2–3 °C. The muscles were chosen due to their different physical–chemical properties, the different metabolic types, and to cover the main anatomical regions of the carcasses (episome—LD and hind leg—SM). The muscles from one half of each carcass were used for fatty acid analysis, and the ones from the other half were used for sensory and textural assessments. The color of the meat was measured on both sides of the carcass before sampling.

2.2. The Fatty Acids Content

To investigate the fatty acids profile, each analyzed muscle was sampled from the carcass and minced to homogenize the sample for analysis. The assessment of fatty acids content was performed using the FOSS 6500 NIR spectrophotometer (manufacturer FOSS Co., Denmark). Freshly ground samples were placed in sterile Petri dishes, weighed (90 ± 5 g), then lyophilized at −110 °C for 24 h, using the CoolSafe ScanVac freeze dryer (manufacturer LaboGene co., Denmark), weighed again (25 ± 1.5 g), then vacuumed and stored in a freezer at a temperature of −80 °C until their analysis. There were assessed the following saturated fatty acids (SFA): C14:0 (myristic acid), C15:0 (pentadecanoic acid), C16:0 (palmitic acid), C17:0 (heptadecanoic acid) and C18:0 (stearic acid). Among the monounsaturated fatty acids (MUFA, ω7, and ω9) there were analyzed: 16:1n-7 (palmitoleic acid, n-7 fatty acid), C18:1n-7 (vaccenic acid cis isomer of oleic acid) and C18:1n-9 (oleic acid); a total of nine polyunsaturated fatty acids (PUFA, ω3 and ω6) were also assessed: C18:2n-6 (linoleic), C18:3n-3 (linolenic/ALA), C20:2n-6 (eicosadienoic), C20:3n-6 (eicosatrienoic), C20:4n-6 (arachidonic/AA), C20:5n-3 (eicosapentaenoic/EPA), C22:4n-6 (docosatetraenoic), C22:5n-3 (docosapentaenoic/DPA) and C22:6n-3 (docosahexaenoic/DHA).

2.3. Health Lipid Indices Calculation

Rabbit meat sanogenic quality was assessed by calculating the amounts of SFA, MUFA, PUFA, the desirable fatty acids (DFA) (DFA = 18:0 + MUFA + PUFA), and the essential fatty acids (EFA) (EFA = C18:2n-6 + C18:3n-3 + C20:4n-6) according to Chen et al. [33]; the Polyunsaturation Index (PI) was calculated as described by Timmons [34]; the atherogenic (AI) and thrombogenic (TI) indices calculation was performed according to Ulbricht and Southgate [35]; the ratio between the hypocholesterolemic and Hypercholesterolemic fatty acids (h/H) was obtained according to Fernandez et al., adapted [36–38]; the Nutritive Value Index (NVI) [4] and the desirable fatty acids (DFA) after Wereńska et al. [39] using Equations (1)–(6):

$$PI = C18{:}2n\text{-}6 + (C18{:}3n\text{-}3 \times 2); \quad (1)$$

$$AI = [(4 \times C14{:}0) + C16{:}0 + C18{:}0]/MUFA + PUFA\ n\text{-}6 + PUFA\ n\text{-}3; \quad (2)$$

$$TI = (14{:}0 + 16{:}0 + 18{:}0)/[(0.5 \times MUFA) + (0.5 \times n\text{-}6\ PUFA) + (3 \times n\text{-}3\ PUFA) + (n\text{-}3\ PUFA/n\text{-}6\ PUFA)]; \quad (3)$$

$$h/H = (C18{:}1 + PUFA)/(C14{:}0 + C16{:}0); \quad (4)$$

$$NVI = (C18{:}0 + C18{:}1)/C16{:}0; \quad (5)$$

$$DFA = \sum MUFA + \sum PUFA + C18{:}0. \quad (6)$$

2.4. Instrumental and Sensory Analysis

For textural measurements, the muscles were individually packaged, vacuumed, and sealed on a vacuum device, then cooked for one hour at a constant temperature of 80 °C in a water bath, with a slight and continuous stirring of the content. Afterward, the samples were cooled to room temperature (20 ± 2 °C) for 30 mins and then kept at 4 °C for more than 30 mins prior to analysis. Between 4 (SD muscles) and 6 prisms (LD muscles) of 1 cm height × 1 cm width × 2 cm length were shaped, with the muscle fibers parallel on the longitudinal axis of the prism. The texture measurement was performed in a Warner–Bratzler cell applied to a Model TA-XT2I texturometer (Stable Micro System, Surrey, UK). The samples were sectioned perpendicularly to the direction of the muscle fibers with a cutting speed of 5 mm/second. The measured parameters were the total shear force (kg/cm^2), firmness ($kg/s \times cm^2$), and area ($kg \times s/cm^2$).

The color measurement of rabbit meat was performed on the Minolta CR3000 portable tristimulus colorimeter via the CIELAB system. Any color can be expressed as a mix of 3 primary colors: red, yellow, and blue. CIELAB is a coordinate system where L = brightness coordinate (gray tones), a = red index, and b = yellow index. The axis for red and green is projected for the term a*. When the value is positive, it shows the direction of the deviation of the yellow tone. When the value is negative, it shows the deviation from the direction of the blue tone. The brightness scale is placed in the center, perpendicularly to the a* and b* axes. Samples of 1.5–2.5 cm thickness were used to avoid the light passage through them and the possible errors that might occur. The samples were exposed to air for 30–40 mins to oxygenate for a more effective reading. The CIELAB system uses two light sources—C, which is equivalent to daylight, and D65, which includes part of the ultraviolet. The calibration was performed so the L, a*, and b* readings fit the values of the standardized palette (L = 98.19; a* = 0.0; b* = 1.93). After each reading, the surface of the device that came into contact with the analyzed pieces of meat was cleaned with ethyl alcohol to avoid possible errors that might occur.

Sensory analysis of rabbit meat was performed after the samples were cooled. They were cut in the same manner and piece sizing and randomly assigned to 23 tasters, trained in advance. The panelists rinsed their mouths with water between the sampling of the meat. The room temperature was 21–22 °C, and ambiental white light was used. The assessment sheets of the sensory characteristics, adapted after Ariño et al., 2007 [40], were filled in using a five-point hedonic scale (scores from 1 to 5), in which 1 represented the not favorable features, while 5 points indicated the characteristics which fully satisfied the tasters (Table 1). For example, the extremely pale color was noted with 1 point, while the intense pink color was noted with 5 points; the global assessment was scored using 1 point for unacceptable meat, 2 points for acceptable meat, 3 points for good meat, 4 points for very good meat and 5 points for exceptional meat.

Table 1. Five-point hedonic scale for sensory characteristics of FG rabbit meat.

Sensory Parameters	Granted Scoring (Points)				
	1	**2**	**3**	**4**	**5**
Color	Extremely Pale	Pale	Pale Pink	Pink	Intense Pink
Fibrous appearance	Weakly highlighted	Lightly highlighted	Medium highlighted	Distinctly highlighted	Strongly highlighted
Smell/ odor	Imperceptible	Weakly perceptible	Medium perceptible	Distinct perceptible	Very perceptible
Taste	Slightly unpleasant	No taste	Tasty enough	Tasty	Very tasty
Flavor	Slightly unpleasant	No flavor	Pleasant	Very pleasant	Extremely pleasant
Intensity of the flavor	Undetectable	Poor	Sufficiently pleasant	Pleasant and strong	Intense pleasant
Juiciness	Dry	Insufficiently juicy	Sufficiently juicy	Juicy	Very juicy
Tenderness	Very stiff	Slightly stiff	Sufficiently soft	Soft	Very soft
Overall assessment	Unacceptable	Acceptable	Good	Very good	Exceptional

2.5. Data Analysis

The results were statistically processed to compute the main descriptors (Mean, SEM—standard error of the mean, CV—coefficient of variation). Data acquired from males and females were compared to assess the amplitude and significance of gender-related differences using the Student's (t) test incorporated within the GraphPad Prism 9.4.1. software (GraphPad Software, Boston, MA, USA).

3. Results

3.1. The Fatty Acids Content and Sanogenic Indices of Flemish Giant Rabbit Meat

Table 2 presents the fatty acids content in LD and SM muscles sampled from rabbits.

Table 2. The fatty acids content (mg/100 g) of Flemish Giant rabbit meat.

Fatty Acids		M/F	LD Mean	LD SEM	LD CV%	*p*-Value	SM Mean	SM SEM	SM CV%	*p*-Value
SFA	C14:0	M	21.12	0.76	16.1	0.035	18.87	0.81	19.15	1.367×10^{-5}
		F	38.63	1.37	15.9	n.s	56.95	1.92	15.11	***
	C15:0	M	3.97	0.13	15.1	0.046	4.97	0.15	13.16	1.525×10^{-6}
		F	7.02	0.15	9.3	n.s	11.02	0.23	9.49	***
	C16:0	M	220.11	3.91	7.95	0.057	272.32	9.16	15.04	6.317×10^{-5}
		F	409.37	5.14	5.61	n.s	614.17	23.48	17.1	***
	C17:0	M	4.91	0.22	19.8	0.021	8.21	0.20	11.08	2.127×10^{-7}
		F	7.96	0.14	7.67	*	13.04	0.12	4.12	***
	C18:0	M	71.49	0.98	6.1	0.037	82.13	1.67	9.07	2.213×10^{-6}
		F	95.58	1.45	6.78	*	141.02	2.64	8.36	***
MUFA	C16:1n-7	M	21.03	0.26	5.51	0.798	28.07	1.26	20.04	1.288×10^{-5}
		F	62.11	0.81	5.82	n.s.	106.04	4.30	18.15	***
	C18:1n-7	M	14.81	0.19	5.61	0.078	15.98	0.39	11.05	1.233×10^{-6}
		F	19.97	0.28	6.16	n.s.	34.16	0.69	9.08	***
	C18:1n-9	M	208.16	5.84	12.54	0.037	270.99	10.34	17.07	8.462×10^{-7}
		F	358.97	4.62	5.76	*	593.17	12.14	9.15	***
PUFA	C18:2n-6	M	180.92	6.24	15.43	0.043	243.19	7.00	12.87	5.235×10^{-6}
		F	256.83	4.01	6.98	*	394.02	6.29	7.14	***
	C18:3n-3	M	14.11	0.20	6.42	0.074	21.1	0.98	20.71	6.159×10^{-6}
		F	23.32	0.58	11.08	n.s	40.02	0.82	9.16	***
	C20:2n-6	M	2.92	0.06	9.54	0.294	3.19	0.06	7.82	1.213×10^{-4}
		F	3.37	0.06	8.26	n.s	5.07	0.11	9.67	***
	C20:3n-6	M	3.89	0.05	5.63	0.081	5.34	0.09	7.16	0.036
		F	3.51	0.04	5.29	n.s	5.11	0.07	6.27	*
	C20:4n-6	M	50.97	0.82	7.19	0.018	49.38	0.90	8.13	0.017
		F	54.93	1.77	14.4	*	55.42	0.84	6.79	*
	C20:5n-3	M	11.17	0.27	10.81	0.197	12.33	0.25	9.17	0.272
		F	8.98	0.25	12.22	n.s.	11.57	0.35	13.38	n.s.
	C22:4n-6	M	15.06	0.22	6.64	0.007	16.14	0.27	7.42	1.707^{-5}
		F	13.95	0.21	6.81	**	15.2	0.21	6.18	***
	C22:5n-3	M	7.79	0.18	10.33	0.637	7.11	0.11	7.06	0.035
		F	9.66	0.31	14.35	n.s	7.92	0.21	11.99	*
	C22:6n-3	M	24.11	0.56	10.38	0.923	22.06	0.48	9.73	0.223
		F	24.69	0.67	12.19	n.s	25.09	0.65	11.51	n.s

LD—*Longissimus dorsi*; SM—*Semimembranosus*, SEM—standard error of mean, CV—coefficient of variation; Student test: ns = not significant, $p > 0.05$; * significant for $p < 0.05$; ** significant for $p < 0.01$; *** significant for $p < 0.001$.

The highest amount of SFA was found in female SM muscles, out of which palmitic acid/C16:0 (614.17 mg/100 g) was the most present. In the MUFA category, the oleic acid/C18:1n-9 (593.17 mg/100 g) was better represented. The most occurring PUFA was the C18:2n-6 (394.02 mg/100 g)(compared to 243.18 mg/100 g in males), followed by the C20:4n-6 (55.42 mg/100 g), with closer values in males (49.38 mg/100 g). In LD muscles, C20:4n-6 reached 54.93 mg/100 g in females vs. 50.97 mg/100 g in males.

For all analyzed fatty acids, higher values were measured in females, especially in the SM muscles, which were richer in fat than in the LD muscles.

The statistical results predominantly highlighted significant differences between genders in SM muscles ($p < 0.001$), compared with LD muscles, where the values were significantly ($p < 0.05$) or even insignificantly different ($p > 0.05$), suggesting the predisposition of females to accumulate more fat in hind limbs, in comparison with the episome.

The sums of several categories of fatty acids and the sanogenic indices are presented in Table 3.

Table 3. Total fatty acids (mg/100 g meat) and sanogenic indices in FG rabbit meat.

Sanogenic Indices	Gender	LD	SM	Mean/Gender	Mean/Breed
Total SFA	M	321.60	386.50	354.05	525.72
	F	558.56	836.20	697.38	
Total MUFA	M	244.01	315.04	279.53	433.37
	F	441.05	733.37	587.21	
Total PUFA	M	310.94	379.84	345.39	412.36
	F	399.24	559.42	479.33	
ΣPUFA/ΣSFA	M	0.97	0.98	0.98	0.84
	F	0.71	0.67	0.69	
ΣPUFA n-6	M	253.76	317.24	285.50	344.61
	F	332.59	474.82	403.71	
ΣPUFA n-3	M	57.18	62.60	59.89	67.76
	F	66.65	84.60	75.63	
Σn6/n3	M	4.44	5.07	4.76	5.03
	F	4.99	5.61	5.30	
EFA	M	246.00	313.67	279.84	346.06
	F	335.08	489.46	412.27	
%EFA	M	28.06	29.01	28.54	26.01
	F	23.95	22.99	23.47	
DFA	M	626.43	777.01	701.72	943.28
	F	935.87	1433.81	1184.84	
%DFA	M	71.47	71.85	71.66	69.40
	F	66.90	67.35	67.13	
NVI	M	1.34	1.36	1.35	1.28
	F	1.16	1.25	1.21	
AI	M	0.31	0.38	0.35	0.42
	F	0.41	0.56	0.48	
TI	M	0.33	0.38	0.35	0.41
	F	0.40	0.54	0.47	
h/H	M	2.21	2.29	2.25	2.01
	F	1.74	1.77	1.76	
PI	M	2.09	2.85	2.47	3.18
	F	3.03	4.74	3.89	
Total fatty acids	M	876.54	1081.38	978.96	1371.44
	F	1398.85	2128.99	1763.92	

LD—*Longissimus dorsi*; SM—*Semimembranosus*; SFA = Saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; EFA = essential fatty acids; %EFA= EFA × 100/Σ Total fatty acids; DFA = desirable fatty acids; %DFA = DFA × 100/Σ Total fatty acids; AI =Atherogenic Index; TI = Thrombogenic Index; h/H =Ratio between the hypocholesterolemic and Hypercholesterolemic fatty acids; PI = Polyunsaturation Index; NVI = Nutritive Value Index.

The sum of SFA was higher in females for both muscles, with more than double the value in SM muscles (836.2 mg/100 g) compared to males (386.5 mg/100 g).

The sum of MUFA (mg/100 g) was higher in females vs. males, as average for both muscles (587.21 vs. 279.53) or in SM muscle (733.37 vs. 315.04) and LD muscles (441.05 vs. 244.01 in males). The sum of PUFA was also higher (on average) in females compared to males (479.33 vs. 345.39).

The total fatty acids were higher in females than in males, on average, for both muscles (1763.92 vs. 978.96 mg/100 g), as well as in SM muscles (2128.99 mg/100 g vs. 1081.38 mg/100 g) and LD muscles (1398.85 mg/100 g in females vs. 876.54 mg/100 g in males).

The Σ PUFA/Σ SFA ratio was higher in males (0.97/1 vs. 0.71/1 in LD and 0.98/1 vs. 0.67/1 in SM; 0.98/1 vs. 0.69/1, on average, for both muscles).

On the contrary, the Σn6/n3 ratio was higher in females (4.99/1 vs. 4.44/1 in LD and 5.61 vs. 5.07 in SM), with average values of 5.30/1 in females and 4.76/1 in males.

AI was higher in females (0.41 vs. 0.31 in LD muscles and 0.56 vs. 0.38 in SM muscles), while the TI also had higher values in females for both muscles (0.33 vs. 0.40 for LD

muscles and 0.38 vs. 0.54 for SM muscles). On average, the values of AI and TI were almost similar (0.35 for vs. 0.48 for AI, respectively, 0.35 vs. 0.47 for TI), depicting the healthier fat composition in male meat compared to females that had higher atherogenicity and thrombogenicity potential.

The h/H indices were lower for females for both muscle groups (1.74/1 vs. 2.21/1 in male LD samples and 1.77 vs. 2.29/1 in male SM samples). The average values (both muscles calculated together) revealed a lower h/H ratio in females, as well (1.76 vs. 2.25 in males), suggesting a stronger hypocholesteremic effect when consumers opt out of male meat in the diet.

The NVI was also smaller in females (1.16 vs. 1.34 in males for LD; 1.25 vs. 1.36 in males for SM). The average values varied between 1.21 (females) and 1.35 (males).

The DFA (mg/100 g) was higher in females (935.87 vs. 626.43 in males for LD and 1433.81 vs. 777.01 in males for SM), with averages of 1184.84 (females) vs. 701. 72 (males). However, when the %DFA was calculated, better proportions were found in males vs. females (71.47 vs. 66.90 in LD muscles and 71.85 vs. 67.35 in SM muscles).

The absolute value of EFA (mg/100 g) was higher in females vs. males (412.27 vs. 279.84), while the relative proportion (%EFA) was higher in males (28.06% in LD and 29.01% in SM muscles) compared with the females (23.95% in LD and 22.99% in SM muscles).

On the other hand, PI was better in females (on average 3.89 vs. 2.47 in males), with wider differences (almost double values) in SM muscles (4.74 vs. 2.85 in males).

3.2. The Instrumental Assessment of Rabbit Meat

3.2.1. The Texture Parameters of Rabbit Meat

The texture analysis data (maximum strength, firmness (consistency)) and the total mechanical work performed to cut the sample (the area under the obtained curve) by applying the Warner–Bratzler test for both muscle groups from rabbits are presented in Table 4.

Table 4. The texture parameters of FG rabbit meat.

Muscles	Parameters	Gender	Mean ± SEM	CV%	*p*-Value
L.D.	Shear force (kg/cm^2)	M	5.62 ± 0.15	11.97	0.0342
		F	5.14 ± 0.15	12.65	*
	Firmness (kg/s × cm^2)	M	2.71 ±0.10	17.14	0.0011
		F	2.11 ± 0.07	14.98	**
	Area (kg × s/cm^2)	M	10.89 ± 0.27	10.95	0.1003
		F	9.49 ± 0.34	15.82	ns
S.M.	Shear force (kg/cm^2)	M	5.48 ± 0.26	20.99	0.4017
		F	5.16 ± 0.22	18.74	ns
	Firmness (kg/s × cm^2)	M	2.37 ± 0.07	12.96	0.2030
		F	2.19 ± 0.08	16.31	ns
	Area (kg × s/cm^2)	M	9.31 ± 0.27	12.92	0.3286
		F	8.57 ± 0.28	14.79	ns

LD—*Longissimus dorsi*; SM—*Semimembranosus*, SEM—standard error of mean; CV—coefficient of variation. Student test: ns = not significant, $p > 0.05$; * significant for $p < 0.05$; ** significant for $p < 0.01$.

The highest average value of total shear force was measured in male LD muscles (5.62 kg/cm^2), while in female samples, it reached 5.14 kg/cm^2. In the SM samples, males also required higher shear force (5.48 kg/cm^2), while in females it reached 5.16 kg/cm^2 ($p < 0.05$).

These data induced the same pattern in the firmness analysis, with the highest values in male LD samples (2.71 kg/s × cm^2) versus 2.11 kg/s × cm^2 in females ($p < 0.01$). In the hindleg, the muscles were also firmer in males (2.37 kg/s × cm^2) than in females (2.19 kg/s × cm^2).

The area recorded by the texturometer (the total mechanical work required to cut the samples) was the highest in LD male muscles (10.89 kg × s/cm^2). In females, it had an

average value of 9.49 kg × s/cm^2. The same dynamics were noticed in SM muscles, with higher values in males (9.31 kg × s/cm^2) than in females (8.57 kg × s/cm^2).

3.2.2. The Color Parameters of Flemish Giant Rabbit Meat

The color parameters of rabbit meat are presented in Table 5. In LD muscles, relatively close average values can be observed for L*/lightness in males (59.12) and females (58.32). The red index (a*) had average values of 2.87 in females and 3.12 in males ($p < 0.05$). The yellow index (b*) was higher in males (3.01) than in females (2.12) ($p < 0.001$).

Table 5. The color parameters of FG rabbit meat.

Muscles	Parameters	Gender	Mean ± SEM	CV	*p*-Value
L.D.	L*	M	59.12 ± 0.95	7.21	0.9912
		F	58.32 ± 0.88	6.77	ns
	a*	M	3.12 ± 0.08	6.82	0.0138
		F	2.87 ± 0.04	5.68	*
	b*	M	3.01 ± 0.03	4.94	0.000002
		F	2.12 ± 0.03	5.83	***
S.M.	L*	M	55.49 ± 0.54	4.28	0.7384
		F	56.16 ± 0.52	4.14	ns
	a*	M	3.21 ± 0.03	4.39	0.8296
		F	3.31 ± 0.03	4.25	ns
	b*	M	2.75 ± 0.03	5.02	0.1268
		F	2.81 ± 0.02	4.41	ns

LD—*Longissimus dorsi*; SM—*Semimembranosus*; SEM—standard error of mean; CV—coefficient of variation. Student test: ns = not significant, $p > 0.05$; * significant for $p < 0.05$; *** significant for $p < 0.001$.

In SM muscles, gender did not significantly affect meat coloring, regardless of the investigated parameter, such as lightness (L* = 56.16 in females vs. L* = 55.49 in males), red color (a* = 3.31 in males vs. 3.21 in females) and yellow color (b* = 2.81 in males vs. 2.75 in females).

In all analyzed situations, the coefficient of variation was calculated within the 4 and 8% range, suggesting good homogeneity in all analyzed traits.

3.3. The Sensory Parameters of Flemish Giant Rabbit Meat

Hedonic scoring of LD muscles revealed higher grades in males than in females for color (2.45 vs. 2.2), fibrous appearance (2.60 vs. 2.53) ($p < 0.001$), smell (3.40 vs. 3.35) ($p < 0.001$), taste (3.65 vs. 3.40) ($p < 0.01$) and flavor (3.40 vs. 3.25). On the contrary, female LD muscles scored better in terms of the intensity of flavor ($p < 0.001$), tenderness ($p < 0.001$), and juiciness ($p < 0.001$) (Figure 1 and Table 6). Overall appreciation resulted in slightly higher scores in males vs. females (3.35 vs. 3.25) ($p < 0.01$).

Table 6. The statistical differences of sensory analysis, by gender, of FG rabbit meat.

Sensory Descriptor	Sensory *p* Values (Males vs. Females)	
	LD	SM
Color	0.8480/ns	0.000105/***
Fibrous appearance	0.000014/***	0.000003/***
Smell	5.9×10^{-8}/***	$1E\times10^{-9}$/***
Taste	0.0028/**	0.0033/**
Flavor	0.1553/ns	0.3088/ns
Flavor intensity	6×10^{-7}/***	0.0004/***
Tenderness	3×10^{-10}/***	2.93×10^{-6}/***
Juiciness	1.55×10^{-6}/***	1×10^{-9}/***
Overall appreciation	0.0019/**	0.0033/**

Student test: ns = not significant, $p > 0.05$; ** significant for $p < 0.01$; *** significant for $p < 0.001$.

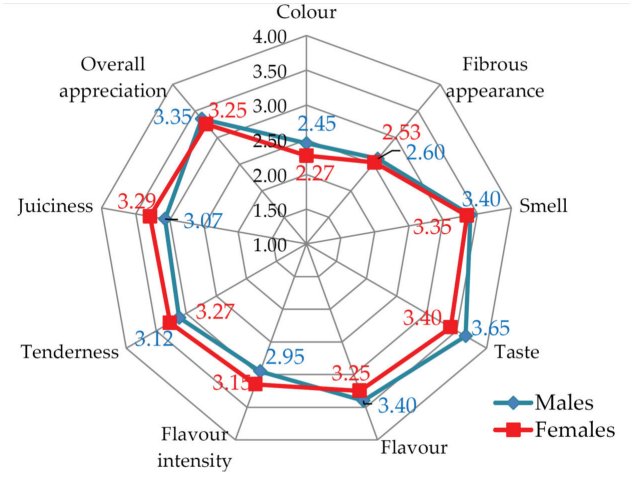

Figure 1. The sensory appreciation of LD muscles.

In SM samples (Figure 2, Table 6), females were better scored for color (3.42 vs. 3.11) ($p < 0.001$), tenderness (2.89 vs. 2.56) ($p < 0.001$), flavor (3.18 vs. 3.11) and juiciness (3.33 vs. 2.78) ($p < 0.001$), while males received better grades for the fibrous appearance (3.01 vs. 2.67) ($p < 0.001$), smell (3.33 vs. 2.78) ($p < 0.001$), taste (3.33 vs. 3.11) ($p < 0.01$), the intensity of flavor (3.56 vs. 3.27) ($p < 0.001$) and overall appreciation (3.33 vs. 3.11) ($p < 0.01$).

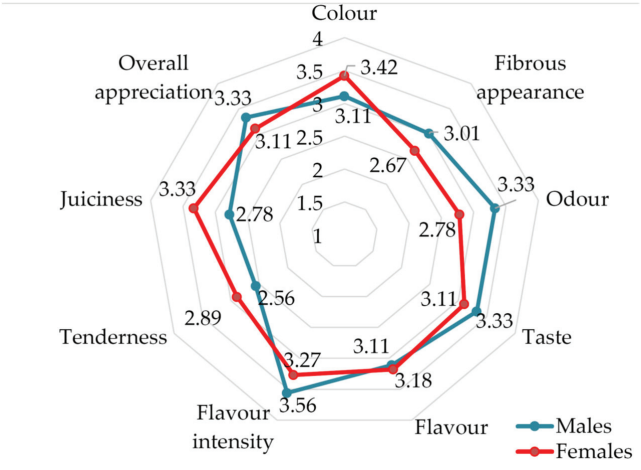

Figure 2. The sensory appreciation of SM muscles.

4. Discussion

4.1. The Fatty Acids Content and Sanogenic Indices of FG Rabbit Meat

The level and quality of dietary nutrients are primordial factors that count toward maintaining a healthy status in human consumers [41].

A dietary PUFA/SFA ratio of 0.45 or higher is recommended for preventing cardiovascular diseases [42]. A better PUFA/SFA ratio was reported in New Zealand rabbit meat (0.9–1.1) [43], Grimaud breeds (0.61–1.03) [44], and gray-colored rabbit breeds (0.92–0.94) [45].

The sanogenity of any dietary fat source can also be measured by the cholesterol neogenesis potential in consumer hepatocytes and is expressed as a ratio of hypocholesterolemic/hypercholesterolemic (h/H) fatty acids. In this respect, the New Zealand White breed had approximately 1.54–1.78 [46].

In our original findings, the PUFA/SFA ratio was 0.84, higher in males (+49%) than in females. However, the n6/n3 ratio was higher for females (+11.3%) vs. males. The h/H was, on average, 2.01, with 27.8% better in males than in females.

The AI and thrombogenicity index (TI) should be lower than 1.0 in foods to prevent atherosclerosis and thrombosis through a healthy diet and lifestyle, respectively [42]. Grimaud breed had better AI (0.52–0.72); however, TI (0.59–1.14) exceeded the recommended level [44]. The AI and TI values obtained in our original study were lower and, consequently, more favorable for consumer health (on average, they reached 0.42). However, if we compare meat origin by gender, female muscles had higher values (+35%) of AI and TI than male ones, suggesting that male meat is healthier. In our research, for all analyzed fatty acids, higher values were found in females, especially in SM muscles, which are usually richer in lipids than LD muscles. Females tend to accumulate more fat than males, with more body fat at any chronological age [47,48].

The biological value of rabbit meat relies on the fatty acids involved in consumer cardiovascular health reinforcement. Rabbit meat is considered a healthy dietary fat source and is recommended for patients with hypertension, hyperlipidemia, and cardiovascular and cerebrovascular diseases [49,50].

The high PUFA/SFA ratio and the presence of essential fatty acids in rabbit meat are favorable for human health [51–54]. The α-linolenic acid (C18:3n-3/ALA) and PUFAs C20:5n-3/EPA, C22:5n-3/DPA, C22:6-n3/DHA received the most attention due to their importance for human health and nutrition [55], as being effective in reducing triacylglycerol in blood and preventing cardiovascular diseases. EPA and DHA reduce inflammation and play a role in decreasing the incidence of childhood allergic diseases. EPA and DHA have biological activities that might influence tumoral cell proliferation and viability; DHA can promote tumor cell apoptosis, possibly by inducing oxidative stress [56,57].

In Western diets, the ratio of n-6/n-3 essential fatty acids is 15/1–16.7/1. Western diets are deficient in n-3 fatty acids and have excessive amounts of n-6 fatty acids compared with the diet on which humans evolved, which had established genetic patterns. Excessive amounts of n-6 PUFA and a very high n-6/n-3 ratio (15/1–16.7/1) promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of n-3 PUFA (therefore, low n-6/n-3 ratio) exert suppressive effects. In the secondary prevention of cardiovascular disease, a ratio of 4/1 was associated with a 70% decrease in total mortality. A ratio of 2.5/1 reduced rectal cell proliferation in patients with colorectal cancer, whereas a ratio of 4/1 with the same amount of n-3 PUFA had no effect. The lower n-6/n-3 dietary ratio in women with breast cancer was associated with decreased risk. A ratio of 2–3/1 suppressed inflammation in patients with rheumatoid arthritis, and a ratio of 5/1 had a beneficial effect on patients with asthma, whereas a ratio of 10/1 had adverse consequences [58]. In our study, the n-6/n-3 ratio was, on average, 5.03, falling, therefore, within the beneficial range.

Rabbit females, in the optimal period for mating, deposit more lipid reserves to provide raw matter for fetal tissue development throughout gestation, then for lactation and offspring viability (based on the metabolism and hormonal equipment specific to females). Health implications should be considered if only female carcasses were sold, but this is not the case in the breeding and sale of rabbit meat. Nevertheless, we would recommend slaughtering females at least 3–4 weeks earlier than males to avoid excessive fat deposition and the development of unfavorable sanogenic indices in meat.

4.2. The Instrumental Assessment of FG Rabbit Meat

4.2.1. The Texture Parameters of FG Rabbit Meat

In the present study, the highest average value of shear force was observed for males vs. females in LD (+9.3%) and SM muscles (+6.2%) (Table 4). The average values for total shear force corresponded to those reported in studies that examined rabbit meat from medium-sized breeds [9,59–63]. The maximum force of 3.41–3.76 kg/cm^2 was measured for rabbits slaughtered at 9 weeks old in Spain [60] in breeds selected multigenerational for meat quality traits. Other traits, such as firmness, were reported from 10.85 kg × s/cm^2 to 14.84 kg/s × cm^2, while the total effort area was reported, on average, at 5.95 kg × s/cm^2 [9,60–62].

Other authors [51] obtained an average value of shear forces that varied, depending on the feed quality, from 3.92 kg/cm^2 to 4.92 kg/cm^2 (slaughtering age: 12 weeks).

In a wider study [9] on the quality of rabbit meat issued from conventional and organic farming systems, an average shear force of 4.58 kg/cm^2 was found in rabbits slaughtered at 90 days (organic farming) and of 3.64 kg/cm^2 in rabbits slaughtered at 63 days (conventional farming). Therefore, the age at slaughter plays an important influence on the texture of rabbit meat. In another study from Spain [62], the shear force reached 3.6 kg/cm^2 in LD muscles (age 4 to 9 weeks), values close to those reported by other authors [60–63]. Also, in other species, shear force was measured between 3.2 and 3.7 kg/cm^2 (lamb) [64], 5.2 kg/cm^2 (pork) [65] and 5.4 kg/cm^2 (beef) [66]. In our findings, rabbit meat had better tenderness than pork and beef and was close to that measured for lamb. However, it is a little forced to compare such findings, knowing that the texture is influenced by multiple factors, such as the genetic origin, age at slaughter, dietary specificity, artificial selection, environmental microclimate, preparation of sample for analysis, and the chosen instrumental method.

4.2.2. The Color Parameters of FG Rabbit Meat

The color parameters of LD muscles (Table 5) had relatively close values for L*/lightness in males (59.12) and females (58.32); the red index (a*) varied between 2.87 (females) and 3.12 (males). Also, a higher yellow index (b*) was observed in males than in females. In SM muscles, the color parameters were very close (females vs. males): L* was 56.16 vs. 55.49; a* was 3.31 vs. 3.21, and b* was 2.81 vs. 2.75. Other authors measured average values of L* for rabbit meat from medium bread, similar to those observed in the present study [5,10–12]. In another study on the conventionally and organically produced rabbit meat, L* varied from 57 to 60, a* index from 2.92 to 3.70, and smaller values were observed for the b* index (0.58 to −1.13) [9].

The mean value of L* of different rabbit breeds ranges from 41.78 to 65.68 [42]. Czech White and Moravian White rabbit breeds had the highest L* of *Biceps femoris* (BF) muscles than Czech gold breeds. Still, the Hyplus rabbits had intermediary L* values compared to other breeds [67], with differences given by the farming system. Another study [68] found meat color differences between the *Longissimus lumborum* and *Biceps femoris* muscles of three rabbit genotypes. The highest L* was reported in the Hyla breed (59.96–63.16). The higher proportion of myoglobin content and the type of myocytes might contribute to more redness a* value, even though it could be affected by many other factors such as exercise, diet, and genetic and environmental conditions [42,69].

In general, the brightness values determined for domestic rabbit meat are relatively close to those determined for turkey meat.

4.3. The Sensory Parameters of Flemish Giant Rabbit Meat

The mean values of sensory analysis parameters of LD muscles and SM muscles from FG rabbit meat are presented in Figures 1 and 2. Overall appreciation has 3–7% higher values for males vs. females (3.35 vs. 3.25 in LD and 3.33 vs. 3.11 for SM muscles). The potential reasons or the significance of these variations relied on the better taste and more

pleasant odor detected in male samples, compared to females, whose muscles were richer in total lipids and probably had a more pronounced flavor of rabbit meat.

Rabbit meat from giant and intermediary size breeds sensory analysis, which is very scarce [40]. A difficulty for sensory analysis of rabbit meat is the small muscle sample size in this type of meat. Moreover, the scoring of panel tests is difficult to interpret in terms of meat quality; for example, a difference of one point for flavor between two persons does not indicate whether this difference is relevant or not. Also, slaughtering parameters can severely affect meat texture and sensory traits due to stunning types of bleeding, extreme voltages, and frequencies that could be preferable to medium voltage and frequency levels (for improving sensory qualities, such as tenderness and juiciness) [70]. The sensory analysis presented in the literature is uneven, with studies pursuing the application of growth, nutrition, slaughter, or cooking strategies to improve the sensory quality of rabbit meat [4,23,40,70].

In our study, as the shear force and firmness measurements had increased values, the fibrous appearance of muscles increased while the tenderness and juiciness decreased (observed through the sensory analysis with trained specialists) on the hedonic scale.

4.4. Recommendation of Rabbit Breeding and Meat Consumption

The biological nutritional value of rabbit meat should be popularized globally, especially in Romania (and in other countries), where a drastic decline in farming and consumption is observed.

In comparison for sanogenic indices, in goose breast muscles, the AI was 0.37, and the TI was 0.69 [71], therefore lower than those calculated for other kinds of meat such as rabbit from medium breeds, whose AI was 0.90 and TI was 1.19 [72]. In chicken, AI was 0.49, and TI was 1.14 [73]. In turkey, the AI was 0.47, and TI was 0.91 [74]. In other mammals, the sanogenic indices varied from beef (AI = 0.60 and TI = 1.86) [75] to pork (AI = 0.47 and TI = 1.12) [76] and to lamb (AI = 0.90 and TI = 0.87) [77].

To ensure the sustainability of rabbit production and protect the global rabbit industry, cost-effective and practical strategies for improving rabbit production and meat quality must be developed. Recently, rabbit farming, like other animal farming, has faced feed shortages due to the impact of climate change, high competition between livestock species, and war conditions [31]. In general, and in Romania, chicken meat is now the most appreciated by consumers [78], at the expense of rabbit meat, which is also much rarer and harder to find. In European countries, the mean market price of rabbit meat at butcheries ranges from 5 to 10 EUR/kg, while broiler is normally priced between 2.50 and 6 EUR/kg. As a result, rabbit meat consumption has gradually decreased, particularly among low-income households. The most common difficulty is the absence of rabbit meat in butcher shops [79], which means that urban inhabitants who enjoy rabbit meat can only purchase it in a few places [80]. This species can efficiently transform the residues of agricultural nature into a product of high nutritional–biological value meat [81]. Regarding the global sustainability in agriculture, agri-food by-products can be added to a variety of foods to increase their bioactive profile, fiber content, and antioxidant capacity [82–85] while maintaining good sensory acceptability, with applicability for rabbit meat, eventually for ready to eat products, like sausages, etc.

Consumers today are encouraged to seek out alternative meat types due to the changes in their eating habits and the increased disease risk associated with conventional meat products [48]. A limited range of innovative, experimental rabbit meat alternative products to raw carcasses are available in the market, including smoked, canned, frozen, cured, sauce-picked, dried, and roasted products, as well as rabbit meat sausages and hamburgers [80], but territorially limited to certain Southern European countries and in China and not on a permanent availability basis.

5. Conclusions

Meat sanogenity was better in males than in females, with higher ratios of unsaturated vs. saturated fatty acids and lower values of the atherogenic and thrombogenic indices. The instrumental analysis of texture revealed less tender meat with better pigmentary properties in males. In thigh muscles, colorimetric investigation revealed closer results between genders. On the sensory overall acceptance, males achieved better scoring than females. However, the latter ones appeared to be better scored when tenderness and juiciness were assessed independently, suggesting less connective tissue in muscle stroma and, possibly, thinner myocytes.

The meat from males showed superior quality characteristics (both sanogenic and sensorial). We would recommend slaughtering females at least 3–4 weeks earlier than males (around 8 months old) to prevent excessive fat deposition in their carcasses and the development of unfavorable sanogenic indices.

As research follow-up, the instrumental data should be completed with structural histological analysis to assess the real composition of the connective tissue in the muscle marbling (is it predominantly collagenic or lipidic?). Also, the data could be refined using more samples and more homogenous carcasses in terms of age, physiological status, and gender repartition.

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Diet Influence on Sperm Quality, Fertility, and Reproductive Behavior in Karakul of Botoșani Rams

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Abstract: This study aims to analyze the influence of an improved diet with vitamins and minerals (VM) on the live weight, body condition, quality of sperm, behavior, and fertility of rams. The biological material comprised two groups of rams (L1—control and L2—VM supplemented), each consisting of 15 individuals. After a complete one-year cycle, they received different dietary treatments at the beginning of the preparation for the reproduction period. Although in the onset of the mounting period (SM), no significant differences were observed for live weight ($p > 0.05$), providing supplemental feeding of a VM complex allowed a better capitalization of body reserves, and, consequently, the rams' groups differed significantly by the end of mating season (FM), for live weight (+4.1%; $p < 0.001$) and body condition score (+15.9%; $p < 0.05$). Adding vitamins and minerals to the L2 diet also improved sperm color ($p < 0.001$), sperm concentration (+11.8%; $p < 0.01$), live spermatozoa (+2.6%; $p < 0.001$), and decreased abnormal spermatozoa proportion (−7.0%; $p < 0.01$). The increase in the scrotum circumference in L2 (+4.57%) suggests that VM supplements improved testosterone secretion, spermatogenesis, and ejaculate volume (+10.20%; ($p < 0.001$), with a positive impact ($p < 0.001$) on mating behavior, on the gestation installation (+11.2%) and on the number of obtained lambs (+14.0%), as well as on the key economic indicators (+13.8% incomes per ram).

Keywords: rams; fertility; Botoșani Karakul breed; semen; spermatozoa

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1. Introduction

In Central and Eastern Europe, the impact of climate change has intensified significantly lately. In a changing environment, the pasture dries up in the summer, and small ruminants are affected even before the breeding season. Nutrition can have a more significant influence on rams' performance and testicular size than the photoperiod. The purpose of nutrition in breeding them is an important aspect that contributes to achieving positive results. Studies indicate that spermatogenic activity, sperm content in the epididymis, and many other traits are higher in rams fed ad libitum. In a study conducted by Oldham et al., 1978 [1], the data obtained show that the efficiency of a balanced diet on testicular activity is important because rams that received a balanced diet had more intense activity and produced more spermatozoa than those with inadequate nutrition.

Research conducted on sheep populations in different arid regions indicates significant effects caused by nutritional deficiencies or climate changes, with a major impact on reproduction. Some of these results highlight the importance of proper and balanced feeding because it positively influences the onset of puberty, ovulation rate, embryo survival,

anestrus length, and the response of sheep to interaction with rams [1,2]. Adequate nutrition can also influence certain traits, such as the fertilization capacity of rams [3], semen quality, and reproductive performance, leading to better economic outcomes [4]. When provided with inadequate food and exposed to climatic factors outside the comfort range, not only will the reproductive capacity be affected, but also the quantity and quality of sperm [5]. Therefore, through nutrition, the goal is to maintain or improve the maintenance of rams to achieve an optimal level of their body condition and best performance. The results of some studies have indicated the beneficial effects of feeding Targhee rams with Zn supplements, although the source of Zn has elicited varied responses in the measured performance characteristics [6].

The fertility of rams represents a management issue influenced by both their health and the quality of their feeding. Bioactive feed components can provide protection against oxidative and inflammatory damage of the male reproductive system. Additionally, this action will improve spermatogenesis and other specific traits related to the reproductive function of rams [6,7]. In the absence of activities supporting the reproductive function, reproductive pathological conditions may arise as a significant barrier to achieving positive results.

At the farm level, the management of rams is complex and heavily influenced by local factors, breed, farm, season, and the farmer's experience [8,9]. Effective management involves genetics, health, nutrition, behavior, economics, and physiological and anatomical changes occurring throughout or outside the breeding season [10,11].

Often, physical, chemical, biological, or technological aspects are not adequately addressed, thus leading to negative effects. Prolonged exposure of rams and ewes to the direct influence of excessively high temperatures can be a stress factor, affecting their behavior, their reproductive function, and sperm production levels [12]. Heat stress induces various physiological, metabolic, endocrine, and molecular disruptions in the body as a response to the effort in maintaining thermal balance [13,14]. To regulate body temperature and ensure survival, certain endocrine changes occur within the body that have a negative association with ram fertility [12]. Prolonged heat stress leads to reduced testosterone concentrations, negatively influencing the spermatogenesis and sexual behavior of rams [10,12,15].

Even a half-degree change in body temperature can reduce spermatogenesis and/or libido [16]. In such circumstances, it is recommended to implement the following activities that enhance thermal comfort during critical periods to mitigate the effects of heat stress: housing the rams in shaded and well-ventilated enclosures, providing antioxidant-enriched diets, and maintaining an appropriate feeding regimen [17].

The purpose of this research was to study the effects of certain technological factors that can be easily monitored on farms and might influence reproductive activity of the Botoșani Karakul breed. Another goal was to identify effective technological methods to mitigate the effects of thermal discomfort, inadequate maintenance, and proper nutrition to ensure improved values for ram fertility. The main objective of this research was to enhance the reproductive performance of rams by implementing a diet that includes a VM (vitamins and minerals) supplement.

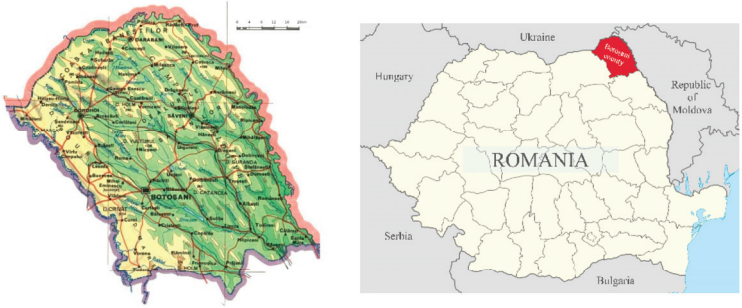
2. Materials and Methods

2.1. Ethical Approval

The activities scheduled during the research were consistent with the ethics of scientific research. When collecting semen and blood samples, considerations from the Ethics and Experiments on Animals were taken into account, and procedures were applied in accordance with the Ethics Committee's regulations of the research and development unit, as well as the recommendations of the Institute of Diagnosis and Animal Health in Romania. Likewise, in the case of handling and collecting biological samples, all ethical requirements were followed to ensure that animals subjected to experimental procedures were not put in discomfort or made to undergo any painful treatments (941/04.10.2021).

2.2. The Research Area and Climatological Conditions

The research area is located in the northeastern part of Romania, being located at 47°44′55″ north latitude and 26°40′10″ east longitude. In this region (Figure 1), climate changes have become obvious, and there is a significant increase in the number of days with daily average temperatures exceeding +35 °C compared to multiannual average (https://www.meteoblue.com, accessed on 12 July 2023).



(**a**) (**b**)

Figure 1. Representation of the research area (**a**) and its location on the map of Romania (**b**).

Current climate changes lead to an increase in hot and dry days during the summer, while winters become quite mild and devoid of snow. In this context, climate changes have a strong and negative impact not only on the environment but also on sheep, affecting their behavior and causing certain disruptions to their physiology.

2.3. Animal, General Procedures, and Data Management

The research was conducted on the sheep population within the Research and Development Station for Sheep and Goats in Popăuți-Botoșani. The biological material used consisted of two groups of adult rams (L1, L2), each comprising 15 rams aged between 2 and 5 years. At the start of the breeding activity, the live weight for L1 was 86.06 ± 2.16 kg, while for L2, it recorded a value of 90.13 ± 2.19 kg. All research activities were carried out over a complete reproduction cycle of one year, and the maintenance and feeding conditions applied to both groups were identic, only differing during the period of sexual rest. The experiment was based on the administration of a VM supplement only to L2 during the preparation period (MP) and the sexual activity period (SM), corresponding to the natural breeding season during autumn (September–November).

During the MP period, the batch L2 received an improved diet. A complex of vitamins and minerals (VM) was additionally provided to support the reproductive activity (Table 1). Daily nutrition included certain minerals (Zn, Fe, Mn, Cu, Co, I, Se) and vitamins (A, D, E). The diets provided to both groups aimed to support body condition, and the addition of VM to L2 ensured the necessary macro- and micronutrients to support the reproductive function and fertility of the rams. In accordance with the experimental protocol, the research also included an evaluation of how body condition influenced the quantity and quality of seminal material in rams, as well as the behavior and fertility of the rams.

Water was provided from safe and abundant sources, and the preparation for mating and the actual mating activity took place during the natural breeding season (September–October). Since the Botoșani Karakul breed is developed and improved for pelts production, the used reproductive system is natural mating (each ram mounts 35 ewes per season). Based on the data obtained in EM, several quality traits of sperm, fertility, and the economic efficiency of the proposed experimental model were determined.

Table 1. Structure of the diet throughout the experimental period.

Dietary Ingredients (kg)	Diet for L1				Diet for L2			
	Rest during Summer	Rest during Winter	Preparation for Mating	Mating	Rest during Summer	Rest during Winter	Preparation for Mating	Mating
Alfalfa hay	-	0.410	0.210	-	-	0.410	0.210	-
Barley straw	1.430	1.610	1.340	1.420	1.430	1.610	1.340	1.420
Hillside pasture	2.400	-	-	2.800	2.400	-	-	2.800
Carrots	-	-	-	0.200	-	-	-	0.200
Barley	0.095	0.060	-	-	0.095	0.060	-	-
Oat	-	-	0.530	0.100	-	-	0.530	0.100
Corn	-	-	-	-	-	-	-	-
VMP	-	-	-	-	0.014	0.019	0.017	0.023
Nutritional characterization								
DM (g)	1786	1781	1783	1777	1800	1800	1800	1800
CP (g)	124.87	120.00	132.00	144.71	124.87	120.00	132.00	144.71
ME-ruminants (MJ)	13.00	12.99	14.64	14.64	13.00	12.99	14.64	14.64
NEM (MJ)	7.30	7.30	8.42	8.47	7.30	7.30	8.42	8.47
Ca (g)	9.33	14.16	9.81	10.54	9.33	14.16	9.81	10.54
P (g)	3.20	3.00	3.53	3.72	3.20	3.00	3.53	3.72
Na (g)	5.73	6.58	5.42	6.49	5.73	6.58	5.42	6.49
Mg (g)	2.50	2.86	2.61	2.50	2.50	2.86	2.61	2.50
Vit.A (IE)	-	-	-	-	8595	11036	10940	14121
Vit.D (IE)	-	-	-	-	1029	1346	1273	1687
Vit.E (mg)	-	-	-	-	39.00	46.00	55.00	65.00
Zn (mg)	-	-	-	-	62.84	82.37	77.60	103.05
Fe (mg)	-	-	-	-	80.00	104.44	99.48	131.29
Mn (mg)	-	-	-	-	47.45	66.97	51.81	77.26
Cu (mg)	-	-	-	-	10.50	14.40	12.05	17.14
Co (mg)	-	-	-	-	0.20	0.25	0.27	0.33
I (mg)	-	-	-	-	0.67	0.77	0.98	1.11
Se (mg)	-	-	-	-	0.26	0.33	0.33	0.42

VMP = vitamin–mineral premix; DM = dry matter; CP = crude protein; NEM = Net energy milk.

2.3.1. General Conditions of the Study

Throughout the entire research period, both batches of rams received appropriate exercise and feeding regimes with similar conditions. Starting from MP, all rams from L2 were accommodated in a separate enclosure; equipped with a roof to limit the effects of solar radiation. Additionally, an air ventilation system was installed in the enclosure to reduce the ambient temperature by approximately 3 °C compared to the thermal level in the accommodation space of L1. Temperature was monitored throughout the preparation and mating periods using a thermometer/hygrometer, specifically the Alanlog, type 76113, which allowed temperature monitoring between −25 °C and +55 °C and humidity ranging from 0% to 100%.

Throughout the research, the fertility of rams was assessed to observe how can it be influenced by the additional administration of a VM (vitamin and mineral) complex, starting from SM.

2.3.2. Body Condition Score and the Behavior Evaluation

Body condition score (BCS) was assessed by palpating muscular mass and fat deposits on the dorsum (upper part of the trunk, loin, and rump). It was graded on a scale from 1 (thin) to 5 (very fat), with half-point increments, using a method developed by Jefferies [18]. BCS was evaluated by two experienced graders who reached a consensus score. Live weight (LW) was measured using an electronic scale with an accuracy of ±100 g.

The behavior of the rams was studied during the mating period using the method described by Goshme [19]. Based on how the rams reacted in the presence of ewes, they were rated on a scale from one to five, according to the following criteria:

Excellent (5): Typical behavior; when introduced into the testing pen, the ram is eager to breed with the ewe, restless, and challenging to restrain, showing a strong desire to mate immediately.

Very Good (4): Optimal sexual reflexes; the ram shows a desire to breed within a maximum of 3 min, although it can be challenging to restrain.

Good (3): The ram sniffs around upon introduction to the pen, performs breeding within the first 5 min, and is relatively easy to control.

Poor (2): The ram sniffs the ewe for a few minutes, and the time taken for breeding extends to 7–10 min. It is more docile and easier to handle.

Very poor (1): Displays delayed and incomplete reflexes, with a breeding time extending to approximately 10 min.

The study of behavior began at the end of the SM period and concluded at EM. The study of sexual reflexes was based on the behavioral manifestations exhibited by the rams during the mating period with the assigned females.

The behavior was assessed individually when bringing the males for mating. The reaction, the desire to mount, the time of approach, and the attitude over the female exhibiting ovulatory heat were observed.

2.4. Semen Analysis

The assessment of the impact of experimental factors on the quantity and quality of semen was conducted on samples collected by experienced individuals using the artificial vagina method in the following three distinct stages from both groups, namely: mount preparation (MP), start mounting (SM), and end of mating (EM). Before collection, the foreskin was cleaned to prevent contamination of the sperm. The technique was performed alternately once a day (Figure 2a) for four consecutive days at the beginning of each phase (MP, SM, and EM).

(**a**) (**b**)

Figure 2. Semen collection (**a**) and scrotal circumference measuring (**b**).

To ensure an even distribution of the time interval between two collections, on the first day, seminal material was collected at 8 a.m. and on the following day at 8 p.m. The collected samples were transported to the laboratory within a maximum of 10 min and stored in a water bath at 37 °C.

2.4.1. Scrotal Circumference (SC)

The scrotal circumference was measured for each male during the collection activities by firmly pulling the testicles down towards the lower part of the scrotum and then placing a measuring tape around the widest point (Figure 2b).

2.4.2. Ejaculate Volume, Color, and pH

The collected volume was measured directly on the graduated flask used for collection, and the color of semen was visually assessed, giving grades from 1 to 4 following a model presented by Jha et al. [20], where the color varied from 1 to 4 (1 = watery, 2 = milky, 3 = yellowish white, 4 = creamy white).

The pH was estimated using an indicator paper (phenolphthalein paper) by matching it against a color scale.

2.4.3. The Evaluation of Specific Characteristics of Semen

Each ejaculate was analyzed separately, and the characteristics that determine the biological quality of the semen were determined. The analysis of specific seminal fluid traits was performed using the Computer-Aided Sperm Analysis System (CEROS II CASA, IMV Technologies, L'Aigle, France), equipped with a high-resolution Hamilton Thorne video camera with a resolution of 782 × 582 and progressive scanning and Trinocular Zeiss Axiolab A5 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and the Windows 10 operating system, and report system Software HT CASAII—Designer, v. 720271 (Hamilton Thorne Inc., Beverly, MA, USA). This system is reliable and user-friendly because the CEROS II processor is separate from the optical component, allowing for precise, repetitive, and automated evaluation of the most important specific traits of seminal fluid. The video camera was connected to the microscope through an adapter, enabling video recordings at a resolution of 800 × 600 pixels and 60 frames per second. Sperm morphology was evaluated at a magnification of 200–400× using phase contrast.

2.4.4. Assessment of Blood Testosterone Level

To determine the testosterone level, blood samples were collected in the first three days of the MP, SM, and EM periods. The blood samples were collected at 8:00 a.m. by making an incision in the jugular vein of each ram (a total of 135 blood samples per group). Blood collection was performed in individual tubes. After collection, the samples were transported to the laboratory, centrifuged, and the obtained serum was frozen at −20 °C until testosterone testing [21]. For testosterone determination, the radioimmunoassay (RIA) method was used, employing TESTO-RIA-CT (DIAsource ImmunoAssays, Ottignies-Louvain-la-Neuve, Belgium) and a commercial kit (KIP1709) with a testing range from 0.1 ng/mL to 17 ng/mL and a sensitivity of 0.05 ng/mL [22,23].

2.5. Statistical Data Processing

Experimental data were input in column type database, submitted to processing within the GraphPad Prism, v. 9.4.1. software (Palo Alto, CA, USA) to obtain statistical descriptors values (mean, standard deviation) and to compare the two groups' performances, using the unpaired *t*-test followed by Welch correction, assuming the standard deviations of the groups were not equal.

3. Results

3.1. Effect of Feed Used on Live Weight (LW) and Body Condition Score (BCS) of Rams

During a complete cycle (from December 2021 to December 2022), even though the maintenance condition of the rams undergoes certain changes, it is essential that during the MP period the factors that have a real influence on their reproductive condition are optimized. During the resting periods, both groups were fed the same diet, which included seasonal forage (Table 1). If the crude protein in the daily diet was provided at an average level of 122.4 g during the rest period, then in the preparation period the protein level increased by 7.84%, reaching a level of 144.71 g during the sexual activity period (Table 1).

Through the daily diet, the aim was to provide an average energy level of 13 MJ ME-ruminants during the resting period, and 14.64 MJ ME-ruminants for the preparation and intense sexual activity period. The energy level for the rest period was determined based on the animals' body weight at the maintenance level, using the animal nutrition optimization program HYBRIMIN Futter 5. For the preparation for breeding and during the mating period, the energy level was slightly higher (+12.5%).

According to results in Table 2, the body condition score significantly improved (+15.9%, $p < 0.05$) in L2 rams, compared to L1 ones and the live weight differed also in a significant manner (+4.7%, $p < 0.001$) due to stimulative feeding.

Table 2. Descriptive statistics for BCS (points) and live weight (kg) for rams.

Assessment Moment	Trait	L1			L2		
		Mean	±St. Dev	CV%	Mean	±St. Dev	CV%
Preparation for mating (MP)	BCS (points)	2.50	0.42	16.80	2.70	0.32	11.85
	LW (kg)	87.49	2.39	2.73	87.92	2.31	2.63
Start mating (SM)	BCS (points)	2.83	0.56	19.79	3.23	0.53	16.41
	LW (kg)	89.09	2.91	3.27	90.49	2.42	2.67
End of mating (EM)	BCS (points)	2.33 ^{a}	0.41	17.60	2.70 ^{b}	0.49	18.15
	LW (kg)	86.06 ^{a}	2.16	2.51	90.13 ^{d}	2.19	2.43

BCS = body condition score; LW = live weight. Means with different superscripts within the same row signify: ^{ab} = $p < 0.05$; ^{ad} = $p < 0.001$

3.2. Physical Evaluation of Reproductive Quality

Statistical data processing indicates small variations between the two groups for SC at the MP, with average values ranging between 31.45 ± 0.56 cm for L1 and 31.25 ± 1.76 for L2, and these differences are not statistically significant. The average coefficient of the variation value indicates a certain level of homogeneity, which is due to the fact that rams from both groups were carefully selected for reproduction.

However, the data obtained after measuring SC at the start of mating (SM) show a significant difference between the groups at $p < 0.05$ and a very significant difference in EM at $p < 0.001$.

The volume of ejaculate (Ev) exhibited variations with different statistical significance during the measurement period. While at the beginning of MP (mount preparation), the difference between the two groups was minimal and not statistically significant, higher values were obtained in L2 for the samples collected during SM (start mounting) and EM (end of mating) (Table 3). In this group, Ev was superior by 17.78% at the time of SM and 10.20% at EM compared to the same traits in L1.

The color of semen varied quite differently between the two groups. At the beginning of the MP period, the baseline color was milky white with shades ranging from white to creamy in both groups, and the differences between the groups were not statistically significant. However, after the experimental treatment and sampling during SM and EM,

the color of ejaculate in the rams from L2 intensified and took on shades towards creamy white, with the difference between the groups being highly significant ($p < 0.001$).

Table 3. Descriptive data for some physical assessments of rams and semen characteristics.

Assessment Moment	Trait	L1			L2		
		Mean	±St. Dev	CV%	Mean	±St. Dev	CV%
Preparation for mating (MP)	SC (cm)	31.45	1.56	4.96	31.25	1.76	5.63
	Ev (mL)	1.20	0.10	8.33	1.25	0.09	7.20
	Color (1–5 degrees)	2.733	0.594	6.57	2.867	0.516	8.14
	pH	7.033	0.188	7.31	7.027	0.166	8.14
Start of mating (SM)	SC (cm)	33.8 ^{a}	1.83	5.40	35.42 ^{b}	1.72	4.01
	Ev (mL)	1.30 ^{a}	0.11	8.46	1.58 ^{d}	0.24	11.19
	Color (1–5 degrees)	2.846 ^{a}	0.520	9.22	3.867 ^{d}	0.352	8.31
	pH	7.058	0.085	9.23	7.100	0.093	8.38
End of mating (EM)	SC (cm)	32.10 ^{a}	1.80	5.61	34.71 ^{d}	1.34	3.86
	Ev (mL)	1.32 ^{a}	0.18	13.64	1.47 ^{b}	0.20	13.61
	Color (1–5 degrees)	2.733 ^{a}	0.458	10.24	3.267 ^{d}	0.458	9.01
	pH	7.020	0.094	10.24	7.060	0.074	9.05

SC = scrotal circumference; Ev = ejaculate volume. Means with different superscripts within the same row signify: ^{ab} = $p < 0.05$; ^{ad} = $p < 0.001$.

The reaction of semen (pH), or the acidity level, is assessed by analyzing the pH value. The determinations (Table 3) on the samples collected at the respective stages indicated average values ranging from a minimum pH of 7.02 ± 0.094 (L1 for EM samples) to a pH of 7.100 ± 0.093 (L2 for SM samples), with no significant differences recorded between the groups.

3.3. Sperm Quality Analyses: Concentration, Live Spermatozoa, Abnormal Spermatozoa, Mobility, Testosterone

Sperm concentration determined in the samples collected in each of the three periods showed that in all situations, the average values were >3.0 × 109 mL^{-1}. The highest values for the total number of sperm per unit volume (Table 4) were recorded in the samples collected at SM and EM. The fact that in the samples collected from L2, the concentration increased from 4.01 ± 0.08 × 109 to average values of 4.41 ± 0.12 × 109 supports the beneficial effect of the additional administered VM complex and its contribution to the increased biological value of seminal fluid. In both cases, the difference between the groups in the samples collected at SM and EM is highly significant ($p < 0.001$).

Live spermatozoa are perhaps one of the most important traits on which the male's fertility depends. Determining this trait in the samples collected at the beginning of the MP (mount preparation) period reveals non-significant differences ($p > 0.05$) between the groups. However, at the EM (end of mating) stage, the proportion of live spermatozoa is higher by 6.86% in L2, with the difference being highly significant ($p < 0.01$).

Abnormal spermatozoa, if present in a large number, can affect fertility, and unfortunately, this deficiency cannot be detected unless the quality of the seminal material is evaluated. The rate of anomalies recorded in the semen is roughly similar in both groups but occurs at different levels. In L1, during the MP, there is a reduction from 3.70 ± 0.15 to 3.59 ± 0.12 in the MP samples. In L2, it is observed that after the MP period, there is a reduction in the proportion represented by abnormal spermatozoa from 3.78 ± 0.10 to 3.53 ± 0.16. When evaluating abnormal spermatozoa in the samples collected at EM, the

average values range between the proportion limits of 3.82 ± 0.12 in L1 and 3.57 ± 0.06 in L2.

Table 4. Evaluation of sperm quality and serum testosterone.

Assessment Moment	Characteristic	L1			L2		
		Mean	±St. Dev	CV%	Mean	±St. Dev	CV%
Mount preparation (MP)	Sperm concentration ($\times 10^9$/mL)	3.98	0.08	2.01	4.01	0.08	2.00
	Live spermatozoa (%)	80.53	2.29	2.84	80.73	1.10	1.36
	Abnormal spermatozoa (%)	3.70	0.15	4.05	3.78	0.10	2.65
	Mobility (%)	83.87	5.05	6.02	85.20	4.07	4.78
	Testosterone (ng/mL)	2.29	0.09	3.39	2.31	0.27	11.69
Start mounting (SM)	Sperm concentration ($\times 10^9$/mL)	4.13 ^{a}	0.17	4.12	4.41 ^{d}	0.13	2.95
	Live spermatozoa (%)	81.87 ^{a}	1.77	2.16	87.80 ^{d}	1.93	2.20
	Abnormal spermatozoa (%)	3.59	0.12	3.34	3.53	0.16	4.53
	Mobility (%)	85.93 ^{a}	4.40	5.12	88.87 ^{b}	2.29	2.58
	Testosterone (ng/mL)	2.47 ^{a}	0.11	4.45	3.18 ^{d}	0.27	8.49
End of mating (EM)	Sperm concentration ($\times 10^9$/mL)	3.46 ^{a}	0.20	5.78	3.87 ^{d}	0.19	4.91
	Live spermatozoa (%)	79.60	0.99	1.24	81.67	2.26	2.77
	Abnormal spermatozoa (%)	3.82 ^{a}	0.12	3.14	3.57 ^{c}	0.06	2.68
	Mobility (%)	85.13 ^{a}	2.61	3.07	88.20 ^{c}	2.01	2.28
	Testosterone (ng/mL)	2.45	0.13	5.31	2.68	0.13	4.85

Means with different superscripts within the same row signify: ^{ab} = $p < 0.05$; ^{ac} = $p < 0.01$; ^{ad} = $p < 0.001$.

Spermatozoa mobility is an important factor that ensures the movement of sperm through the female reproductive tract after sexual contact. Typically, when mobility is weak, it is associated with low fertilization rates in many mammal species, including rams. According to the obtained data, it is observed that mobility had very good values in all analyzed stages. This is due to the fact that the food provided to all rams met their daily nutritional requirements, and the provided sexual rest was balanced.

At the MP stage, no significant differences are observed between the two groups. However, after the introduction of VM supplements into their diet, mobility recorded higher values in L2 (Table 4). In the samples collected at SM, which is after a 60-day period during which the rams in L2 received VM supplements, mobility differed between the groups, being 87.80 ± 1.93% in L2 and 81.87 ± 1.77 in L1, with the difference being significant ($p < 0.05$).

Testosterone levels decreased in L1 during the MP and SM intervals, while in L2, they increased from the beginning of the preparation period (MP) from 2.31 ± 0.27 ng/mL to 3.18 ± 0.27 ng/mL. At the start of the reproductive activity, the testosterone level was highly significant ($p < 0.001$).

3.4. Behavior Type and Fertility of Rams

The study of behavioral patterns during the SM period was based on a realistic assessment of the manifestation of typical reflexes, the desire to mount, and the average time that elapsed from introduction into the pen until the mounting took place (Table 5).

Table 5. The type of behavior in rams.

Manifested Behavior	Grade	L1		L2	
		n	%	n	%
Excellent—Typical behavior; eager to breed upon introduction into the testing pen, restless, challenging to restrain, showing an immediate desire to mate.	5	3	20.00	9	60.00
Very good—Optimal sexual reflexes, the ram shows a desire to breed within a maximum of 3 min, although it can be challenging to restrain.	4	6	40.00	4	26.66
Good—Upon introduction to the pen, the ram sniffs around, breeding occurs within the first 5 min, and it is relatively easy to control.	3	4	26.66	2	13.34
Poor—Sniffs the ewe for a few minutes, the time taken for breeding extends, the ram is more docile, and easier to handle.	2	2	13.34	0	0
Very poor—Displays delayed and incomplete reflexes, mounting time extends to approximately 10 min.	1	0	0	0	0
Total		15	100	15	100
Statistical data (Mean ± Stdev)		$3.66^{a} \pm 0.95$		$4.46^{b} \pm 0.55$	

Means with different superscripts within the same row, between groups, signify: ^{ab} = $p < 0.05$; n = the number of rams; grade = the grade offered when the rams' behavior is evaluated.

Data analysis indicates the existence of an uneven distribution of the number of rams that received scores associated with good reproductive quality, higher than 3 points. The average score obtained by L2 was approximately 17.937% higher than L1, and it was statistically significant ($p < 0.05$).

The fertility of rams is an important aspect in sheep farming activities. Ram fertility is influenced by seasonal factors, and only those that undergo an adaptation period can achieve very good results. Analyzing this trait is crucial because it depends on both the number of mounted and pregnant females and the number of lambs obtained from each ram used for reproduction. The fact that each ram in L2 mated more females than those in L1 (+10.10%) and that the number of lambs obtained at birth was higher by 12.27% in L2, compared to L1, confirms that the fertility of the rams was influenced by the experimental factors (Table 6).

The higher value of fertility in the second batch (L2) is confirmed by the fact that the rams in this batch mated with approximately 93.10% of the total number of ewes assigned in the breeding program for the Botoșani Karakul sheep breed, while those in the first batch (L1) mated with only 83.69% of the total number of ewes.

Table 6. Ram fertility in relation to mating ewes and the lambs obtained.

	Pregnant Ewes				Lambs Obtained		
Batch	**Mean**	**±St. Dev**	**CV%**	**% of Mounted Ewes**	**Mean**	**±St. Dev**	**CV%**
L1	29.29^{a}	2.29	7.78	83.69	29.41^{a}	2.24	6.46
L2	32.59^{d}	1.78	5.57	93.10	33.53^{d}	1.67	5.15

Means with different superscripts within columns, between groups, signify: ^{ad} = $p < 0.001$.

3.5. The Effect of the VM Supplement on Economic Indicators

Since the research was conducted in a research station that also serves as a technology transfer facility for other farmers, an economic analysis was performed for the main

production indicators (Table 7). The accounting values reveal the fact that all costs related to maintenance, feeding, electricity, and additional VM administration were only 6.18% higher in Batch 2 (L2).

Table 7. Economic aspects and variable total costs.

Economic Indicators	Measure Unit	L1	L2	The Difference between Groups	
				±	%
Fertilized ewes	n	440	489	49	+10.02
Obtained lambs	n	441	502	61	+12.15
Costs/ram/day	€	3.25	3.36	0.11	+3.27
Costs/ram/year	€	1186.25	1264.40	78.15	+6.18
Income from lambs	€	33,075	37,650	4575	+13.8
Income per ram	€	2205	2510	305	+13.8

n = number of rams; L1 = group 1, L2 = group 2.

However, these additional costs that resulted from the experimental factors that were applied are compensated by the economic indicators, such as the increased number of ewes mated in Batch 2 (+10.02%) and a higher number of born lambs (+12.15%).

While the cost per ram per year increased by 6.18% due to specific feeding investments in the L2 group, the generated income also increased to a greater extent (13.8%) when compared to the L1 control group. This justifies the use of stimulating feeding in breeder rams.

4. Discussions

4.1. Live Weight (LW) and Body Condition Score (BCS)

The determination of LW (live weight) performed at the beginning of the MP and SM periods indicates that the differences between the batches are small and statistically insignificant ($p > 0.05$), suggesting that the VM supplements did not cause significant variations. However, it is worth mentioning that at the end of the season (EM), rams in Batch 2 had a higher LW by 4.7 kg compared to Batch 1. This can be explained by the better mobilization of body reserves in Batch 2 during the previous periods and the contribution of the additional VM complex. Therefore, after the end of breeding season (EM), the reduction of body weight was better managed by the rams in Batch 2, as the difference in LW was superior and highly significant ($p < 0.001$).

In practice, these differences are due to the fact that the diet for batch L2 was aimed at providing an additional level of certain bioactive and semen-forming substances. Energy and protein deficits are well-known causes of reproductive disorders in domestic animals [10]. The supplementation of the diet in batch L2 had a positive effect and better supported the effort during mating. Under similar conditions but with the provision of an additional daily VM diet, it was observed that in L2, live weight increased by only 2.8% from the beginning of mating, while in L1, it increased by only 1.8%. The increased energy and crude protein facilitated the replenishment of body reserves, having a positive effect on BCS (Body Condition Score), as during the preparation period for mating, the body condition improved from 2.50 points to 2.83 points in L1. In L2, the addition of bioactive substances to the diet not only led to higher LW but also improved BCS, as a BCS of $\geq$3 points were achieved at the beginning of the SM period.

What is interesting is that although the rams had the same mating program, the loss of live body mass due to the effort made during the mating period is different. While at the beginning of the mating period (MP), the live weight (LW) between the batches was relatively close (87.49 $\pm$ 2.39 kg in L1 and 87.92 $\pm$ 2.31 kg in L2), at the end of the mating

period, there is a difference of 4.48%. The evaluation of the body condition at the end of the mating period (EM) indicates a significant difference ($p < 0.05$) between the two groups.

In the case of rams, the lack of mineral and vitamin complexes (MV) can lead to the onset of pathologies that will affect reproductive activity, frequently affecting fertility. Similarly, feeding rations with excess salt can have negative effects on growth rates in young animals and on the reproductive function of rams and ewes, as it can lead to hormonal imbalances, such as testosterone, FSH, LH, and leptin [24].

The results obtained from the evaluation of SC (Table 3) indicate a certain evolution in the mean values obtained during the period when these determinations were made. Considering that both groups of rams received similar conditions throughout the research, we can conclude that these trends in mean values for the analyzed traits are due to the experimental treatment applied only to L2. The increased SC in the L2 rams suggests better support for the spermatogenesis process, with a positive effect on ram fertility. Additionally, at L2 during the SM, the average SC value was higher by 4.57%, which also led to a 10.20% increase in the volume of seminal fluid collected at that time.

According to these data, the annual evaluation of breeding rams should include an SC assessment because the size of the scrotum is considered an important trait in selecting breeding rams [25]. This statement is supported by the fact that in other research, a positive genetic correlation was found between scrotal circumference and semen volume, as well as sperm mobility [26].

The volume of ejaculate (Ev) was one of the traits determined immediately after collection. The statistical analysis of the data shows variations in the volume during the three collection periods. Regardless of the collection moment, the highest average values were obtained from the L2 rams (Table 3). While the differences between groups were very small and not significant during the MP phase, in the SM period, average values of 1.58 ± 0.24 mL for L2 and 1.30 ± 0.11 mL for L1 were recorded, with a highly significant difference ($p < 0.001$). At the onset of the breeding season (MP), the volume increased by over 17.72% in L2, and the difference between groups was of the same level of statistical significance ($p < 0.001$). At the end of the reproductive season (EM), based on samples collected in the last days, a significant difference ($p < 0.05$) in Ev was observed between the two groups. Essentially, the differences in semen volume were supported by the additional VM complex administered to L2.

Similar data have been reported by other research groups. For example, in research conducted on sheep in Mexico, results confirmed that supplementing the daily diet with organic zinc (Zinpro Performance MineralsTM) at a rate of 70 ppm/day above the basal diet (containing 19.6 ppm Zn) significantly influenced not only sperm concentration but also the volume, which increased from 0.69 mL to 0.97 mL [27].

The color was a distinct element, and the final data indicate an intensification of color in L2 and the appearance of different color shades between the groups. This change in the basic color and the appearance of several modifications lead to very significant differences ($p < 0.001$) and are due to the increased sperm concentration. Between the groups, the color of the sperm showed a non-significant variation in samples collected during MP ($p > 0.05$), with average scores of 2.733 ± 0.594 for L1 and 2.867 ± 0.516 for L2 (from yellowish to slightly creamy). Samples evaluated after collection from SM and EM showed that L2 had semen with a color associated with good quality (from milky white to creamy). This indicates that the administration of VM more effectively supported the spermatogenesis process. In both assessments, it was found that the differences between the groups were highly significant ($p < 0.001$).

Furthermore, the appearance of these differences also indicates a much better quality of semen color in L2 as a result of the VM supplements that significantly improved many of the seminal material traits, including color. Similar data have been reported in other research as well. For instance, in Bangladeshi rams, the color of semen varied significantly ($p < 0.05$) among rams, ranging from 1.9 ± 1.0 to 4.0 ± 0.0 (from yellowish to creamy). Some rams significantly ($p < 0.05$) exhibited semen of good quality (from milky white to

creamy), while others significantly ($p < 0.05$) presented atypical color after undergoing the same experimental design [28].

pH as an indicator of sperm acidity or alkalinity, is an important trait in the analysis of semen quality in mammals. The obtained mean values fall within relatively narrow ranges, and there are no significant differences between the two groups. The pH of the seminal fluid remained at a constant level very close to pH = 7, a level that falls within the variability limits cited in other bibliographic sources. For the pH of semen determined in various ram breeds, the range of variation is reported to be between 5.9 and 7.3 [29,30]. In the case of Dorper ram groups, the mean values for pH semen ranges from 6.7 ± 0.3 to 6.9 ± 0.2 for samples collected with an artificial vagina and also for those collected using an electro-ejaculator [31]. From these data, it can be assumed that using an electro-ejaculator during collection did not overly stimulate the accessory glands, which would normally lead to a more alkaline semen pH [29].

The mean values obtained for both groups indicate that the pH falls within the cited limits and those determined in Karakul Botoșani sheep breed [32]. The fact that semen pH is at a level that supports the content of epididymis and accessory gland secretions at an almost constant level is eloquent evidence that the spermatogenesis process is proceeding normally, with mean values at the same level in any of the three periods, and the observed differences were not statistically significant ($p > 0.05$).

4.2. The Quality of Sperm

For a real evaluation, at least two, and occasionally three, semen analyses are necessary, each obtained after a certain interval of several weeks. Only in these conditions can conclusive data be obtained to certify the semen quality of a male [33]. In line with this suggestion, in the present research, it was decided that all determinations be made at different times (MP, SM, and EM) placed in the vicinity of the period in which rams are prepared for mating.

Sperm concentration is influenced by the diet and the additional administration of bioactive substances in L2. The determinations made on the collected samples indicate a highly significant increase ($p < 0.001$) in the number of sperm in L2 in the samples collected during SM and EM. In samples collected during MP, the difference between the two groups is only 1%, and it has no statistical significance ($p > 0.05$). This can be explained by the fact that both batches received the same experimental treatment until the onset of MP. Therefore, in conditions where the experimental treatment was similar until the beginning of MP, it is clear that a daily supplementation of feed with VM played an extremely important role, positively influencing not only the quantity but also the concentration of the collected seminal material during SM.

This is possible because vitamins and some mineral salts are associated with increased male fertility by providing the appropriate nutrients for the reproductive system. The role of vitamins (A, D, E, etc.) is not fully understood and still represents an area where future research will bring new findings. However, since 1972, [31] it has been demonstrated that a vitamin A deficiency in mammals induces several negative effects (inhibition of spermatogenesis, reduced testicle size, and decreased testicle volume), affecting male fertility. Currently, very few farmers supplement the daily ration of rams with carrots or other feeds rich in carotene around the breeding season. As for the role of calcium (Ca), caution is needed because excessive calcium intake can disrupt reproductive function by inhibiting the absorption of various microelements (P, Mg, Zn, Cu, etc.) in the intestines. The dietary calcium-phosphorus ratio should be at least 2:1 and should not exceed 7:1. Ratios lower than 2:1 can increase the prevalence of urinary calculi [34].

Live spermatozoa are a trait influenced by various micro and macronutrients, much like sperm production. This was the reason for using the VM complex, and the results obtained have demonstrated that the experimental design was appropriate. The proportion of live spermatozoa is quite similar at the beginning of the MP, with 80.53 ± 2.29 in L1 and 80.73 ± 1.10 in L2, with no significant differences noted. However, after the MP period, the

effect of VM leads to highly significant differences ($p < 0.001$) in the total number of live spermatozoa between the batches. By the end of the breeding season (EM), the proportion of live spermatozoa in L2 is 2.53% higher (79.60 $\pm$ 0.99 in L1 and 81.67 $\pm$ 2.26 in L2), with the difference being significant ($p < 0.01$).

The administration of the VM complex played a significant role in achieving these significant differences. Many scientific articles affirm that the beneficial effect of certain salts and vitamins on spermatogenesis is extremely important. For example, vitamin E supports sperm production in rams primarily through its antioxidant effect. The results obtained in the research conducted on Botoșani Karakul sheep breed rams are consistent with other scientific studies where similar values have been obtained. For instance, in an extensive scientific study where rams were treated twice a week with 5 mg sodium selenite and 450 mg vitamin E for 1 month, both the quality and quantity of sperm were significantly affected by the treatments. In the end, improvements were noted in ejaculate volume and mass activity. This treatment significantly influenced ($p < 0.01$) sperm concentration, which increased in the treated rams compared to the control group, and the proportion of dead and abnormal spermatozoa was lower in the treated groups [35].

Abnormal spermatozoa represent the most serious factor affecting male fertility. A high proportion of spermatozoa showing irregularities and non-conformities significantly affects the fertility of ewes. The obtained results highlight certain differences. The fact that the average values were higher in L1 suggests that the VM complex was effective and contributed to obtaining seminal material with a considerably reduced proportion of abnormal spermatozoa. Confirmation is provided by the fact that by the end of the breeding season (EM), the proportion of abnormal spermatozoa in the seminal fluid collected from L1 was approximately 6.54% higher than in L2, and this difference was statistically significant ($p < 0.01$).

It is likely that the administration of VM supplements, particularly the introduction of vitamin A, played a major role in achieving these differences and improving fertility. The effect of vitamin A and its metabolites has been highlighted in other scientific studies, demonstrating that consistent administration of this vitamin plays an essential role in improving the reproductive performance of rams [34–37]. Among the effects associated with vitamin A administration are improvements in spermatogenesis, sperm quality, libido, and stimulation of testosterone secretion. Other scientific studies specify that vitamin E plays a crucial role because it is associated with antioxidant enzymes and thus contributes to maintaining the functional competence of sperm exposed to oxidative stress. This vitamin also contributes to increased sperm viability and reduces lipid peroxidation when subjected to oxidative stress inducers [38].

Mobility is ensuring the migration of spermatozoa through the female reproductive tract and aiding them in penetrating the ovum. When mobility is reduced, it is associated with low fertilization rates in many mammalian species, including rams. Therefore, mobility is one of the most important parameters used to assess the in vitro quality and function of ram sperm [39]. Determining the mobility of spermatozoa in samples collected during the breeding season (SM) indicates higher values in L2. The fact that the difference between the average mobility values determined in the two batches was 3.41% ($p < 0.05$) suggests that the administration of VM had a positive influence on maintaining this trait at a higher level in L2 due to the inclusion of the VM complex in their diet.

Testosterone is a hormonal product secreted by the Leydig cells in the testicles. Essentially, the entire process of spermatogenesis is based on cellular events and is dependent on the level of testosterone in the testicles. In the absence of the testosterone receptor or androgens, spermatogenesis fails to progress beyond the meiotic stage [40]. To increase ram fertility, management actions should be introduced to support an increase in the blood testosterone level during periods within the intense sexual activity season of rams.

Blood sample analysis indicates similar average concentration levels per ml during the breeding season's preparation phase (MP), with no statistical significance between batches. However, an analysis of the data obtained from samples collected during the SM phase

indicates a higher level of testosterone in the rams from L2. In L1, the average value was 2.47 ± 0.11 ng/mL, while in L2, it was 3.18 ± 0.27 ng/mL, and the difference was highly significant ($p < 0.001$). The same statistical significance was obtained in the determinations made on samples collected during the EM phase ($p < 0.001$).

The results confirm that, in the case of the L2 rams, the additional feed conditions with VM and housing in covered and ventilated spaces have facilitated a better expression of the hormonal secretion process. Our results are in line with the trend of values cited in the scientific literature, which address similar aspects. Therefore, Casao et al. in 2010 [41] concluded that throughout a calendar year, there are certain monthly variations in serum testosterone, with a decrease after the winter solstice and a minimum level reached in May (testosterone) or June and July (melatonin). After this reduction, there is an increase, with a maximum level reached during the natural autumn season ($p < 0.01$). The content of testosterone in the seminal plasma of rams reached a minimum in May, subsequently increasing to a maximum level of 35.52 ± 8.71 ng/mL in November ($p < 0.01$). Both melatonin and testosterone values showed a seasonal variation ($p < 0.01$), with low levels outside the breeding season and high concentrations during the natural breeding season.

The lower values recorded outside the breeding season (December to June) are likely due to the lower temperatures during the winter season. There is scientific evidence confirming that prolonged heat stress leads to a decrease in testosterone concentrations in the blood of rams, with negative effects on spermatogenesis and sexual behavior. [10,12,15].

4.3. Male Sexual Behavior and Fertility of Rams

The study of the sexual behavior of animals, the approach, and all the changes that precede fertilization have aroused the interest of ethologists. To achieve positive results, the ram must be young, strong, healthy, and well-nourished. These individuals are the ones actively engaged in reproduction [32,42].

The results obtained confirm the hypotheses suggested by other scientific studies, which highlight the wide variability in the type of behavior exhibited by rams. Based on the scores obtained for the evaluation of the type of behavior displayed, an average score of 3.66 ± 0.95 was observed for L1 and 4.46 ± 0.55 for L2, with a significant difference ($p < 0.05$). The results show that all the rams in L2 received scores of three points (13.33%), four points (26.66%), and five points (60%) in the behavior evaluation.

If we only analyze the group of rams that scored less than 3 points, we observe that in L1, this group represented a proportion of 13.34%, while in L2, all 15 rams exceeded this minimum score. Achieving such differences suggests that those in L2 exhibited more intense behavior associated with being very good reproducers. This claim is further supported by values obtained for other breeds and under different conditions. For example, some studies have demonstrated that behavioral and physiological responses to positive stimuli or aversive handling were not directly linked at the group level, as some individuals responded more intensively to one type of stimulus while others responded differently to another type. The perception of individual behavior is based on a relatively wide combination of exhibited behavior types, influenced by the individual temperament of each ram and how each animal responds either to isolation from or the presence of a female.

The fertility depends on the relationship between parents, namely, the number of ewes assigned to a ram. The experimental plan followed the breeding program for Botoșani Karakul sheep breed, which recommends a ratio of 1 ram to 35 ewes. This ratio falls within the range specified by other bibliographic sources, which recommend proportions of one adult ram to 50 ewes during the autumn breeding season or one ram to 25–33 ewes during the off-season. Maintaining these specific ratios does not affect ram fertility.

The virility and breeding capacity of the rams were superior in L2 because each ram mated with approximately 32 out of 35 ewes, with a highly significant difference compared to L1 ($p < 0.001$). In terms of the total number of lambs obtained from the mated ewes, a highly significant difference between the groups is observed ($p < 0.001$), as the rams in L2 produced 12.27% more lambs from the inseminated ewes.

All the data confirm that the additional supplementation of VM had a positive influence on ram fertility. Some bibliographic sources state that the inclusion of certain minerals in the diet or their reproductive applications has improved the quality of seminal fluid, ram fertility, and their mating behavior. It has enhanced the antioxidant status, increased serum testosterone levels, and reduced abnormal spermatozoa [43].

5. Conclusions

Rams' dietary stimulation with a supplement of liposoluble vitamins (A, D, E) and of certain micro-elements (Zn, Fe, Mn, Cu, Co, I, Se) significantly improved the overall body condition and, mostly, the reproductive performance due to improvement of ejaculate volume, of semen quality, of testosterone level, of mating behavior, and of fertility traits.

According to our findings, an income increase of almost 14% was generated by the supplemental feeding of rams, based on generated costs of about 6%, economic values accepted by sheep breeders.

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Data Availability Statement: Detailed data that generated the results presented in this study are available on request from the corresponding author.

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Influence of Guar Meal from Pig Compound Feed on Productive Performance, Nitrogen Metabolism, and Greenhouse Gas Emissions

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Abstract: Guar (*Cyamopsis tetragonoloba*) is an annual legume tolerant to drought. Guar meal (GM) is a protein- and carbohydrate-rich co-product generated after the mechanical separation of the endosperm from the germ and hull of guar seed. GM has received considerable interest in animal feed as an alternative to soybean meal (SM). In this study, we aimed to assess the nitrogen (N) balance indicators, performance, carcass traits, and main greenhouse gas (GHG) emissions resulting from enteric fermentation (E-CH_4) and manure (M-CH_4 and N_2O). Two tests were performed: (i) a biological trial on 45 pigs (15 animals/group) and (ii) a digestibility test in metabolism cages (N = 15, 5 replicates/group). Three different diets were given to the pigs: one diet was based on 0% GM (SM diet); in the second, GM-50%, GM replaced 50% of the SM; and the third was GM-100%, in which GM fully replaced the SM. The GM and SM diets were analyzed for their proximate composition. A model based on prediction equations was used to estimate the GHGs. GM up to 10% in the diets of finishing pigs did not significantly impact growth performance or carcass traits, although a slight increase in neutral detergent fiber (NDF) was observed. GM up to 10% improved N digestibility ($p < 0.0001$), net protein utilization ($p < 0.0001$), the biological value of protein, coefficients of metabolizability, and the coefficient of the total tract's apparent digestibility. Irrespective of its dietary proportion, GM decreased total nitrogen output (TNO, $p = 0.11$). A highly significant impact was noted for N_2O and E-CH_4 (for DM, $p < 0.0001$), as well as a significant impact for E-CH_4, expressed as g CO_2 Eq ($p = 0.007$), and g CO_2 Eq. LU (livestock unit, $p = 0.005$), also reported as ADG ($p = 0.024$). Manure, M-CH_4, was not significantly influenced. In conclusion, GM can replace up to 100% SM and is thus a valuable byproduct that does not alter animal performance and can positively impact N_2O and E-CH_4.

Keywords: greenhouse gas emissions; guar meal; nitrogen; performance; pigs

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1. Introduction

The livestock sector is a notable consumer of natural resources. Classical diets for pigs are based on a mix of maize and soybean meal (SM) as the primary energy and protein-rich feedstuff. However, there is a growing discrepancy between production, availability, and demand [1,2]. Only non-genetically modified (non-GM) soybean is permitted in the

European Union. With this background, supply chain disturbances and reduced packing plant capacity have caused considerable difficulties [3]. Romania is a country that relies on the importation of SM at a fluctuating price. In addition, the frequency of drought might lead to an increased gap between the feed supply and the nutritional needs of animals.

The identification of well-known feedstuffs and the use of locally accessible vegetable resources are required to address feed deficits. High priority has been given to the search for solutions to improve existing feed resources efficiently. The availability of non-conventional forage resources is increasing, although most of these resources need to be more palatable [4]. Guar (*Cyamopsis tetragonoloba*) is an atmospheric nitrogen (N)-fixing annual legume that is drought-tolerant and environmentally friendly. Guar meal (GM) is a co-product of the guar gum industry that is not genetically altered and is characterized by an elevated protein level (around 50%), as well as carbohydrates. GM is composed of germs and hulls that remain after the mechanical separation of the endosperm from the germ and guar seed hulls [5,6]. The nutritional value of GM was described in detail by Biel and Jaroszewska [7]. The protein concentrations in GM range from 33 to 60% and have favorable amino acid profiles. On the other hand, anti-nutritive components such as guar gum (mannan), saponins, and trypsin inhibitors limit GM's usage in animal feed [8].

The anti-nutritional properties of GM, as well as its low palatability, have led to doubts about the use of this byproduct. However, thermic treatment and limiting the levels of inclusion can eliminate these drawbacks. According to Abdel-Wahab et al. [9], a reduction in the levels of certain anti-nutritional factors (e.g., β-mannan and saponin) can positively affect the health and performance of buffalo. In addition, this co-product is a natural feed ingredient as no chemicals or preservatives are used to obtain it. Previous studies highlighted changes in some growth, meat quality, and health parameters when using GM in poultry [10–12], cattle [13], sheep [14,15], goat [16], and buffalo [9]. Karpiesiuk et al. [2] and Hasan et al. [4] investigated the effects of using dietary GM supplementation as a cost-cutting technique on the performance and nutrient metabolism of pigs. Nonetheless, it remains critical to research the effects of using the GM co-products that remain after guar gum extraction on greenhouse gas (GHG) production.

Pig farming is growing steadily in terms of its complexity, industrialization, and intensification. The gases produced in the pig house impact not only the health and efficiency of the pig sector but also human health and quality of life. Relevant hazardous gases include gaseous N and its compounds such as nitrous oxide (N_2O) and ammonia (NH_3). Consequently, GHG emissions linked to global warming potential (GWP) have provoked public concern around the globe. Pork is one of the most commonly consumed meats [17]. According to Bälter et al. [18], food of animal origin emits more GHGs than feed of vegetable origin. Pig farming has a large impact on GHG production, especially enteric CH_4 (E-CH_4) and manure-based CH_4 (M-CH_4) and N_2O in manure, while carbon dioxide (CO_2) emissions are considered zero since plants reuse this gas through photosynthesis [19,20]. According to FAO [21], pigs produce lower E-CH_4 emissions (avg. 11%) than ruminants (more than 90%), but there are higher CH_4 emissions in their manure (more than 69% compared to ruminants, which produce less than 8%). The manure storage of N_2O is more pronounced among chickens (avg. 66%) and pigs (avg. 20%) compared to that among ruminants (less than 7%) [21].

The reduction in GHG emissions via animal feed and manure management is a significant problem for sustainable pig farming. Biogenic CH_4 is a volatile organic compound produced by bacteria in pigs' digestive tracts (E-CH_4) and feces through the anaerobic breakdown of organic matter [22,23]. The GWP of CH_4 is 25 times greater than that of CO_2 [24]. The microbial process in the N cycle and manure carbon content, along with the time required for storage and treatment, determines the amount of N_2O emitted during storage and treatment [19,25]. The GWP of N_2O is 298-fold greater than that of CO_2. N_2O originates only from manure [26] and accounts for about 26% of N_2O production.

Taking into consideration the factors mentioned above, this study aimed to test two hypotheses: 1. The total substitution of SM with GM will affect the growth performance

and carcass characteristics of pigs; 2. GM has the potential to reduce N from manure and the main GHG emissions (CH_4 and N_2O) in growing–finishing pigs.

2. Materials and Methods

The trials took place in the IBNA Balotesti experimental Biobase located in Ilfov county in the southeastern part of Romania. This area is located in the central-eastern part of the Walachia Plain and is characterized by a temperate–continental climate, with dry and hot summers and cold winters.

Two experiments were carried out following protocol 7976/12/12, authorized by the Ethical Committee of the IBNA Balotesti and pursuant to Romanian Law No. 199/2018, which complies with the EU Directive 2010/63/EU on animal research.

GM is a protein- and carbohydrate-rich co-product generated after the mechanical separation of the endosperm from the germ and hull of natural guar seed that is broken and heat-treated (roasted for 3 min at 120–130 °C) to improve digestibility and palatability. GM has high nutritional value and a similar or reduced cost to SM. Section 3.1 provides a detailed comparison between GM's composition and that of soybean meal.

2.1. Animals and Housing

Experiment 1. Experimental design.

This study used a total of forty-eight healthy, crossbred finishing Topigs pigs ((female Large White × Hybrid (Large White × Pietrain) × male Talent (mainly Duroc)), (2 replicates/group; 8 pigs/pen), 69.73 ± 0.77 kg initial body weight (BW), 120 ± 5 days old, with a similar sex ratio (mixed, with 4 ♀and 4 ♂in each pen), and ear-tagged individually. The pigs were randomly assigned to three feeding groups for 35 days in a grow–finish shelter with strict environmental controls (21 °C; 60% relative humidity). The pigs received experimental diets for 35 days.

Experiment 2. N digestibility.

Following the procedure outlined in the law, using individual steel cages in an atmosphere-controlled room, a metabolic test was conducted over 21 days (7 days for accommodation) to assess N metabolism. A total of 15 barrows (Topigs hybrid pigs, BW avg. 89.6 kg ± 2 kg) were selected and split into three groups (5 replicates/group). Previous research has shown that three to six animals per group are statistically adequate for digestibility trials [27]. The pigs were individually housed and weighed. The digestibility trial weeks were divided into two balance periods (4 sampling days per period).

2.2. Treatments

To evaluate growth performance and carcass traits and estimate the main GHGs (CH_4 and N_2O), the GM and SM were analyzed for ether extract (EE), crude protein (CP), crude fiber (CF), amino acids, and minerals before they were included in the diets.

The composition and nutritional characteristics of the diets are outlined in Table 1. Throughout both experiments, the diets remained the same. Three feeding treatments that met the nutritional requirements of the Topigs hybrid were formulated: (1) The control group (SM); (2) GM-50%, where 50% of the SM was replaced with GM; and (3) GM-100%, in which the SM was completely replaced with GM. The diets included crystalline amino acids, DL-methionine and L-Lysine-HCl, to meet the requirements for all three diets, as well as calcium carbonate and monocalcium phosphate to provide the Ca and P requirements. The fiber level was about 3.8% higher in the GM-100% diet vs. that in the SM and GM-50% diets; NDF was +8.8% higher in GM-50% vs. the SM diet, with an increase of 17.06% in the GM-100% vs. SM diet.

Table 1. Experimental diet composition based on two levels of GM that replaced 50% or 100% of the SM in the diets.

Items (g * kg feed^{-1})	SM	GM-50%	GM-100%
Maize	499.1	520.3	517.3
Wheat	150.0	150.0	150.0
Rice bran	110.0	110.0	110.0
Soybean meal (44%)	120.0	50.0	-
Guar meal	-	50.0	100.0
Sunflower meal	80.0	80.0	85.0
DL-methionine	0.9	0.6	0.1
L-Lysine-HCl	2.4	2.1	1.2
Calcium carbonate	16.7	16.2	15.4
Monocalcium phosphate	5.9	5.8	6.0
Sodium chloride	4.0	4.0	4.0
Choline premix	1.0	1.0	1.0
Vitamin and trace mineral mixture [1]	10.0	10.0	10.0
Analyzed composition (g * kg^{-1} as feed bases)			
DM	881.0	882.0	883.0
CP	155.2	151.6	155.9
EE	38.8	39.9	40.5
Crude fiber	47.6	47.7	49.5
NDF	139.5	153.0	168.2
ADF	57.5	63.0	70.5
Ca	8.0	8.0	8.0
P	6.0	6.0	6.0
Lys	8.8	8.8	8.8
Met + Cys	6.7	6.7	6.7
Calculated composition (g * kg^{-1} as feed bases) [2]			
ME, Mj as feed basis	12.6	12.8	12.9
N	24.83	24.26	24.94
Lys d	7.3	7.4	7.6
Met + Cys d	5.6	5.6	5.7

Abbreviation: dry matter (DM), crude protein (CP), metabolizable energy (ME), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), lysine (Lys), methionine + cysteine (Met + Cys), calcium (Ca), phosphorus (P), digestible (d). Diets 2 and 3 were formulated with the addition of GM to replace 50% (GM-50%) and 100% (GM-100%) of the SM in the control diet (SM) and to adjust the CP, respectively. [1] The vitamin–mineral premix administrated per kg of feed: (i) 6000 IU vitamin A; 800 IU vitamin D3; 20 IU vitamin E; 1 mg vitamin K3; 1 mg vitamin B1; 3.04 mg vitamin B2; 10 mg vitamin B3; 6.3 mg vitamin B5; 1.5 mg vitamin B6; 0.03 mg vitamin B7; 0.3 mg vitamin B9; 0.02 mg vitamin B12; 30 mg Mn; 80 mg Fe; 25 mg Cu; 100 mg Zn; 0.22 mg I; 0.22 mg Se; 0.3 mg Co; 60 mg antioxidant. [2] To calculate N content, we used a factor of 6.25; the ME was calculated using regression equations based on the chemical characteristics (ME = 5.01 DP + 8.93 EE + 3.44 GF + 4.08); for the calculation of digestible amino acid levels, the feed chemical composition and theoretical coefficients were determined by IBNA Balotesti. Feed was provided twice a day at 08:00 and 14:00 h. Water was available ad libitum.

2.3. Measurement and Sampling

The BW of each pig was recorded on an electronic scale at the start and end of the biological and digestibility trials. The pigs were fasted overnight before being weighed, and blood was collected to minimize postprandial nutrient contents. The average daily gain (ADG) and feed conversion ratio (FCR) were computed based on BW. We used the equations of Diaz et al. to calculate the Kleiber ratio (KR), relative growth rate (RGR, %) based on ADG, and metabolic BW ($MBW^{0.75}$), along with BW and age [28].

The carcass traits (backfat thickness, *Longissimus dorsi* area, and lean meat percentage) were determined on the left side using a PIGLOG 105 ultrasonic apparatus (SFK-Technology, Denmark) fitted with a formula for assessing meat percentage:

$$Y = 64.39 - 0.28 \text{ Fat-1} + 0.14 \text{ LD muscle thickness} - 0.55 \text{ Fat-2}$$

where LD is the *Longissimus dorsi* muscle. Fat-1 was measured 7 cm sideways behind the last rib from the middle dorsal line, whereas fat-2 was measured 10 cm from the last rib to the cranial section and 7 cm sideways from the middle dorsal line.

During the digestibility trial, samples were collected 4 days/week (two balance periods) from 08.00 h to 09.00 h to determine the N contents of the feces and urine. After weighing the total urine collected, 10% aliquots of urine were subsampled and stored at 4 °C for analysis. H_2SO_4 at a concentration of 25% was added to each urine container to decrease the pH and diminish nitrogenous component volatilization. In the same way, after weighing the total feces samples, a quantity of about 10% ± 0.4 g was subsampled and stored at 4 °C prior to the N analyses. The samples were processed according to the procedure outlined by Hăbeanu et al. [29]. In the same manner, a blank digest was used. The samples were digested in the presence of catalyzers with H_2SO_4 and then distilled and titrated. Class A glassware was employed for transvasation, dilution, and storage. The total N production was estimated after measuring N intake and excretion.

Blood samples (~6 mL) were taken via jugular venipuncture in heparin tubes (2 replicates per animal). Then, the samples were centrifuged for 15 min at 3000 rpm (iFuge D06, Neuation Technologies Pvt. Ltd., Gujarat, India), and the plasma obtained was placed in Eppendorf safe-lock tubes and stored in a freezer at −20 °C until urea nitrogen (PUN) analysis (Spotchem EZ SP-4430 chemistry analyzer).

2.4. Analytical Laboratory Procedure

The proximate composition of the GM, SM, and diets was assessed at IBNA Balotesti in duplicate for dry matter (DM), CP, EE, CF, and their fractions compared to neutral detergent fiber (NDF) and acid detergent fiber (ADF), using the procedures outlined in Commission Regulation (EC) no. 152 (OJEU, 2009). Briefly, the semiautomatic traditional Kjeldahl technique, using a Kjeltek auto 1030 Tecator (SR EN ISO 5983-2:2009 [30] AOAC 2001.11 [31]), was employed to determine the CP. CF extraction was performed using an intermediate filtration method (standard SR EN ISO 6865:2002 [32]). Van Soest extractions were used to assess NDF and ADF according to SR EN ISO 16472:2006 [33] and SR EN ISO 13906:2008 [34]. A Raw Fiber Extractor FIWE 6 (Velp Scientifica, Usmate Velate, MB, Italy) was used for the analysis.

The amino acid composition was determined in duplicate via high-performance liquid chromatography, using HPLC Surveyor Plus Thermo Electron equipment (Waltham, MA, USA) fitted with a HyperSil BDS C18 column (Thermo Electron, Waltham, MA, USA) using silica sized at 250 mm × 4.6 mm × 5 µm, as previously described by Varzaru et al. [35] and Gheorghe et al. [36].

In the digestibility trial, from the total quantity of collected feces, 10% ± 0.4 g subsamples were taken for analysis.

The samples were digested with H_2SO_4 in the presence of catalyzers to assess the N concentration, followed by distillation and titration. The semiautomatic Kjeldahl method was carried out on a Kjeltec Auto 1030 Analyzer (Hillerod, Denmark). The reagents for mineral concentration were supplied by Merck (Darmstadt, Germany). Class A glassware was used for transvasation, dilution, and storage. Stock solutions traceable to standard reference material (NIST) were used for calibration. N retained (NR), total N output (TNO), and N digestibility were determined by measuring N intake (on a DM basis) and N excretions according to the methods described by Hăbeanu et al. [29] and using equations described by Hlatini et al. [27]. The coefficient of the total tract apparent digestibility (CTTAD), the coefficient of metabolizability (CAM), the biological value of protein (BVP), and the net protein utilization were calculated using the following equations:

$$\text{CTTAD} = (\text{N intake} - \text{fecal N output})/\text{N intake} \quad (1)$$

$$\text{CAM} = \text{N intake} - \text{N fecal output} - \text{N urinary output}/\text{N intake} \quad (2)$$

$$\text{BVP} = \text{N retained}/\text{N digested} \quad (3)$$

$$\text{NPU} = \text{N retained}/\text{N intake}. \quad (4)$$

Blood samples were taken from the jugular vein, placed in heparin tubes (in duplicate), and then centrifuged for 15 min at 3000 rpm to separate the plasma. We used a chemical analyzer (Spotchem EZ SP-4430) to test the N and urea N in the plasma (PUN).

2.5. CH_4 (E-CH_4 and M-CH_4) and N_2O Emissions

In this study, to obtain models for estimating GHGs, the input and experimental output data were incorporated into prediction equations established by the IPCC [19,37] and prior works. The TNO assessed in our feeding trial was integrated into the equation for N_2O prediction proposed by Philippe and Nick [26]:

$$N_2O = \text{TNO} * 0.2/100 * 44/28 \quad (5)$$

where 0.2% is the conversion factor of the N excreted into N_2O (IPCC, 2006) [19] in manure storage pits under animal housing, and 44/28 is the ratio of the molar mass of N_2O compared to that of N.

EvaPig® software, version 2.0.3.2, created by the French National Institute for Agricultural Research, Metex Nvistago, and the French Association of Zootechnie, was used for compound feed verification.

In this work, to determine the E-CH_4, expressed as the CO_2 equivalent (g Eq.CO_2 * day^{-1}), we applied Philippe and Nick's equation [26]:

$$\text{E-}CH_4 = 0.012 * \text{dRes} * \text{DM intake} \quad (6)$$

where dRes are digestible residues.

The IPCC's [19,37] equations were used to estimate manure CH_4 emissions:

$$CH_4, \text{manure} = \text{VS} * \text{B0} * \text{MCF} * \text{fSMD} \quad (7)$$

where VS means solid volatile excretions per day resulting from organic solid manure substances, calculated using the IPCC's [37] Tier 1 method with the following equation:

$$\text{VS} = \text{VSt} * \text{BW} * 1000^{-1} \text{ (kg * day}^{-1}\text{)}. \quad (8)$$

Here, VSt = 4.9, where B0 denotes the maximum CH_4 production in the pigs' manure (0.45 m^3 CH_4 * kg^{-1} VS * 0.67, which is the factor used for converting m^3 into kg; IPCC [37]); MCF is the CH_4 conversion factor for the waste management system used, depending on the climate region, expressed in %; MCF (4% for solid storage in a dry, temperate region; IPCC [37]); and fSMD is the fraction of manure used by each management system. In our study, the solid storage system applied to the entire amount of manure (fSMD = 1).

2.6. Statistical Analyses

IBM SPSS (2011, version 20) was used to describe the experimental data recorded and predicted statistically. A completely randomized experimental design was used to assign the animals to three dietary treatments, with each group having a similar sex ratio of 24 ♀:24 ♂(mixed, with 4 ♀and 4 ♂in each pen). In our model, in the first experiment, the diet was considered a fixed factor, the pen was the experimental unit for measuring intake, and the pigs served as the experimental units for determining the other variables assessed. In the balance trial, each pig was considered an experimental unit. A Shapiro–Wilk test was used to verify data distribution. The impact of diet was regarded as statistically significant if the *p*-value was less than 0.05. The means determined to be significant were separated using an LSD test. Replicate effects were not included in the study as the *p*-value was higher than 0.05. A Pearson test was used to determine the measure of correlation. A strong

correlation was defined as a coefficient value > 0.7, and a moderate correlation was defined as a value below 0.69 and up to 0.5 (Akoglu cited by [38]); higher values were defined as significantly affected.

The group size was established based on Charan and Kantharia [39], where E = Total number of animals − Total number of groups, and E = the degree of freedom of the analysis of variance. In our study, the E value was 45, which is higher than 20. Therefore, including more animals would not increase the probability of obtaining significant findings.

3. Results

3.1. Feedstuff Chemical Composition

Data on the chemical composition of protein-rich sources showed that GM has higher levels of analyzed CP (+15.4%), EE (+43%), and limitative amino acids (AA) (+34% lysine and +42% Met + Cyst) (Table 2). The lysine level was found to be higher in both feedstuffs. The diets were formulated to include 50 g or 100 g GM/kg feed. The addition of GM into the diet increased the NDF and ADF levels. These fiber fractions potentially impacted the responses of the animals.

Table 2. Comparison of the compositions of SM and GM.

Nutrients, %	SM [1]	GM [1]
Dry matter	87.74	90.73
CP	44.0	52.02
EE	1.69	2.98
Fiber	6.29	7.73
NDF	12.44	39.92
ADF	7.45	20.49
Main AA		
Lysine	2.75	4.21
Met.	0.64	1.11
Cyst.	0.67	1.28
Met. + Cyst.	1.31	2.38
Minerals		
Ca	0.20	0.70
P	0.60	0.60

[1] Salmonella, aflatoxins, and PCBs like dioxin below the detection limit.

Throughout the experiments, we did not observe any health problems or the refusal of feed among the animals.

3.2. Growth Performance in the Biological Trial

Irrespective of the GM inclusion level, the carcass traits and growth performance were not significantly affected (Figure 1).

3.3. N Digestibility

Table 3 presents the intake data, mean values, and SEMs for the balance indicators. While N, fiber, and ADF decreased ($p > 0.05$), the NDF fraction increased in the group fed GM, regardless of the percentage of inclusion. This result was reflected in the fecal N composition, with a significant decrease observed in the experimental groups compared to the SM group. However, no influence on urinary N was observed ($p > 0.05$). Therefore, although a decrease in TNO was determined, this decrease was not significant. A significantly higher impact was obtained for N excretion in the % intake, N digestibility, NPU, CAM, and CTTAD indicators.

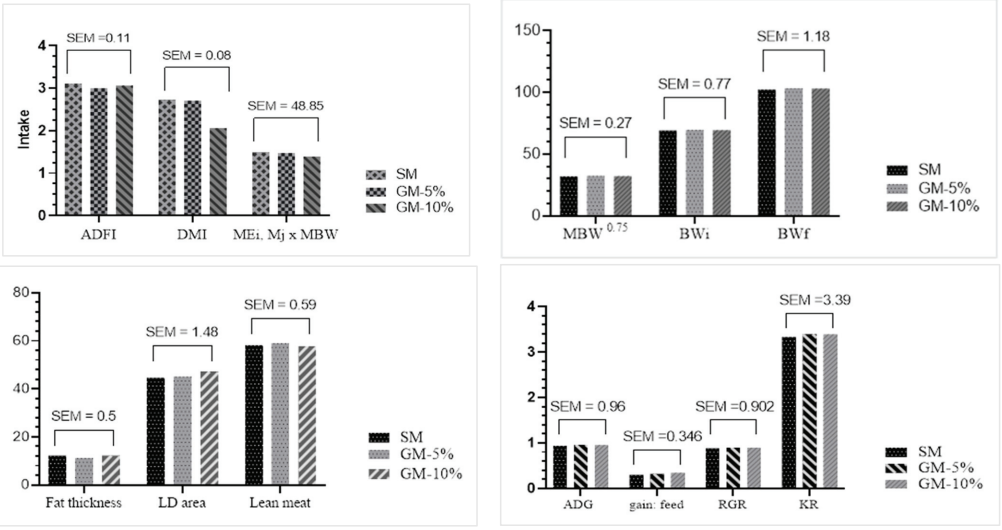

Figure 1. Intake and descriptive statistics of the growth parameters and carcass traits of the fattening pigs fed two levels of a GM diet that replaced 50% or 100% SM (SM diet). $p > 0.05$, with no significant difference between the mean. The measurements were performed with PIGLOG 105 on live animals to determine their carcass traits. The number of observations was 48. Abbreviations: average daily feed intake (ADFI, Kg); dry matter intake (DMI, Kg); body weight (BW, Kg); metabolic BW ($MBW^{0.75}$); average daily gain (ADG, Kg); relative growth rate (RGR, %); Kleiber ratio (KR, Kg); LD, *Longissimus dorsi*; fat thickness (mm); LD area (mm); lean meat (%); standard error of the mean (SEM).

Table 3. N metabolism of the fattening pigs fed two levels of a GM diet that replaced 50% or 100% of the SM diet.

Items [1]	SM	GM-50%	GM-100%	SEM	*p*-Value [2]
Intake, g * day^{-1}					
Feed	2550	2360	2168	86.92	0.18
N	76.96	74.84	72.21	2.44	0.12
Fiber	147.7	146.9	143.6	4.75	0.14
NDF	413.9	399.2	287.8	16.02	0.29
ADF	148.0	141.3	132.8	4.66	0.07
N balance, g * day^{-1}					
Fecal N	8.73 ^{a}	7.08 ^{b}	6.86 ^{b}	0.25	0.01
Urinary N	33.34	33.27	29.86	1.18	0.13
TNO	42.07	40.35	36.72	1.41	0.11
NR	34.89	34.49	35.48	1.06	0.12
N excretion of % intake	54.65 ^{a}	53.80 ^{b}	50.67 ^{c}	0.34	0.007
N digestibility, %	88.63 ^{a}	90.49 ^{b}	90.46 ^{b}	0.16	<0.001
NPU	45.34 ^{a}	46.19 ^{b}	49.33 ^{c}	0.34	<0.001
BVP	51.0 ^{a}	51.2 ^{a}	54.5 ^{b}	0.34	0.05
CTTAD	0.89 ^{a}	0.90 ^{b}	0.90 ^{b}	0.001	0.01
CAM	0.45 ^{a}	0.46 ^{a}	0.49 ^{b}	0.003	<0.001
PUN, mg/dL	26.61	26.84	27.27	0.62	0.19

[1] The number of observations N = 15 (5 replicates per group). Abbreviations: total N output (TNO); N retained (NR); net protein utilization (NPU); biological value of protein (BVP); coefficient of total tract apparent digestibility (CTTAD); coefficient of metabolizability (CAM), plasma urea nitrogen (PUN). [2] $^{a–c}$ Means values with different superscripts in the same row differ, ignificantly ($p < 0.05$), distinctly significant ($p \leq 0.01$), highly significant ($p \leq 0.0001$). Values without letters—insignificant effect ($p \geq 0.05$). SEM—standard error of means.

3.4. CH_4 (E-CH_4 and M-CH_4) and N_2O Emissions Estimated

Table 4 outlines the level of the main GHGs ge nerated via the enteric fermentative process and in the manure. When expressed as DMI bases, N_2O decreased significantly in the GM-50% and GM-100% groups vs. the SM-fed group. A significant decrease was observed for E-CH_4, expressed as the g of CO_2 Eq * day^{-1} (9% lower in the GM-50%-fed group and 21% lower in the GM-100%-fed group compared with the SM diet) and reported as the livestock unit (LU), considered to be 500 kg LW (live weight) based on Philippe and Nick [26] (10% and 22% lower in the GM-50% and GM-100% groups compared to the SM group). The highest influence ($p < 0.0001$) was obtained when evaluated using the DMI bases (8% and 15% lower in the experimental groups vs. that in the SM group). Conversely, the dietary addition of GM did not significantly influence M-CH_4.

Table 4. Mean GHG level (g CO_2 Eq. N_2O, E-CH_4, and M-CH_4) ± SEM for the fattening pigs fed two levels of GM that replaced 50% or 100% of the SM.

Items [1]	SM	GM-50%	GM-100%	SEM	*p*-Value [2]
		Intake, g * day^{-1}			
DMI	2216.0	2078.0	1929.0	7.62	0.19
TNO	42.07	40.35	36.72	1.41	0.11
N ex.	61.80	58.20	51.10	2.14	0.12
N_2O (g CO_2 Eq * day^{-1})	39.41	37.80	34.40	1.32	0.13
N_2O (g CO_2 Eq. LU^{-1} * day^{-1})	193.3	182.75	167.35	9.1	0.09
N_2O (g CO_2 Eq. ADG, kg^{-1} * day^{-1})	42.78	39.44	36.45	1.7	0.19
N_2O (g CO_2 Eq. DMI, kg^{-1} * day^{-1})	14.41 [a]	13.88 [b]	13.36 [c]	0.08	<0.001
E-CH_4 (g CO_2 Eq * day^{-1})	41.87 [a]	38.09 [ab]	33.08 [b]	1.24	0.007
E-CH_4 (g CO_2 Eq. LU^{-1} * day^{-1})	205.39 [a]	184.15 [ab]	160.96 [b]	6.91	0.005
E-CH_4 (g CO_2 Eq. ADG, kg^{-1} *day^{-1})	45.46 [a]	39.76 [ab]	35.04 [b]	1.77	0.024
E-CH_4 (g CO_2 Eq. DMI, kg^{-1} * day^{-1})	15.31 [a]	14.02 [b]	12.90 [c]	0.18	<0.001
dRes (g, as DM bases)	51.05 [a]	46.73 [b]	43.01 [b]	0.61	0.007
VS (g * day^{-1})	501	507	504	5.7	0.71
M-CH_4 (g CO_2 Eq * day^{-1})	151.13	152.91	152.16	1.74	0.92
M-CH_4 (g CO_2 Eq. LU^{-1} * day^{-1})	738.69	738.73	738.66	0.01	0.25
M-CH_4 (g CO_2 Eq. ADG, kg^{-1} * day^{-1})	162.64	160.43	159.88	3.17	0.93
M-CH_4 (g CO_2 Eq. DMI, kg^{-1} * day^{-1})	55.72	58.51	61.65	1.91	0.39

[1] The number of observations N = 15 (5 replicates per group). Abbreviations: daily N excretion rate (N ex.); volatile solids (VS); dRes: digestible residue; [1] considering the global warming potential of 25 for CH4. LU means livestock unit = 500 kg LW (used in [14]). [2] [a–c] Means values with different superscripts in the same row differ, ignificantly ($p < 0.05$), distinctly significant ($p \leq 0.01$), highly significant ($p \leq 0.0001$). Values without letters—insignificant effect ($p \geq 0.05$). SEM–standard error of means.

3.5. Relationship between Input and Output Parameters

The Pearson coefficients from Table 5 show a strong correlation between specific input and output parameters. For example, the level of dietary GM is strongly positively correlated with the % N digestibility and NPU and negatively correlated with the N excretion of the % intake ($p < 0.0001$) and has a high-to-moderate correlation with E-CH_4 ($r = -0.48$; $p < 0.01$). However, for the other input parameters, such as the ADFI, DMI, fiber, and the fractions of ADF and NDF, the N intake presents a strong positive relationship with the TNO, NR, N_2O, and E-CH_4 ($p < 0.0001$) and a negative moderate correlation with BVFP (with r ranging between 0.47 and 0.6 except for NDF).

Table 5. Pearson correlations between input and output parameters.

Pearson Correlation (r)	TNO, g/day	NR, g/day	% N Dig	N Excretion of % Intake	NPU	BVFP	N_2O, Eq CO_2	E-CH_4, Eq CO_2	M-CH_4, Eq CO_2
Level of GM	−0.287	0.043	0.832 ***	−0.886 ***	0.886 ***	0.758 ***	−0.286	−0.487 **	0.045
ADFI	0.991 ***	0.972 ***	0.124	0.473 **	−0.473 **	−0.565 **	0.991 ***	0.937 ***	0.163
DMI g/zi	0.990 ***	0.974 ***	0.130	0.467 **	−0.467 **	−0.560 ***	0.990 ***	0.934 ***	0.164
Fiber intake, as DM bases	0.972 ***	0.992 ***	0.183	0.381 *	−0.381 *	−0.479 **	0.972 ***	0.898 ***	0.166
ADF intake, as feed bases	0.998 ***	0.955 ***	0.019	0.533 **	−0.533 **	−0.603 ***	0.998 ***	0.965 ***	0.154
NDF intake, as feed bases	0.849 ***	0.968 ***	0.456 *	0.105	−0.105	−0.246	0.850 ***	0.711 ***	0.173
N intake, as feed bases	0.989 ***	0.980 ***	0.101	0.452 *	−0.452 *	−0.535 **	0.989 ***	0.933 ***	0.160
N intake, as DM bases DM intake	0.988 ***	0.980 ***	0.098	0.449 *	−0.449 *	−0.530 **	0.988 ***	0.933 ***	0.160

* $p \leq 0.005$—significant difference; ** $p \leq 0.01$—distinct significant difference; *** $p \leq 0.0001$—highly significant difference.

4. Discussion

GM is a concentrated protein source derived as a co-product of galactomannan extraction from guar seed. GM could be considered an appropriate feedstuff for animal feeding since the basic components of animal diets are frequently used in human nutrition. Furthermore, GM is nutritionally comparable to SM and is a reasonably inexpensive and readily accessible feed material that is also processed in large quantities for gum extraction.

This study showed the potential of GM to replace classical SM feedstuff, with a focus on nitrogen metabolism due to the relationship between TNO and N_2O. For the first time, we report data predicting GHG emissions (N_2O and CH_4) resulting from the use of GM.

In previous research, dietary guar meal was not sufficiently studied as an alternative to traditional soybean meal [4]. Previous studies attempted to identify the ideal levels of dietary GM that could be used without adversely impacting performance [40], and few studies have investigated the impact of guar inclusion on carcass characteristics [41]. Anti-nutritional factors are widely recognized to restrict the amount of GM that can be included in the diet. For example, feeding animals can produce certain issues due to anti-trypsin and very viscous non-starch polysaccharides such as galactomannan polysaccharides, which raise the viscosity of the digesta and limit digestive enzyme activity as well as decrease nutritional digestibility [42]. These problems can be reduced by processing the meal; the anti-trypsin factor can be inhibited by toasting and by reducing the level of dietary inclusion. Pigs, chickens, and laboratory animals (rats and mice) have all been proven to suffer adverse effects from galactomannan, which is present in residual guar gum from meal [43]. Guar gum has been shown to inhibit rat and pig glucose absorption, which negatively impacts growth performance. However, research indicates that eating the gum's galactomannans, as found in guar meal, may enhance gut health by reducing the colonization of pathogenic bacteria in the gut. Furthermore, GM contains trypsin inhibitors involved in protein digestion.

In the present study, performance and carcass traits did not yield a significant decrease when including GM in the diet. Even though GM increased the NDF fraction in the diet, we predicted that the pancreas' considerable increase in total enzymatic activity with age would yield an improvement in feed intake and nutrient digestibility. In the literature, most studies focused on broiler chickens. Lee et al. [44] observed a decrease in the BW and feed efficiency of chickens fed with high concentrations of GM, likely due to the existence of residual guar gum in the GM. According to Owusu-Asiedu et al. [45], the ADG and ADFI in pigs decreased with cellulose and guar gum during the first 7 days of being fed a high-NSP diet. Increased non-starch polysaccharide (NSP) content in the pigs' diets directly affected growth rate and voluntary feed intake. Pigs and their microbiota may

adjust to high-fiber diets over a longer period of time. A higher percentage of energy was also digested in the large intestine because of increased dietary NSP, which similarly decreased the total-tract energy digestibility and voluntary feed intake. In 2018, Karpiesiuk et al. [2] partially replaced SM with GM (25, 50, and 75%) in a pig-fattening diet. Pigs in the group fed diets containing 25% GM protein obtained the best performance, as evidenced by the lowest feed conversion ratio and the highest growth rate. On the other hand, Hasan et al. [4] observed a negative impact on ADFI and ADG when using GuarPro F-71 in young pigs' diets as a 75% replacement for SM, which showed a linear decrease as guar inclusion increased but not a decrease in feed efficiency, in conflict with our results and those of Heo et al. [46]. GM diets had no noticeable impact on ADFI in growing–finishing pigs [47]. The ADFI of Yorkshire Landrace pigs decreased when GM was added to their diets. There is a lack of information in the literature about how GM affects the quality of swine meat. Karpiesiuk et al. [41] added GM with 50% and 75% protein to pig diets and reported that the addition of GM may have negatively impacted performance; however, meat quality was not affected (unpublished data).

Milczarek et al. [40] proposed using a dietary GM level of 4% to obtain good performance and improve the carcass composition of broiler chickens, as well as the physicochemical qualities of their muscles. Conversely, in terms of carcass composition, dressing percentage, and carcass muscularity, chickens fed diets with a proportion of GM higher than 12% performed noticeably worse.

Nitrogen (N or its gaseous form, N_2) and carbon (C) are components vital to life. Firstly, N_2 must be transformed by nitrifying bacteria to enter the feed in food chains as part of the N cycle and be used by plants and animals as a nutrient. Some of the consumed N is lost through organic or inorganic excretion. In anaerobic environments, nitrification and denitrification processes produce manure-based nitrous oxide (N_2O). Oxygen encourages the formation of N_2O [20,23,26].

The farm's manure storage releases both N_2O and CH_4. C is the fourth most frequent element in the Earth's crust. Global energy and the C cycle are driven by methanogenesis. The second-most common GHG after CO_2, CH_4 is produced by animals through enteric fermentation and their manure.

This increase is responsible for around 20% of the current trend in global warming. Presently, between 500 and 600 GT of the world's yearly CH_4 emissions come from various habitats [47]. CH_4 remains in the atmosphere for nine to fifteen years and is over 25 times more effective than CO_2 at retaining atmospheric heat [48]. The raising of livestock plays a substantial role in the build-up of CH_4 in the atmosphere. As our understanding of GHGs continues to evolve, feeding factors remain incompletely explored.

To decrease the main greenhouse gases, various dietary approaches were investigated. These approaches included high fiber contents, weight increases, feed efficiency, rates of protein and fat deposition, slaughter weight, and carcass lean yield [49–51]. Kpogo et al. [49], for example, previously investigated the impact of multicarbohydrase enzymes on GHG in the diet using wheat millrun as a co-product. Even though adding 30% wheat millrun to pig diets increases their fiber content, the authors did not observe a significant effect on GHGs. Furthermore, adding multienzymes to wheat millrun diets did not significantly affect emissions. In 2022, Hăbeanu et al. [38] highlighted that the intestinal microbial community influences pigs' growth and intestinal health. Feeding pigs a higher level of fiber led to higher levels of the total bacteria identified, which influenced the total volatile fatty acids (VFAs). The authors noted an important decrease in E-CH_4 in pigs fed high-fiber diets featuring the addition of mustard and grapeseed cake.

The findings of the present study did not agree with those obtained by Kpogo et al. [49], which showed that N digestibility was not enhanced. Conversely, Chen et al. [52] found that utilizing a cocktail of non-starch polysaccharide enzymes in a corn–miscellaneous meal diet improved nutrient digestibility; nonetheless, the authors did not observe a significant influence on N_2O and CH_4 emissions throughout the experiment. Our data regarding E-CH_4 are less comprehensive than those obtained by Hăbeanu et al. [38]. However, if we

take into consideration the data reported to LU, our data are similar to those obtained by Philippe et al. [17,26].

There is no evidence in the literature to suggest a low correlation between DMI, feed intake, N intake, and other parameters on M-CH_4 emissions.

5. Conclusions

The results of this study did not support our first hypothesis, which predicted that the performance of the pigs would decrease if GM completely replaced SM. Replacing 100% of the SM in the diet with GM can positively alter certain indicators of N metabolism. For N_2O (based DM) and E-CH_4, the positive effects of dietary GM were greater when utilizing a GM-100% diet. This result supports our second hypothesis, although the impact of the anaerobic process in the manure on M-CH_4 was less pronounced. This particular type of GHG decreases if organic matter is more digestible.

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Effect of Dietary Calcium Propionate Inclusion Level and Duration in High-Risk Newly Received Stocker Calves: Growth Performance, Body Fat Reserves, and Health

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Abstract: This study utilized fifty bull calves of the Continental × British crossbreed, with an average body weight of 147.0 ± 1.67 kg (BW), in a completely randomized design. The objective was to examine the impact of varying levels and duration of calcium propionate (CaPr) supplementation on the growth performance, body fat reserves, serum metabolites, and hemogram of high-risk newly received stocker calves. These calves were individually housed and fed a received diet for 56 d. The calves received the following treatments: (1) no CaPr (CTL), (2) 20 g CaPr/calf/d, (3) 40 g CaPr/calf/d, (4) 60 g CaPr/calf/d, and (5) 80 g CaPr/calf/d at 14, 28, 42, and 56 d after their arrival. Supplementing with 20 g CaPr from 28 to 56 d after arrival increased average daily gain (ADG) and BW ($p < 0.05$), and DMI was not affected ($p > 0.05$). This was reflected at 28 d with increases ($p < 0.05$) in the ADG/DMI ratio and longissimus muscle area (LMA), and at 56 d in back fat thickness (BFT) and fat thickness at the rump (FTR). Also, with 20 g, blood urea nitrogen decreased ($p < 0.05$), and increases were observed in the activity of gamma glutamyltransferase, monocytes (quadratic trend, $p < 0.07$), and granulocytes % (quadratic effect, $p < 0.03$). However, as the level of CaPr increased during the first 14 d after arrival, daily water intake, creatinine, total cholesterol, mean corpuscular hemoglobin concentration (linear effect, $p < 0.05$), globulin, calcium, and mean corpuscular volume (linear trend, $p = 0.08$) increased, while alkaline phosphatase (linear trend, $p = 0.07$) and lymphocytes (linear effect, $p = 0.05$) decreased. Finally, the different levels of CaPr supplementation did not produce any significant effects or differences ($p > 0.05$) in the remaining serum metabolites and hemogram ($p > 0.05$). Ultimately, the inclusion of 20 g CaPr/calf/d in the diet for 28 d in newly received stocker calves increased ADG, ADG/DMI ratio, and LMA. If extended to 42 or 56 d, the increases in ADG persisted, but there was also a rise in body fat reserves (BFT and FTR) at the expense of a reduction in the ADG/DMI ratio. Furthermore, the different supplementation levels did not impact the reference range for most serum metabolites or the health of stocker calves.

Keywords: beef calf; gluconeogenic precursor's; reception diet; serum metabolites; hemogram

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1. Introduction

Factors such as recent weaning, handling, transportation, commingling, and exposure to a new environment, along with a lightweight condition (<200 kg), can cause acute stress in calves. Calves in such circumstances are considered high risk [1,2], often leading to water and feed deprivation [3]. These stress factors have a negative impact on the energy balance [4], resulting in a reduction of body fat reserves. Consequently, this negatively affects the immune system [5], leaving the animal vulnerable to infectious agents. As

a result, morbidity rates can increase by 16.2%, and mortality also increases during the reception period (first 6 to 8 weeks) [6,7].

Energy is one of the most critical nutrients for the animal's immune system [6], making its storage in the form of body fat a valuable indicator of the nutritional status and health of calves. Energy is stored in the body as lipids [8], which, when catabolized, are highly efficient in energy production. However, the low dry matter intake (DMI), approximately 0.9 to 1.5% of body weight (BW), during the first 14 d after arrival [9,10], complicates the correction of nutritional deficiencies. This could further compromise immune function [11] and reduce body fat reserves. As a result, a significant portion of the consumed energy is directed towards the production of antibodies and immune system proteins. However, this also means that less energy is available for tissue deposition or average daily gain (ADG).

Due to this this altered feeding pattern, the performance of newly received stocker calves is typically optimized by using diets with a higher grain concentration (≥60% concentrate) [12]. However, this approach leads to a 17% increase in morbidity rate and a 24% increase in the number of days requiring medical treatment (morbidity severity) [13]. On the other hand, increasing the proportion of forage in the diet decreases the morbidity rate by 1.3% [14] and improves DMI by 9% [15], but it also reduces ADG by 8.3% [14]. Despite the decrease in morbidity among the calves, it fails to compensate for the economic loss associated with the reduced productivity of calves fed with high forage proportions [4,16,17].

In light of all the aforementioned factors, it becomes imperative to explore ingredients that can increase the availability of dietary energy to enhance both growth performance and body fat reserves without compromising the health of calves. It has been observed that gluconeogenic precursor calcium propionate (CaPr) alters energy metabolism when supplemented in ruminant diets. Specifically, it has two significant effects: (1) It alters rumen fermentation by improving ruminal DM digestibility, increasing the proportion of ruminal propionate, and reducing methane production [18,19], and (2) it enhances the action of insulin on glucose (GLU) metabolism [20], promoting an increase in energy status through enhanced GLU synthesis via gluconeogenesis in the liver [21]. In this context, Carrillo-Muro et al. [22,23], studying finishing diets for lambs, found that 10 g of CaPr/lamb/d for 28 d increased dry matter intake (DMI) by 1 to 13%, ADG by 28%, ADG/DMI ratio by 17 to 25%, and BW by 5 to 7%. If the supplementation was extended to 42 d, it resulted in a 30% increase in body fat reserves. Nevertheless, there is currently no available information regarding the effects of the levels and duration of CaPr inclusion on the growth performance, body fat reserves, serum metabolites, and hemogram of high-risk, newly received stocker calves.

Hence, in this study, we hypothesized that augmenting the available energy in the diet through CaPr supplementation in high-risk newly received stocker calves could potentially enhance growth performance, body fat reserves, and serum metabolites, without adverse effects on the hemogram. Moreover, we aimed to ascertain whether the magnitude of these effects is linked to the level and duration of CaPr supplementation. Therefore, the objective of the present study was to investigate the impact of different levels (0, 20, 40, 60, and 80 g CaPr calf/d) and durations of CaPr inclusion (0, 14, 28, 42, or 56 d) in high-risk newly received beef calves concerning their growth performance, body fat reserves, serum metabolites, and hemogram.

2. Materials and Methods

The protocol (protocol # 2023/05/19) received approval from the Animal Welfare Committee at the Unidad Académica de Medicina Veterinaria y Zootecnia at the Universidad Autónoma de Zacatecas (UAMVZ-UAZ). The handling and management of the calves strictly adhered to the guidelines set forth by the Officials Mexicans Standards. The experiment was conducted over two consecutive months at the Torunos Livestock Preconditioning Center, within the experimental area, located in Fresnillo, Zacatecas, Mexico (north-central Mexico), and owned by Grupo Exportador Pa Lante S.P.R. de R.L. Through-

out the duration of the experiment (June to July 2023), the ambient air temperature averaged 22.4 °C, with a minimum of 12.2 °C and a maximum of 28.3 °C.

2.1. Animal Housing and Management Health

The calves used in this experiment fit the definition of high risk due to their lightweight condition (<200 kg) and because their health and management history is unknown [2]. Eighty-seven calves were weaned and transported approximately 120 km (4 h by truck) from an order buyer facility in Milpillas de la Sierra, Valparaiso, Zacatecas, to the Torunos Livestock Preconditioning Center. The calves arrived at the preconditioning center on 1 June 2023 and experienced a 5% reduction in weight during transit. Upon arrival, the calves were housed in pens, with two calves per pen, and provided access to water and long-stem alfalfa hay overnight. The following morning (0600 h), the calves underwent the following procedures: (1) metaphylatic antimicrobial treatment (Emicina® líquida, Zoetis, Ciudad de Mexico, Mexico); (2) eleven-way clostridial, *Mannheimia haemolytica*, and *Pasteurella multocida* type A and D vaccination (Biovac 11 Vías®, Biozoo, Jalisco, Mexico); (3) deworming with 4% ivermectin (Master LP®, Ourofino Salud Animal, São Paulo, Brazil) and pour on cypermethrin (Cypermil Pour On®, Ourofino Salud Animal, São Paulo, Brazil); (4) each calf was assigned a unique ear tag with an individual number; and (5) individual initial body weight (IBW) was recorded. Thirty-seven calves were excluded from the experiment due to their low IBW or temperament issues, resulting in a final group of 50 bulls for use in the experiment (n = 50). These fifty bull calves were primarily 50% Charolais, 25% Simmental, and 25% Angus crossbred with an average IBW of 147.0 ± 1.67 kg and 5 months of age. The IBWs of the calves were recorded, and they were accommodated in 50 soil-surfaced pens (3.14 m × 5.25 m).

2.2. Diet Management and Feed Samples

For the 56 d of the experimental phase, a basal diet was used, with the proportion of concentrate gradually increasing (1) 50% concentrate was provided from d 0 to 14; (2) 60% concentrate from d 15 to 28; and (3) 70% concentrate from d 29 to 56. On transition days, the basal diet was provided at 90% of the amount delivered the day before. The basal diets were formulated to meet NRC [24] recommendations for nutrients (Table 1). Throughout the study, the calves had unrestricted access to the basal diet and fresh water. The basal diet was divided into three daily feedings at 0800, 1200, and 1800 h in a 20:20:60 proportion, respectively. To ascertain the correct feed quantity for delivery, the feed bunks were assessed at 0730, 1130, and 1730 h daily, with residual feed being collected and weighed to determine dry matter intake (DMI). Adjustments in daily feed delivery were made at the afternoon feeding. Additionally, the calves' individual weights were recorded at the start of the experiment (IBW), at intermediate points (14, 28, 42 d), and at the end of the experiment (56 d). The calves were under daily observation for any signs of bovine respiratory disease, which encompassed symptoms such as labored breathing, nasal or ocular discharge, depression, anorexia, and lethargy. Animals expressing symptoms were removed from experiment. Furthermore, daily samples of the basal diet were collected and analyzed in triplicate to determine the following: (1) DM%, which was achieved by drying the samples for 24 h at 100 °C in a forced air-drying oven; (2) crude protein (CP) (FP-528 LECO nitrogen analyzer) [25]; (3) neutral detergent fiber (NDF) (fiber Ankom analyzer); and (4) Ether extract (EE) (extractor of Ankom^{xt15}).

Table 1. Composition and nutritional profile (DM basis) of basal diet offered to bull calves during the experiment (g kg^{-1} DM).

Ingredients	Concentrate in Basal Diet, % [a]		
	50.0%	**60.0%**	**70.0%**
Alfalfa hay mature	250.0	200.0	150.0
Oats hay	250.0	200.0	150.0
Cracked corn	280.0	380.0	480.0
Soybean meal (44% CP)	105.0	105.0	105.0
Liquid molasses cane	50.0	50.0	50.0
Vegetable fat	21.5	21.5	21.5
Sodium bentonite	10.0	10.0	10.0
Sodium sesquicarbonate	15.0	15.0	15.0
Calcium carbonate	8.0	8.0	8.0
Monocalcium phosphate	2.0	2.0	2.0
Urea	5.0	5.0	5.0
Salt	2.5	2.5	2.5
Microminerals: Co, Fe, I, Mn, Zn, Se, and Cu [b]	0.5	0.5	0.5
Vitamins: A, D, and E [c]	0.5	0.5	0.5
Chemical composition, g kg^{-1} DM [d]			
Dry matter	872.4	862.4	852.4
Crude protein	148.8	146.5	144.1
Ether extract	43.6	45.8	48.0
Neutral detergent fiber	348.4	298.7	249.0
Calcium	9.3	8.9	8.5
Phosphorus	2.9	2.8	2.8
Ca/P ratio	3.2	3.2	3.0
Calculated net energy, Mcal/kg [d]			
Maintenance	1.6	1.7	1.8
Gain	1.0	1.1	1.2

[a] 50% concentrate = fed from d 0 to 14; 60% concentrate = fed from d 15 to 28; 70% concentrate = fed from d 29 to 56. [b] Microminerals: Co (0.5 g), Fe (50 g), I (2.5 g), Mn (50 g), Zn (50 g), Se (0.2 g), and Cu (15 g). Excipient q.s. 1000 g. [c] Vitamins A (5,000,000 IU), D (2,000,000 IU), and E (10,000 IU). Excipient q.s. 1000 g. [d] Based on the tabular values for individual feed ingredients (Ca, P, net energy for maintenance and gain) [24], with the exception of DM, CP, NDF, and EE (Ankom procedures), which were determined in our laboratory.

The diet provided in this study was carefully monitored to ensure that aflatoxin levels were well below the established safety limits for animal feed. This precautionary measure was taken to safeguard the animals' health and welfare. By maintaining feed quality within safe limits, we aimed to minimize any potential influence of aflatoxins on the study results.

2.3. Experimental Design and Treatments

A completely randomized design was employed to investigate the effects of varying levels and durations of CaPr supplementation in calves. The treatments consisted of (1) No CaPr (CTL), (2) 20 g CaPr/calf/d, (3) 40 g CaPr/calf/d, (4) 60 g CaPr/calf/d, and (5) 80 g CaPr/calf/d. These treatments were administered for a duration of 56 d, with evaluations conducted at 0, 14, 28, 42, and 56 d after arrival (Figure 1). The source of CaPr used was Nuprocal® (Nutryplus, Queretaro, Mexico), originating from the same batch, containing propionic acid and calcium sulfate (chemical composition 20% calcium and 69% of propionic acid). Each individual CaPr dose was meticulously weighed using a precision balance (Pioneer-PX523, Ohaus Corp., Parsippany, NJ, USA). To ensure that the treated group consumed the full dosage, the doses were mixed with 100 g of the basal diet, provided at 0800 and 1600 h. Any remaining portion of the diet was administered to the calves once they consumed their initial portions.

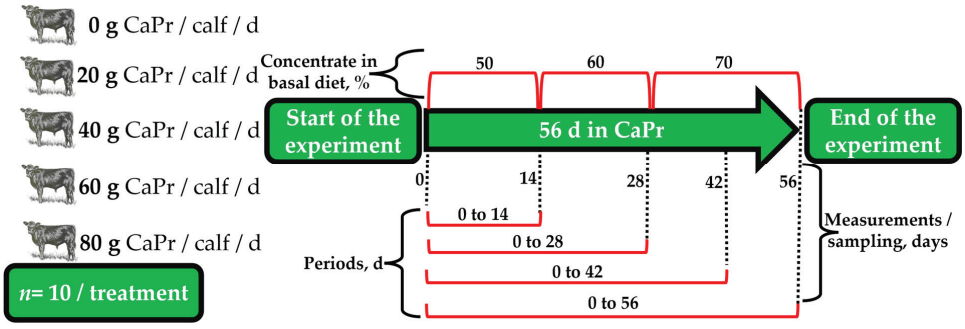

Figure 1. Completely randomized design, level (0, 20, 40, 60, or 80 g CaPr/calf/d), and duration (0, 14, 28, 42, and 56 d) of calcium propionate (CaPr) supplementation in high-risk newly received stocker calves.

2.4. Growth Performance

By utilizing the individual data collected data during feeding trial, we computed the following averages for various time intervals (d 0 to 14, d 0 to 28, d 0 to 42, and d 0 to 56): (1) ADG = [(Weight out—Weight in/Days on period] expressed as kg/d; (2) DMI = (Feed offered—Feed refused), which was weighed and recorded daily, expressed as kg/d; (3) ADG/DMI ratio = (ADG/DMI); and (4) Daily water intake (DWI) = (Water offered − Water refused), which was determined and recorded daily, expressed as L/d. For this purpose, a drinking cup with a capacity of 30 L was graduated to determine intake.

2.5. Serum Metabolites and Hemogram

Serum metabolite and hemogram samples were processed at the Laboratorio de Análisis Clínicos Veterinarios of the UAZ-UAMVZ. Blood samples were collected from five randomly selected calves from each treatment, on d 0, 14, 28, 42, and 56. Coinciding with individual weighing, at 0700 h, and before the first feeding of the d, blood was drawn from the jugular vein. The blood samples (6.0-mL BD Vacutainer con EDTA K2-Dikysa) were later analyzed for a complete blood count (CBC) using an automatic cell counting machine (Exigo veterinary haematology analyser, Boule Medical AB, Sweden). The following parameters were determined: total white blood cells (WBC), lymphocytes (LYM), lymphocytes % (LYM%), monocytes (MON), monocytes % (MON%), granulocytes (GRA), granulocytes % (GRA%), platelets (PLT), mean platelet volume (MPV), red blood cells (RBC), red blood cells distribution width test % (RDW%), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). In addition, the blood serum was collected by centrifugation (2500× *g* for 30 min; 4 °C), and metabolites were quantified using an automated analyzer (FUJI DRI-CHEM NX500; Fujifilm, Tokyo, Japan). The following parameters were determined: activity of alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), and aspartate aminotransferase (AST); levels of albumin (ALB), blood urea nitrogen (BUN), calcium (Ca), creatinine (CRE), GLU, total bilirubin (TBIL), total cholesterol (TCHO), triglycerides (TG), total protein (TP), sodium (Na^+), potassium (K^+), and chlorine (Cl^-). The globulin fraction (GLO) is a calculated value obtained by subtracting the ALB concentration from the TP concentration [26].

2.6. Body Fat Reserves and Longissimus Muscle Area

The following measurements were made: (1) Longissimus muscle area (LMA), measured in cm^2; (2) back fat thickness (BFT), which represents subcutaneous fat over the longissimus dorsi muscle, measured in mm, wherein both measurements were taken between the 12th and 13th ribs; and (3) fat thickness at the rump (FTR) at the p8 site, measured in mm, which was assessed. This measurement is located over the gluteus muscle on the

rump and is determined at the intersection of a line drawn through the pin bone, parallel to the chine, and perpendicular to the third sacral crest [27]. These measurements were obtained through ultrasonography every 0, 14, 28, 42, and 56 d. The measurements were consistently performed by the same operator using a real-time scanner equipped with a linear array transducer of 3.5 MHz (Aloka Prosound 2 instrument).

2.7. Statistical Analyses

Statistical analysis was performed using the SAS® OnDemand free software, which is freely available. A normality test was performed using the UNIVARIATE procedure. The data related to growth performance were analyzed assuming a completely random design, with each calf serving as the experimental unit. The GLM procedure of SAS was used, involving a model that took into account the effects of treatment (CaPr level). A one-way ANOVA was performed, and Bartlett's test was used to assess variances homogeneity. The statistical model used can be expressed as $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, where Y_{ij} corresponds to response variable, μ is overall mean effect, T_i is the treatment effect (i = 1, 2, 3, 4, 5), and ε_{ij} is the residual error term. When significant effects were detected, mean comparisons were conducted using the Tukey method with the LSMEANS instruction. Orthogonal polynomials were applied to evaluate linear and quadratic responses among the different levels of CaPr. Statistical significance was determined when the *p*-value was $\leq$0.05, and a trend if the *p*-value was >0.05 and $\leq$0.10.

3. Results

3.1. Growth Performance

In the initial 14 d, most of the studied variables remained unaffected by the varying levels ($p > 0.05$; Table 2). Nevertheless, a noticeable effect was observed concerning DWI during this timeframe, with a 4.6% increase as the inclusion level of CaPr went up (linear effect, $p = 0.04$). As for DMI, reductions were observed from 28 to 56 d as the level of inclusion increased (linear effect, $p < 0.05$). The lowest consumption was recorded on d 56 with 80 g, but the other levels were comparable to the CTL ($p < 0.05$). Starting from d 28, distinctions became apparent with 20 g, showing an 11.8% increase in ADG (quadratic trend, $p = 0.07$) and a 4.6% increase in BW, compared to the CTL ($p < 0.05$). This trend persisted through 42 and 56 d, with the highest increase occurring on d 42, resulting in a 13.3% increase in ADG and a 4.9% increase in BW ($p < 0.05$). Nonetheless, it was also observed that as the level of inclusion increased, ADG decreased on 42 and 56 d (linear effect, $p < 0.05$), and the same trend was noted for BW on d 56 (linear trend, $p = 0.06$). This was reflected in a 16.7% increase in the ADG/DMI ratio on d 28 ($p < 0.05$) with 20 g. However, on 42 and 56 d, no significant differences were observed between the treatments ($p > 0.05$).

Table 2. Effect of dietary calcium propionate (CaPr) inclusion level and duration on the growth performance of high-risk newly received stocker calves ($n = 10$/treatment).

Item	Calcium Propionate Levels [1]					SEM [2]	Effects (*p*-Value)	
	0	20	40	60	80		Linear	Quadratic
				Body weight, kg				
Initial	145.3	147.8	143.4	146.4	152.3	1.67	0.93	0.92
Day 14	173.1	177.8	167.9	172.2	175.4	1.85	0.59	0.96
Day 28	192.3 ^{b}	201.1 ^{a}	189.0 ^{b}	190.2 ^{b}	190.6 ^{bc}	1.65	0.41	0.45
Day 42	209.1 ^{b}	219.3 ^{a}	200.1 ^{b}	202.6 ^{b}	198.9 ^{bc}	2.81	0.18	0.54
Day 56	232.8 ^{b}	239.1 ^{a}	223.3 ^{b}	220.2 ^{b}	214.5 ^{bc}	2.70	0.06	0.45
				Average daily gain, kg				
D 0 to 14	2.0	2.2	1.8	1.8	1.7	0.13	0.20	0.70
D 0 to 28	1.7 ^{b}	1.9 ^{a}	1.6 ^{b}	1.6 ^{b}	1.4 ^{bc}	0.07	0.12	0.07
D 0 to 42	1.5 ^{b}	1.7 ^{a}	1.3 ^{b}	1.3 ^{b}	1.1 ^{bc}	0.08	0.03	0.20
D 0 to 56	1.5 ^{b}	1.6 ^{a}	1.4 ^{b}	1.3 ^{b}	1.1 ^{bc}	0.07	0.01	0.25

Table 2. *Cont.*

Item	Calcium Propionate Levels [1]					SEM [2]	Effects (*p*-Value)	
	0	20	40	60	80		Linear	Quadratic
				Dry matter intake, kg/d				
D 0 to 14	4.0	4.0	3.9	3.9	3.9	0.07	0.18	0.98
D 0 to 28	4.9	4.8	4.7	4.4	4.3	0.16	0.04	0.54
D 0 to 42	5.1	5.1	4.9	4.7	4.6	0.17	0.05	0.57
D 0 to 56	5.5 ^{a}	5.4 ^{a}	5.2 ^{ab}	5.0 ^{ab}	4.6 ^{b}	0.17	0.05	0.80
				Daily water intake, L/d				
D 0 to 14	23.5	23.9	24.1	24.6	23.7	0.38	0.04	0.87
D 0 to 28	23.3	23.8	23.4	23.5	23.2	0.25	0.88	0.45
D 0 to 42	25.1	25.1	24.7	25.5	24.2	0.42	0.63	0.36
D 0 to 56	26.7	26.8	26.0	26.8	24.9	0.59	0.84	0.53
				ADG/DMI ratio				
D 0 to 14	0.48	0.53	0.44	0.46	0.42	0.035	0.34	0.74
D 0 to 28	0.36 ^{b}	0.42 ^{a}	0.36 ^{b}	0.37 ^{b}	0.32 ^{bc}	0.019	0.74	0.28
D 0 to 42	0.34	0.36	0.34	0.33	0.30	0.016	0.40	0.26
D 0 to 56	0.32	0.32	0.32	0.30	0.30	0.019	0.32	0.42

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] SEM = standard error of the mean. ^{a,b,c} Means a row with different superscripts differ ($p < 0.05$) according to Tukey's test.

3.2. Enzymatic Activity

The activity of ALP decreased with the increasing levels of CaPr (linear trend, $p = 0.07$; Table 3). It was higher in the CTL and lower with 80 g ($p < 0.05$). However, in the case of GGT, its activity was higher with 20 g CaPr/calf/d, showing a significant 37.3% increase (quadratic trend, $p = 0.06$) when compared to the CTL. The different levels of CaPr supplementation did not produce any effect on the activity of AST ($p > 0.05$).

Table 3. Overall effect of level calcium propionate (CaPr) inclusion on enzymes activity of high-risk newly received stocker calves, sampled on d 0, 14, 28, 42, and 56 ($n = 5$/treatment).

Item [2]	CaPr Levels [1]					Reference Range	SEM [3]	Effects (*p*-Value)	
	0	20	40	60	80			Linear	Quadratic
ALP, U/I	379.4 ^{a}	281.1 ^{ab}	265.9 ^{ab}	256.1 ^{ab}	210.7 ^{b}	0–488 [28]	37.33	0.07	0.16
GGT, U/I	16.6	22.8	19.5	17.6	16.3	6.1–17.4 [28]	2.06	0.98	0.06
AST, U/I	74.7	85.3	86.1	77.1	61.6	48–100 [29]	7.61	0.82	0.21

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] Enzyme activities have been expressed as ALP = alkaline phosphatase, GGT = gamma glutamyltransferase, AST = aspartate aminotransferase. [3] SEM = standard error of the mean. ^{a,b} Means a row with different superscripts differ ($p < 0.05$) according to Tukey's test.

3.3. Serum Metabolites

The concentration of GLO increased with the level of CaPr (linear trend, $p = 0.08$; Table 4), with higher levels observed with 40 to 80 g of CaPr ($p < 0.05$). BUN was lower with 20 g, higher with 80 g, and similar with the other levels and CTL ($p < 0.05$). Additionally, increasing the level of CaPr increased the concentration of Ca (linear trend, $p = 0.08$), and CRE (linear effect, $p = 0.003$) and TCHO (linear effect, $p = 0.02$) was higher with levels above 40 g ($p < 0.05$). The different levels of CaPr supplementation did not result in any significant effect or difference in ALB, GLU, TBIL, TG, TP, and electrolytes ($p > 0.05$).

Table 4. Overall effect of level calcium propionate (CaPr) inclusion on serum metabolites of high-risk newly received stocker calves, sampled on d 0, 14, 28, 42, and 56 (n = 5/treatment).

Item [2]	CaPr Levels [1]					Reference Range	SEM [3]	Effects (p-Value)	
	0	20	40	60	80			Linear	Quadratic
TP, g/dL	5.9	6.6	6	6.1	6.5	6.74–7.46 [28]	0.22	0.93	0.18
ALB, g/dL	3.9	3	2.9	2.8	2.6	2.8–3.8 [29]	0.5	0.13	0.46
GLO, g/dL	3.1 ^{b}	3.4 ^{b}	3.7 ^{ab}	3.8 ^{ab}	3.9 ^{a}	3.0–3.48 [28]	0.2	0.08	0.23
BUN, mg/dL	11.3 ^{ab}	10.6 ^{b}	10.9 ^{ab}	10.9 ^{ab}	12.8 ^{a}	10–25 [30]	0.49	0.39	0.87
CRE, mg/dL	0.75	0.79	0.8	0.83	0.87	1–2 [28]	0.028	0.003	0.61
TBIL, mg/dL	0.23	0.28	0.28	0.27	0.25	0.01–0.5 [28]	0.018	0.15	0.21
TCHO, mg/dL	65.5 ^{b}	65.5 ^{b}	71.3 ^{ab}	74.4 ^{ab}	82.3 ^{a}	80–120 [30]	4.02	0.02	0.22
TG, mg/dL	35.1	22.9	22.8	26.9	29.6	0–14 [28]	5.97	0.4	0.21
Ca, mg/dL	10.1	10.6	10.9	11.3	14.8	8.3–10.4 [30]	1.8	0.08	0.46
GLU, mg/dL	106.4	104	100.6	91	90.3	45–75 [28]	7.31	0.36	0.43
				Electrolytes, mEq/L					
Na^+	121.2	124	122.4	124.5	123	132–152 [28]	1.9	0.35	0.8
K^+	4.4	4.6	4.3	4.4	4.2	3.9–5.8 [28]	0.1	0.81	0.57
Cl^-	85.7	87.6	86.5	87.1	84.7	97–111 [28]	1.65	0.7	0.7

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] TP = total protein, ALB = albumin, GLO = globulins, BUN = blood urea nitrogen, CRE = creatinine, TBIL = total bilirubin, TCHO = total cholesterol, TG = triglycerides, Ca = calcium, GLU = glucose, Na^+ = sodium, K^+ = potassium, Cl^- = chlorine. [3] SEM = standard error of the mean. ^{a,b} Means a row with different superscripts differ ($p < 0.05$) according to Tukey's test.

3.4. Body Fat Reserves and Longissimus Muscle Area

BFT, FTR, and LMA were not affected by the different levels during the initial 14 d ($p > 0.05$; Table 5). Regarding LMA, at 28 d, its maximum value was observed, with a 23.9% increase with 20 g (quadratic effect, $p = 0.05$) compared to the CTL. However, the most significant increase in BFT ($p < 0.05$) was observed from d 42 onwards, and for FTR, these increases persisted up to d 56. These increases were most pronounced with 20 g of CaPr (quadratic effect, $p < 0.05$), showing a 24.9% increase in BFT and a 21% increase in FTR compared to the CTL.

Table 5. Effect of dietary calcium propionate (CaPr) inclusion level and duration on the body fat reserves and longissimus muscle area of high-risk newly received stocker calves (n = 10/treatment).

Item	Calcium Propionate Levels [1]					SEM [2]	Effects (p-Value)	
	0	20	40	60	80		Linear	Quadratic
				Back fat thickness, mm				
Initial	2.7	2.6	2.9	2.6	2.4	0.21	0.95	0.76
Day 14	2.2	2.3	2.0	2.2	2.1	0.12	0.51	0.68
Day 28	2.5	3.0	2.7	2.8	2.6	0.15	0.43	0.27
Day 42	3.4	4.3	3.8	3.7	3.4	0.21	0.85	0.05
Day 56	3.3	4.1	3.5	3.4	3.1	0.18	0.76	0.05
				Fat thickness at the rump, mm				
Initial	2.6	2.6	2.5	2.4	2.3	0.19	0.54	0.83
Day 14	2.5	2.6	2.4	2.4	2.2	0.18	0.50	0.72
Day 28	3.0	2.9	2.7	2.9	2.9	0.2	0.66	0.71
Day 42	3.8	4.3	3.9	4.3	4.0	0.27	0.43	0.89
Day 56	3.6	4.4	3.9	3.6	3.7	0.20	0.55	0.03
				Longissimus muscle area, cm^2				
Initial	26.7	27.7	26.6	27.3	26.5	1.18	0.69	0.28
Day 14	33.0	33.9	33.9	31.7	34.5	1.65	0.63	0.47
Day 28	34.7 ^{c}	43.0 ^{a}	38.0 ^{b}	39.1 ^{b}	38.2 ^{b}	1.45	0.26	0.05
Day 42	36.3	41.3	37.1	40.4	35.2	1.53	0.31	0.67
Day 56	37.7	43.4	39.9	38.4	35.9	1.75	0.87	0.13

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] SEM = standard error of the mean. ^{a,b,c} Means a row with different superscripts differ ($p < 0.05$) according to Tukey's test.

3.5. White Blood Cells

As the level of CaPr increased, the value of LYM decreased (linear effect, $p = 0.05$; Table 6). LYM% was 16.7% higher in the CTL (quadratic effect, $p = 0.02$). MON increased by 21.4% with 20 g (quadratic trend, $p = 0.07$). GRA increased by 12.9% with 20, 40, and 60 g, and GRA% increased by 19.5% with 20 g (quadratic effect, $p < 0.03$). The different levels of CaPr supplementation did not have any significant effect or difference in WBC and MON% ($p > 0.05$).

Table 6. Overall effect of level calcium propionate (CaPr) inclusion on white blood cells of newly received stocker calves, sampled on d 0, 14, 28, 42, and 56 ($n = 5$/treatment).

Item [2]	CaPr Levels [1]					Reference Range	SEM [3]	Effects (p-Value)	
	0	20	40	60	80			Linear	Quadratic
WBC, $\times 10^3/\mu L$	8.9	9.3	8.9	8.3	8.2	4–12 [31]	0.54	0.36	0.37
LYM, $\times 10^3/\mu L$	5.1	5	4.5	4.3	4	1.6–5.6 [32]	0.31	0.05	0.93
LYM, %	54	53	51	48	47	45–75 [32]	1.77	0.32	0.02
MON, $\times 10^3/\mu L$	0.7	0.9	0.8	0.7	0.7	0–0.8 [33]	0.06	0.45	0.07
MON, %	7.9	8.6	8.4	8.5	8.2	2–7 [33]	0.34	0.33	0.43
GRA, $\times 10^3/\mu L$	3.1	3.5	3.5	3.5	2.8	1.8–6.3 [34]	0.27	0.57	0.03
GRA, %	38	45	44	38	41	15–45 [34]	1.7	0.55	0.02

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] WBC = total white blood cells, LYM = lymphocytes, LYM% = lymphocytes %, MON = monocytes, MON% = monocytes %, GRA = granulocytes, GRA% = granulocytes %. [3] SEM = standard error of the mean.

3.6. Platelets and Red Blood Cells

As the level of CaPr increased, so did the values of MCV (linear trend, $p = 0.08$; Table 7) and MCH (linear effect, $p = 0.02$). The different levels of CaPr supplementation did not produce any significant effect or difference in PLT, MPV, RBC, RDW%, HGB, HCT%, and MCHC ($p > 0.05$).

Table 7. Overall effect of level calcium propionate (CaPr) inclusion on platelets and red blood cells of newly received stocker calves, sampled on d 0, 14, 28, 42, and 56 ($n = 5$/treatment).

Item [2]	CaPr Levels [1]					Reference Range	SEM [3]	Effects (p-Value)	
	0	20	40	60	80			Linear	Quadratic
PLT, $\times 10^3/\mu L$	243	254	268	222	298	193–637 [34]	21.08	0.61	0.19
MPV	7.2	7.3	7.1	7.4	7.2	4.5–7.5 [34]	0.25	0.59	0.56
RBC, $\times 10^6/\mu L$	10.1	9.9	9.6	9.6	9.4	5.1–7.6 [34]	0.25	0.11	0.71
RDW, %	26	25.4	26	25.6	26.1	16–20 [34]	0.48	0.67	0.52
HGB, g/100 mL	11.6	11.8	11	11.7	10.9	8.0–12.0 [34]	0.25	0.98	0.72
HCT, %	34.3	34.8	34	34.6	31.9	22.0–32.0 [34]	0.97	0.89	0.74
Red blood cell index									
MCV, fL	34.1	34.1	35	35.7	36.3	38–50 [32]	0.78	0.08	0.67
MCH, pg	11.5	11.7	12	12	12.5	14–19 [35]	0.26	0.02	0.86
MCHC, g/dL	34	33.9	34	34.7	34.5	38–43 [35]	0.64	0.43	0.61

[1] Treatments consisted of oral administration of CaPr at a dose of 0, 20, 40, 60, or 80 g/calf/d at four feeding periods of 0 to 14, 0 to 28, 0 to 42, or 0 to 56 d. [2] PLT = platelets, MPV = mean platelet volume, RBC = red blood cells, RDW% = red blood cells distribution width test %, HGB = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration. [3] SEM = standard error of the mean.

4. Discussion

4.1. Growth Performance

The literature on increasing energy intake in calves receiving diets through gluconeogenic precursors is quite limited. However, various publications concur that with receiving diets providing increasing levels of net energy for gain (NEg) from concentrates, DMI, ADG, and ADG/DMI ratio significantly improve [36–40]. Moreover, different authors

with various additives agree that the most significant improvements in productive behavior are observed during the first 30 d of reception [41–43]. On the other hand, when CaPr is supplemented in the diet and reaches the rumen, it undergoes hydrolysis at an acidic pH, resulting in the formation of Ca^{2+} and propionic acid [21]. Additionally, in the rumen: (1) the pattern of volatile fatty acids is altered [19]; (2) methane production decreases; (3) digestibility of dry matter increases; (4) fermentation efficiency improves [18]; (5) insulin response capacity in GLU metabolism improves [20]; and (6) body fat reserves increase [23]. As a cumulative result of these mechanisms, there is a promotion of energy status achieved through increased GLU synthesis via gluconeogenesis [21]. This results in improvements in DMI, ADG, ADG/DMI ratio, and BW during the finishing phase [22,23].

The 20 g CaPr level did not affect DMI at any point during the reception period. However, starting from d 28 up to d 56, there was a reduction in DMI with the increasing CaPr levels (40 to 80 g), reaching as low as 19.6% with 80 g. Similarly, when gluconeogenic precursor crude glycerin is included in the beef cattle diet at increasing proportions, DMI decreases [44,45]. Hales et al. [46] also described that DMI reduction in beef cattle, as crude glycerin concentration is increased, seems consistent throughout literature. A similar pattern has been observed with increasing levels of concentrate in the diets of receiving calves. There are maximal DMI levels with 60 to 72% concentrate and reductions with 90% concentrate during the first 28 d [12,36,47]; likewise, Crawford et al. [40] mention that DMI reduces with increasing NEg concentration in calf reception diets at 56 d after arrival. In agreement, but in finishing diets for lambs, Carrillo-Muro et al. [22] observed reductions in DMI of 14.3% with the higher levels of 30 g CaPr, whereas with 10 g, DMI increased by 1.1% during the first 28 d [23]. However, Tomczak et al. [39] with increasing NEg concentration in calf reception diets did not observe differences in DMI.

Newly received calves commonly face the stress of dehydration [3], so they should be offered water immediately upon arrival [48]. In this study, DWI was not affected when 20 g of CaPr was included, but it increased by 4.6% within the first 14 d as the inclusion level increased. In contrast, Carey et al. [45] added crude glycerin to the drinking water of receiving calves and did not observe any effects on DWI. However, Lofgreen et al. [49] reported that DWI was 16.6% higher at 28 d with diets containing less concentrate (20%).

Starting from day 28, there were increases of 11.8% in ADG and 4.6% in BW, with 20 g of CaPr, reaching 13.3% ADG and 4.9% BW at day 42 compared to CTL. Nevertheless, as the levels of CaPr supplementation increased, ADG decreased. In line with these findings, in lambs with finished diets, Carrillo-Muro et al. [22] observed reductions of 16.0% in ADG and 2.8% in BW with the highest levels of CaPr, ranging from 20 to 30 g. Conversely, with 10 g of CaPr during the first 28 days, they observed an increase of 26.8% in ADG and 4.7% in BW [23]. Additionally, Lofgreen et al. [49] reported that ADG improved after the first 7 d of calf arrival and continued to increase until d 28, especially with higher values of NEg. Similarly, Lofgreen [37], using a diet containing 75% concentrate, detected a 6.5% increase in ADG compared to a 50% concentrate diet. Also, Crawford et al. [40] mention that the ADG increased with increasing NEg concentration in calf reception diets; for the first 28 d, it did not differ, and at 56 d, it was greater with the highest level of NEg. In contrast, Pritchard and Méndez [47] reported increased ADG during the first 28 d of the reception period in calves fed diets with lower energy content compared to a diet containing 60% concentrate. However, Tomczak et al. [39], with increasing NEg concentration in calf reception diets, did not observe differences in ADG and BW.

As for the ADG/DMI ratio, a notable improvement of 16.7% was observed during the first 28 d when 20 g of CaPr was included. Conversely, this ratio decreased with the CTL and the other levels. Similarly, Carrillo-Muro et al. [22], in lambs with finished diets, noted a 5.9% improvement in the ADG/DMI ratio with the lowest CaPr level, 10 g, and a substantial 25% improvement during the initial 28 d with 10 g [23]. Fluharty and Loerch [12] found that in receiving calves, increasing concentrate proportions (70, 75, 80, and 85%) resulted in a more significant increase by d 14. Also, Lofgreen [37] detected a 17.5% increase in the ADG/DMI ratio with 75% concentrate compared to 50% concentrate.

Likewise, Tomczak et al. [39], Spore et al. [38], and Crawford et al. [40] mentioned that the ADG/DMI ratio increased with higher NEg concentration in calf reception diets. Contrastingly, Pritchard and Méndez [47] reported an increase in the ADG/DMI ratio during the first 28 d of the receiving period in calves fed diets with lower energy compared to a diet with 60% concentrate.

The reduced growth performance of received beef calves with higher levels of 40, 60, and 80 g CaPr can be explained by the decrease in DMI. This reduction in DMI is attributed to the hepatic oxidation theory (HOT), as described by Allen [50]. The HOT theory explains the role of the ruminant liver in signaling and controlling satiety through temporal patterns of various oxidative products, including propionic acid. Signals are transmitted from the liver to the brain via afferents in the vagus nerve and are influenced by hepatic oxidation and ATP generation [51,52]. Additionally, the elevated levels of CaPr may have increased insulin secretion, leading to a reduction in DMI. Insulin reaches the brain and binds to its specific receptors on neurons, resulting in reduced DMI [53,54].

4.2. Enzymatic Activity

Blood samples were analyzed to measure enzyme activity, specifically ALP, GGT, and AST, to assess whether different levels of CaPr supplementation could affect liver and kidney functions or metabolic processes in these organs. It was noted that ALP activity exhibited a decrease with increasing CaPr levels, although the values remained within the normal reference ranges (0 to 488 [28]). According to Otter [33], physiologically higher ALP activity occurs in young growing cattle and is predominantly associated with bone growth and osteoblast proliferation. Based on this, it can be inferred that received beef calves with 80 g of CaPr may experience a reduction in bone growth. As for GGT, its activity was highest with 20 g of CaPr, showing a notable increase of 37.3%, slightly exceeding the reference range (6.1 to 17.4 [28]). This could be attributed to increased liver activity in these calves [55]. However, a study by Ladeira et al. [56], which used different proportions of glycerin in bulls, did not observe any effects on GGT. As for AST, its activity with various levels of CaPr supplementation did not produce any effects and remained within normal ranges (48 to 100 [29]). These results align with previous studies by Ladeira et al. [56] in bulls and de Freitas et al. [57] in ewes, both of which used different proportions of glycerin and found no observable effects on AST. In contrast, Silva et al. [58], in a study involving crude glycerin in beef cattle, observed increases in AST levels with higher inclusion rates. Carlson [59] notes that AST is a nonspecific indicator of tissue damage and can be elevated in cases of muscle injury or necrosis, particularly in recumbent animals. Importantly, it is worth noting that the enzyme activity values for ALP, GGT, and AST observed in this study remained within the normal, non-pathological range. This suggests that there were no indications of liver or kidney damage, as well as no notable metabolic improvements associated with CaPr supplementation in calves' reception.

4.3. Serum Metabolites

The TP, which primarily includes ALB and GLO, serves as a significant solid component of serum and acts as an indicator of an animal's nutritional status [60]. In the current study, the concentrations of TP and ALB remained unaffected by CaPr supplementation. However, it is noteworthy that TP levels were slightly below the reference range (6.74 to 7.46 [28]), while ALB values were within the normal range (2.7 to 4.2 [29]). In terms of TP, these findings align with a study by de Freitas et al. [57] in ewes, which investigated the effects of different proportions of crude glycerin supplementation and similarly found no significant effects on TP. Nevertheless, the concentration of GLO was slightly elevated with CaPr levels of 40 to 80 g, exceeding the reference range (3.0 to 3.48 [28]). This can occur due to increased levels of ALB, GLO, or both. Dehydration is the sole cause of hyperalbuminemia, where both ALB and GLO levels increase. However, when hyperproteinemia is observed in the absence of dehydration, it is typically associated with hyperglobulinemia. Common causes of hyperglobulinemia include chronic antigenic stimulation and liver

disease. Chronic antigenic stimulation can be attributed to various conditions such as traumatic reticuloperitonitis, liver abscesses, or chronic pneumonia [26]. Based on this, it can be assumed that with higher levels of 40 to 80 g CaPr, there may be chronic liver inflammation.

As for the BUN levels, they are typically used to estimate nitrogen excretion and utilization efficiency [61]. In ruminants, BUN concentrations are influenced by various factors, including CP intake, rumen degradability, and liver and kidney function [62]. Notably, supplementation with 20 g of CaPr resulted in reduced serum BUN levels, although all these values remained within the normal range (10 to 25 [30]). Similarly, Crawford et al. [40] mention that the BUN decreased with increasing NEg concentration in calf reception diets. Carrillo-Muro et al. [22] observed in lambs with finished diets that with higher levels of 20 CaPr, BUN increased. Furthermore, de Freitas et al. [57] in ewes and Carey et al. [45] in newly received beef calves observed increases in BUN with the highest proportions of crude glycerin. Waggoner et al. [63] pointed out that calves with immunological issues exhibit lower N retention, probably due to increased muscle catabolism to obtain proteins and enhance the immune response. The elevated BUN concentration observed in newly received cattle could be caused by mobilization of protein stores to compensate for inadequate DMI [64], or from immune requirements for amino acids [65]. Based on the aforementioned principles, it can be inferred that supplementation with 20 g CaPr in high-risk newly received stocker calves promotes nitrogen utilization and reduces muscle protein catabolism. Conversely, the opposite occurs with elevated levels of 40 to 80 g or 0.

The concentrations of CRE observed In the study fell within the normal range (1 to 2 mg/dL) reported by Kaneko et al. [28]. This indicates that the renal glomerular filtration rate for CRE was adequate and remained unaffected by the presence of CaPr. However, it is notable that as the level of CaPr increased, CRE concentrations also rose. This contrasts with findings from Crawford et al. [40] with increasing NEg concentration in calf reception diets and de Freitas et al. [57] in ewes, who used different levels of glycerin and did not observe effects on CRE. Otter [33] noted that CRE can be low in emaciated beef cattle or those with low muscle mass or elevated in heavily muscled animals. These results suggest that a higher level of CaPr (40 to 80 g) may promote increased muscle deposition over fat. This observation aligns with the higher values of BFT and FTR observed with a CaPr level of 20 g.

In the current study, TBIL concentrations were not affected by CaPr supplementation and remained within the normal range (0.01 to 0.5 [28]). TBIL serves as important indicator of liver function; it typically increases during severe lipidosis [66,67] and decreases in concentration when the liver is healthy. Therefore, based on these TBIL values, it can be concluded that different levels of CaPr do not have any negative effects on liver function.

Serum lipids mainly comprised TCHO and TG. TCHO levels fell below the reference range (73 to 280 [30]) but exhibited an increase as the level of CaPr supplementation rose, with only the 80 g CaPr falling within the range. No significant treatment effects were observed on TG, but all values were above the reference range (0 to 14 [28]). The increase in TCHO levels with the 80 g CaPr level could be attributed to the heightened production of propionic acid in the rumen, subsequently leading to increased TCHO production in the liver. The decrease in serum TCHO levels in this study indicates an energy deficit, whereas increases in TCHO typically occur in response to the ingestion of energy-rich, lipid-containing foods [68]. As crude glycerin inclusion increased, the same conclusion was drawn by Silva et al. [58] in beef cattle and de Freitas et al. [57] in ewes, where they observed increases in TCHO. However, in lambs with finished diets, Carrillo-Muro et al. [22], in their study of lambs fed finishing diets, did not observe significant effects on TCHO and TG levels with levels of 10, 20, or 30 g CaPr/lamb/d. Similarly, de Freitas et al. [57] did not find significant effects with different proportions of crude glycerin inclusion. Ndlovu et al. [69] pointed out that TCHO concentration reflects the energy metabolism in the liver, particularly lipid export in the form of very-low-density lipoproteins.

The CaPr used in this study, chemical composition of 20% calcium and 69% propionic acid, may have provided additional calcium beyond the calves' nutritional requirements,

which were already met by the basal diet. Consequently, as the levels of CaPr inclusion increased, blood calcium concentration also showed an increase, with all levels exceeding the reference range (8.3 to 10.4 [30]). This aligns with what Russell and Roussel [26] mentioned, stating that hypercalcemia is fairly rare in ruminants and usually occurs as a result of the administration of Ca solutions or gels.

GLU concentrations are considered metabolic indicators of nutrient intake in beef cattle [70]. No significant treatment effects were observed on GLU. Likewise, Crawford et al. [40] reported no observed differences in GLU with increasing NEg concentration in calf reception diets. However, it is worth noting that all GLU values recorded in our study were above the reference range (45 to 75 [28]). Consistent with these findings, in lambs fed different levels of CaPr in their diets, Carrillo-Muro et al. [22] did not observe any effects on GLU with levels of 10, 20, or 30 g CaPr. Similarly, Silva et al. [58] in beef cattle and de Freitas et al. [57] in ewes did not observe effects on GLU with different proportions of crude glycerin inclusion. In contrast, Ladeira et al. [56] used different proportions of glycerin in bulls and observed that GLU decreased as the level of inclusion increased. The GLU concentrations within the reference range for all treatments are indicative of adequate DMI intake, since circulating GLU is influenced by nutrient availability [71]. Likewise, Oosthuysen et al. [72] reported that elevated blood GLU concentrations suggest improved energy status associated with better utilization of dietary nutrients.

The different levels of CaPr supplementation did not produce any effects or differences in electrolytes (Na^+, K^+, and Cl^-). Similarly, Crawford et al. [40] reported no significant differences in these electrolytes in their study involving increasing NEg concentration in calf reception diets. However, the values of Na^+ and Cl^- were below the ranges of 132 to 152 and 97 to 111, respectively [28]. Conversely, K^+ levels were within the reference range of 3.9 to 5.8 [28]. The low values of Na^+ and Cl^- in the calves might be attributed to the diet not entirely meeting their nutritional requirements. Radostits et al. [73] note that the most common causes of hyponatremia are the lack or inadequate level of Na^+ in the diet, and alterations in Cl^- concentration are generally associated with proportional changes in Na^+ concentration, resulting from shifts in relative water balance [59]. Another common reason for reduced Na^+ and Cl^- levels in reception calves is diarrhea, which, however, did not occur in this study [59]. Regarding K^+, Crawford et al. [40] did not report significant differences in K^+ levels in their study of increasing NEg concentration in calf reception diets. However, K^+ deficiency is commonly linked to stressed beef cattle experiencing dehydration and the loss of K^+ from tissues [48].

4.4. Body Fat Reserves and Longissimus Muscle Area

Evaluating lipid reserves in newly received calves can offer valuable insights into their nutritional status. Energy is stored in the body in the form of lipids [8], primarily TG [74]. When catabolized, lipids are highly efficient in energy production, yielding up to 9.4 Mcal/kg, whereas carbohydrates produce 4.2 and proteins 5.6 Mcal/kg [75,76]. Therefore, energy from body fat reserves can be nearly twice as much as that derived from muscles. During periods of limited energy availability, the body's fat reserves are the first to be mobilized through the process of adipose tissue lipolysis, releasing TG [77]. Several factors influence body fat reserves, including (1) reproductive potential [78]; (2) negative energy balance [79]; (3) feeding level; and (4) nutrient composition [80].

The findings regarding BFT, FTR, and LMA align with expected patterns of tissue deposition at various stages of the growth curve in young animals. Initially, growth prioritizes the development of bone and muscle, with fat accumulation occurring subsequently. Diets with higher energy or protein content tend to stimulate fat deposition [81,82]. Consequently, supplementation with 20 g of CaPr resulted in a 23.9% maximum increase in LMA up to d 28, followed by a 24.9% increase in BFT from d 42 onwards, and a 21% increase in FTR up to d 56. In line with these results, Carrillo-Muro et al. [23] observed in lamb finishing diets that, with the lowest level of 10 g CaPr, BFT increased by up to 30% by d 42 as the inclusion period extended. Similarly, Crawford et al. [40] with increasing NEg concentration in calf

reception diets, observed that the highest level of NEg increased BFT and FTR at 74 d after arrival. Klinger et al. [83] observed similar outcomes when comparing limit feeding a high NEg diet compared with a high roughage diet during a 77 d backgrounding trial. In contrast, no significant effects were observed on LMA. This aligns with findings from Carrillo-Muro et al. [23], Martínez-Aispuro et al. [84], Lee-Rangel et al. [85], and Mendoza-Martínez et al. [86], which were observed in lambs with finishing diets over 42 d, as well as with the results from Crawford et al. [40] in calf reception diets over 74 d.

4.5. White Blood Cells

The values of WBC were unaffected by the different levels of CaPr inclusion, and these values fell within the normal WBC range (4 to 12 [31]). LYM decreased with increasing CaPr levels, yet these values remained within the normal range (1.5 to 5.6 [32]). MON counts slightly exceeded the range (0 to 0.8) [33]. However, the fact that these values remained within the normal range could indicate an overall enhancement in the calves' immune status or reduced infection rates. In contrast, Silva et al. [58] and Lopez et al. [87], in beef cattle supplemented with crude glycerin, did not observe effects on any of these WBC variables. However, de Freitas et al. [57] in ewes, with different proportions of crude glycerin supplementation, observed a reduction in WBC and LYM with the highest level, and no changes in MON.

4.6. Platelets and Red Blood Cells

With increasing CaPr levels, the values of MCV and MCH also increased. However, it is worth noting that MCV was below the typical range (38 to 50 [32]). The different levels of CaPr supplementation did not produce any significant differences in PLT, MPV, RBC, RDW%, HGB, HCT%, and MCHC. Nevertheless, RBC, RDW%, and HCT remained above the range (22 to 32 [34]), while MCHC levels remained below ([38 to 43 [35]). Similarly, for Silva et al. [58] in beef cattle and de Freitas et al. [57] in ewes, no effects were observed with different proportions of crude glycerin inclusion on these variables. Yet, de Freitas et al. [57] noted an increase in MCV with the highest levels. The values of HCT% were not influenced by the different CaPr inclusion doses; nevertheless, these percentages remained above the typical HCT% range (22% to 32%; [34]), suggesting that the beef cattle experienced slight dehydration throughout the experiment.

5. Conclusions

The gluconeogenic compound CaPr can be successfully integrated in reception diets for high-risk, newly received stocker calves. When administered at a daily level of 20 g CaPr/calf for 28 d, it significantly enhanced growth performance. This improvement was evident through increased ADG, an enhanced ADG/DMI ratio, and an increase in LMA. However, when supplementation was extended to 42 or 56 d at this level, ADG continued to increase, and it also led to an elevation in body fat reserves (BFT and FTR), resulting in a reduction in the ADG/DMI ratio. Furthermore, with the different levels of CaPr supplementation, serum metabolites and hemogram remained within the reference ranges. However, it is important to note that further studies with a larger number of high-risk newly received stocker calves are necessary to comprehensively evaluate the economic and health aspects before recommending the practical use of CaPr in such scenarios.

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Institutional Review Board Statement: This experiment was conducted at the Torunos Livestock Preconditioning Center, in the experimental area, situated in Fresnillo 99150, Mexico, property Grupo Exportador Pa Lante S.P.R. de R.L. The experiment reported herein was approved of Bioethics and Animal Welfare Committee of UAMVZ-UAZ, with protocol # 2023/05/19.

Data Availability Statement: The information published in this study is available on request from the corresponding author.

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Effect of Using Ensilaged Corn Wet Distillers' Grains Plus Solubles (WDGS) as a Partial Replacement for Concentrated Feed for Wet Lot Fed Fatteners during Fattening on Growth Performance, Carcass Characteristics and Pork Quality

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Simple Summary: Wet Distillers' Grains plus Solubles (WDGS) is characterized by a high concentration of crude protein and crude fat on a dry matter basis (DM), which makes them a valuable feed source for pigs. Because of that, WDGS can replace some amounts of the protein components and cereal grains of feed. Preservation based on ensiling improves the nutritional value of WDGS through enhancing its digestibility. This study reveals that utilizing 20% WDGS may have a detrimental effect on feed intake. WDGS did not affect growth performance or the quality and the nutritional value of the pork.

Abstract: The purpose of this study was to determine the nutritional suitability of WDGS in pigs' feeding and production. Pigs were liquid fed and divided into 3 groups. Pigs in the control group were fed diets based on cereal grains, while the experimental groups were also given 10% or 15% WDGS, which partially replaced their cereal grains. During this study, the average daily gains (ADG), feed intake, chemical composition of meat, fatty acid profile of meat, and quality parameters of the carcass and meat were examined. The highest statistical weight gains were detected for the group WDGS 10% during the first stage of the fattening period. No statistical differences were detected for the final body weight, carcass traits, chemical composition of the meat or the composition of fatty acids such as SFAs, PUFAs, and MUFAs, with the exception of eicosenoic acid (C20:1n9). Pigs fed on 10% WDGS exhibited lower peroxidation of lipids (TBARS) than the control group or WDGS 15%. Similarly, water holding capacity (WHC) was the lowest for the group WDGS 10%. Of the meat coloration, redness (a*), yellowness (b*), and chroma (C*) were affected by the WDGS' inclusion, where the highest values were observed for the group WDGS 10%. In conclusion, WDGS can be utilized in the liquid feeding of pigs for up to 15% of their DM.

Keywords: WDGS; liquid feeding; fatteners; growth performance; carcass characteristics; meat quality

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1. Introduction

The bio-ethanol industry delivers ever-increasing amounts of by-products, which are of three general types: condensed corn distillers solubles (CCDS), dried distillers grains plus solubles (DDGS), and wet distillers grains plus solubles (WDGS) [1–3]. Of all the ethanol by-products DDGS and WDGS are the ones that possess the greatest importance for the feed industry and livestock feeding [4]. Currently, DDGS is the main by-product utilized by the animal feed industry that can often be found in commercial mixtures for

farm animals. Its usability as animal feed is highly dependent on the effectiveness of the drying process. As such, any fluctuations in energy resources' prices affect the final production costs of DDGS. Because of that, it may be more profitable for farmers to utilize WDGS instead of DDGS whenever possible [5,6]. Therefore, there is a growing trend in the utilization of WDGS as animal feed, for cattle in particular but also for swine. Unlike DDGS, WDGS is characterized by a rather low dry matter content of around 35–45%, which subsequently may lead to spoilage even after 2 days of storage at 32 °C [7]. Because of that WDGS should either be freshly fed or ensilaged [2]. Nowadays, more and more producers decide to implement wet lot feeding, also known as liquid feeding, into pig production systems. Liquid feeding offers many advantages such as the increased digestibility of the feed, lowered labor intensity and the increased allowance of wet by-products, e.g., milk, whey, or WDGS in the pigs' diets [8], also decreased aggression, which creates better welfare conditions for the animals [9]. Furthermore, fermenting liquid feed also provides a habitat for probiotic lactic acid bacteria, which improve pigs' welfare by reducing *Salmonella* spp. proliferation in the guts [10].

Fresh WDGS exhibits a high nutritional value. The average crude protein concentration is between 25 and 30% DM and average crude fat concentration is 7–12% DM [6,11]. However, WDGS exhibit insufficient amounts of many limiting amino acids for pigs, including lysine, methionine, arginine and threonine, which should be additionally supplemented in the feed [12]. The composition of fat in WDGS also deserves attention, because the high content of PUFA, MUFA, carotenoids and α-tocopherols may affect the physicochemical properties of the obtained meat and fat [13–16]. Therefore, determining the beneficial dose of WDGS in the feed is crucial in the process of universal introduction of this product to pig nutrition.

Due to the fact that WDGS has a feeding value similar to that of DDGS and corn grain, it can potentially be used as a substitute to many feedstuffs rich in energy or protein in pigs' diets. While it has not been confirmed for WDGS, it has been reported that pigs' diets containing 15% DM of DDGS lead to feed refusals and overall reductions in feed intake [17], which can be safely assumed may also occur when WDGS are administered beyond those levels as well. Suffice to say, similarly to DDGS, WDGS should constitute up to 10% DM of a fattener diet [18] so as to avoid its detrimental effects. As such, WDGS may be a suitable component for ensiling and incorporating it into the liquid diets of hogs. Ensiling is an extremely important process for the proper preservation of feedstuff that may spoil or become inappropriate for animal feeding. Ensiling also provides the possibility to utilize feeds that may have been unheard of in pig feeding and nutrition. Apart from WDGS, feeds such as grass [19], corn grains [20] and other agricultural by-products [21] can be easily ensilaged and used as pig feed. It is highly likely that with time those feeds will become even more popular and omnipresent in the liquid feeding of pigs.

Considering the above, the aim of this research was to determine if ensilaged WDGS could be utilized as a suitable component and a substitute to cereal grains or protein feedstuff for fatteners in a wet lot feeding system. The effectiveness of WDGS on animal performance was determined based on physiochemical analyses of meat and animal production yields

2. Materials and Methods

All pigs were handled in accordance with the regulations of the Polish Council on Animal Care and the EU Directive 2010/63/EU of 22 September 2010 [22]. The experiment did not require approval from the 2nd Local Animal Research Ethics Committee in Warsaw as it was performed under the production conditions of a pig-producing farm.

2.1. Animals and Housing

The experiment was conducted on (n = 30) 3-breed Danish fatteners: ♂Duroc × ♀Landrace × Yorkshire. The animals were randomly and equally divided into 3 treatment groups based on sex (barrow to gilt ratio 1:1) and body weight (BW). Each one of the

groups contained 10 fatteners (5 barrows and 5 gilts). Pigs from each group were housed in group pens with mounted plastic grating (1/3 of the total surface of the pen was grated with each head getting 1 m^2 of floor area) in an environmentally controlled building in accordance with Regulation of the Minister for Agriculture and Rural Development (2010) [23]. The experiment was conducted under the following conditions: building was kept at a temperature of 18–19 °C, and relative humidity of up to 70%. Growing fatteners were given liquid feed with a diet containing two different dosages of WDGS at 10% and 15% DM (originally 20% DM). The animals were liquid-fed utilizing automatic feeding and water from the bowl waterer *ad libitum*.

The fattening period was divided into two stages. The 1st stage started when fatteners weighed around 35 kg and finished with fatteners reaching 60–70 kg, while the 2nd stage started when fatteners weighed between 60 and 70 kg and finished with a final body weight of around 130 kg. Before the fattening period started, pigs were subjected to the introductory period where ensilaged WDGS was steadily increased in the pigs' diets. Originally, during the 1st stage of fattening, pigs were to be fed either 10% or 20% of ensilaged WDGS. However, it was observed that the pigs fed diets containing 20% WDGS exhibited lowered feed intake. Because of this fact, the WDGS dosage was lowered to 15%. It is noteworthy that the WDGS dosages of 10% and 15% were stable and used throughout the entire experiment. The weighing of pigs during the experiment was performed individually and every month. Feed intake was collectively calculated for each of the feeding groups, which was later used to determine the feed conversion ratio (FCR).

All pigs were slaughtered in accordance with the standard procedures of the slaughterhouse. All pigs were supervised by a qualified veterinarian.

2.2. Feed Diets

Fatteners were liquid-fed with the use of water as a diluent. The dilution ratio of feed to water was 1 to 2, 1 to 1.6 and 1 to 1.5 for the control group, WDGS 10% group and WDGS 15% group, respectively. Feed mixtures were balanced based on the nutritional requirement of the pigs. The main ingredients of the diet were: barley, triticale and rye middlings, rapeseed and soybean meals, and mineral-vitamin premix with amino acid additives. WDGS was ensilaged prior to the diet mixing, for which the experimental groups contained WDGS at 10% and 15% DM (Table 1). WDGS served as a substitute for grain middling and feeds rich in protein, e.g., soybean post-extraction meal. WDGS exhibited on average 23.4% crude protein, 6.8% crude fat, 11.1% crude fiber and 2.8% crude ash.

2.3. Sampling Conditions

The samples of feed were collected immediately after preparing the liquid feed mixture and kept at +4 °C, until they were dried at 105 °C or frozen at −20 °C, depending on the methodologies. Samples of *Musculus longissimus dorsi* (MLD) were collected 24 h post mortem and stored at +4 °C with the intention of commencing physicochemical analyses while samples destined for chemical analysis were stored at −20 °C.

2.4. Chemical Analyses

Nutritional Value

The chemical composition of both animal feed and meat samples of *Musculus longissimus dorsi*), were determined in compliance with AOAC (2012) [24]: dry matter concentration via drying the samples at 105 °C to constant weight, crude ash via incineration at 550 °C for 6 h, crude protein (Nx6.25) utilizing the micro-Kjeldahl technique (Kjeltec System 1026 Distilling Unit, Foss Tecator, Hilleroed, Denmark), crude fat after extraction with petroleum ether via the Soxhlet method, crude fiber through acid and alkaline hydrolysis via the Henneberg and Stohmann method.

Table 1. Average composition and nutritional value of DM of the diets during the entire fattening period.

Item	Experimental Groups		
	Control	WDGS 10%	WDGS 15%
	% DM Diet		
Barley	20.5	20.5	22.5
Triticale	10	10	10
Rye	50	45	40
Rapeseed meal	5	5	5
Soybean meal (>46 CP)	11	7	5
Corn WDGS	-	10	15
Plant oil	1.0	-	-
Premix *	2.5	2.5	2.5
Nutritional value of 1 kg DM diet			
Metabolic Energy (MJ)	14.50	14.40	14.40
Crude Protein (%)	19.31	19.19	19.20
Crude Fat (%)	2.61	2.76	2.84
Crude Fiber (%)	5.70	5.35	5.79
Crude Ash (%)	4.30	5.00	5.30

* Composition of premix: lysine—12.10%; methionine—2.65%; threonine—5.05%; tryptophan—0.25%; calcium—20.50%; phosphorus—1.80%; sodium—5.00%; iron—4000 mg; manganese—2400 mg; zinc—2600 mg; copper—800 mg; iodine—55.0 mg; selenium—13.50 mg; vitamin A—260,000 IU; vitamin D3—69,000 IU; vitamin E—4700 mg; vitamin K3—68 mg; vitamin B1—68 mg; vitamin B2—170 mg; vitamin B6—105 mg; vitamin B12—830 mcg; vitamin C—1000 mg; folic acid—27.00 mg; pantothenic acid—410 mg; niacinamide B3—690 mcg; biotin—3450 mg; choline chloride—10,000 mg; Aroma, antioxidant: 1b (E320-BHA, E321-BHT, E324—Ethoxyquin)—550 mg/kg; Enzymes: 4a E-1640 6—phytase (EC 3.1.3.2.6 n-5000 FTU/g)—17,500 FTU/kg, (E1600 endo 1,4-beta-xylanase, EC 3.2.1.8—22,000 VU/g; 425,000 VU/kg, endo 1.3 beta-glucanase EC 3.2.1.6—30,000 VU/g, 57,000 VU/kg); raw material composition: calcium carbonate, monocalcium phosphate, (monophosphate) sodium chloride 1.8.1.9, herbal mix 10 g/kg.

2.5. Fatty Acid Composition

The fatty acid composition in extracted fat samples from MLD were analyzed using the gas chromatography flame ionization detection method (GC/FID) according to PN-EN ISO 12966-1:2015 + AC:2015-06 [25], PN-EN ISO 12966-2:2017-05 pkt. 5.2 [26], PN-EN ISO 12966-4:2015-07 [27]. The following fractions were determined: saturated fatty acids (SFA)—C10:0, C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, C23:0; monounsaturated fatty acids (MUFA)—C16:1, C17:1, C18:1 cis 7, C18:1 cis 9, C20:1 cis 9; polyunsaturated fatty acids (PUFA)—C18:2 cis 6, C18:3, C20:2. In addition to fatty acid composition, the atherogenicity index—AI, thrombogenicity index—TI and S/P saturation according to Ulbricht and Southgate (1991) [28] were determined with the following formulas:

$$\mathrm{AI} = \frac{4 \times \mathrm{C14:0} + \mathrm{C16:0}}{\sum \mathrm{MUFA} + \sum \mathrm{PUFA}} \quad (1)$$

$$\mathrm{TI} = \frac{\mathrm{C14:0} + \mathrm{C16:0} + \mathrm{C18:0}}{0.5 \times \sum \mathrm{MUFA} + 0.5 \times \sum \mathrm{PUFA}n6 + 3 \times \sum \mathrm{PUFA}n3 + \frac{\sum \mathrm{PUFA}n3}{\sum \mathrm{PUFA}n6}} \quad (2)$$

$$\mathrm{S/P} = \frac{\mathrm{C14:0} + \mathrm{C16:0} + \mathrm{C18:0}}{\sum \mathrm{MUFA}cis + \sum \mathrm{PUFA}} \quad (3)$$

Also, the hypocholesterolemic fatty acids (DFA) to the hypercholesterolemic fatty acids (OFA) ratio was calculated based on the formula by Fernández et al. (2007) [29].

$$\text{DFA/OFA} = \frac{\text{C18:1} + \text{C18:2} + \text{C18:3} + \text{C20:4} + \text{C20:5} + \text{C22:6}}{\text{C14:0} + \text{C16:0}} \quad (4)$$

2.6. Oxidative Status and Lipid Peroxidation

Oxidative status and lipid peroxidation of the meat was determined using the TBARS assay kit (DTBA-100) by QuaniChrom™ (BioAssay Systems; Hayward, CA, USA). TBARS assay utilizes thiobarbituric acid as a reagent, which detects products of lipid decomposition and peroxidation known as malondialdehydes (MDA). The analysis was performed according to manufacturer's protocol. First, 200 µL of ice-cold phosphate-buffered saline (PBS) was added to 20 mg of pork samples. Then, samples were quickly homogenized so that the 100 µL of the precipitate of each sample could be pipetted out into empty Eppendorf tubes. To each of the vials 200 µL of 10% solution of trichloroacetic acid was added and incubated on ice for 5 min. Afterwards, samples were centrifuged for 5 min at 14,000 rpm, from which 200 µL of supernatants and 200 µL of TBA reagents were pipetted out into new vials to be incubated at 100 °C for 60 min. After that, samples were read in a spectrophotometer (INFINITE M NANO; TECAN™, Männedorf, Switzerland) at A = 535 nm.

2.7. Pork Quality

The color of the muscle was evaluated with a colorimeter (CR-400/410, Konica Minolta; Tokyo, Japan) in accordance with the CIE L*a*b* system. The measurements were performed at three random locations per sample of a 2 cm thick slice of MLD. Hue (b*/a*) and chroma ($\sqrt{}$(a*2 + b*2)) were determined based on a formula provided by Mordenti et al. (2012) [30].

In order to measure drip loss, samples of MLD weighing around 300 g were separately placed into the plastic bags and stored at +4 °C for 24 h. Afterwards, the exudate was emptied from the bags so that it could be weighed, which was later expressed as a relative percentage of the weight sample [31].

WHC was performed on the homogenized samples of meat, which weighed around 300 mg each, in accordance with the methodologies of Grau and Hamm (1952) [32] and Pohja and Ninivarra (1957) [33].

Thermal drip loss was determined using samples of homogenized pork meat weighing between 20 g and 40 g, which then were tightly packed into glass weighing dishes. Subsequently, the samples were submerged and kept at 70 °C for 15 min in a heated bath. After that time, meat samples were taken out of the weighing dishes and left for 24 h so as to allow the water to drip out in order to weight the difference and express it as a relative percentage of the weight sample [34].

2.8. Statistical Analysis

All the data were subjected to heterogeneity and equal distribution tests using Shapiro–Wilk's test and equality of variances using Levene's test. Afterwards, the data were tested by one-factor analysis of variance (ANOVA) or Kruskal–Wallis test if the requirements for the analysis of variance were not met. Tables present the results as mean values ± standard deviations (SDs), standard errors of the means (SEM) and the statistical significance of the group (*p*-value). Results above $p > 0.05$ were considered to be insignificant. The differences between groups were analyzed using RIR Tuckey's test for ANOVA or the Dunn–Bonferroni test for Kruskal–Wallis.

For the determination of average daily gains, carcass traits, chemical composition of meat, oxidative status of meat and the quality of meat, one-way ANOVA was performed followed by RIR Tuckey's test. However, since the conditions for ANOVA were not met for the fatty acid composition, a non-parametric alternative Kruskal–Wallis test was performed followed by Dunn-Bonferroni test.

All statistical analyses were performed with Statistica ver. 12 software.

3. Results

3.1. Animal Performance and Slaughter Performance

During the first stage of the fattening period fatteners fed 10% WDGS showed significantly higher average daily gains (ADG) than pigs fed 15% WDGS, yet, pigs from the control group displayed similar ADG to the two former groups ($p < 0.05$). For the second stage of the fattening period, no statistical differences between the feeding groups were detected. However, a subtle tendency of increasing ADG was observed for the WDGS 15% group ($p > 0.05$). Throughout the entire fattening, no statistical differences between the feeding groups were detected ($p > 0.05$) (Table 2).

Table 2. Average daily gains (ADG) of fatteners fed WDGS during the fattening period.

Item	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
ADG of first stage of fattening period [g]	1218 ^{ab} ± 94.0	1292 ^{b} ± 108.3	1127 ^{a} ± 156.8	26.238	0.0302
ADG of second stage of fattening period [g]	1312 ± 142.1	1401 ± 127.3	1454 ± 122.0	26.777	0.0854
ADG of the entire fattening period [g]	1260± 108.7	1341 ± 104,3	1275 ± 97.8	20.371	0.2322

Numerical values in the same row marked in pairs with letters ab differ at $p \leq 0.05$.

Of all the groups, the WDGS 15% group exhibited higher FCR than the control or WDGS 10% groups (Figure 1).

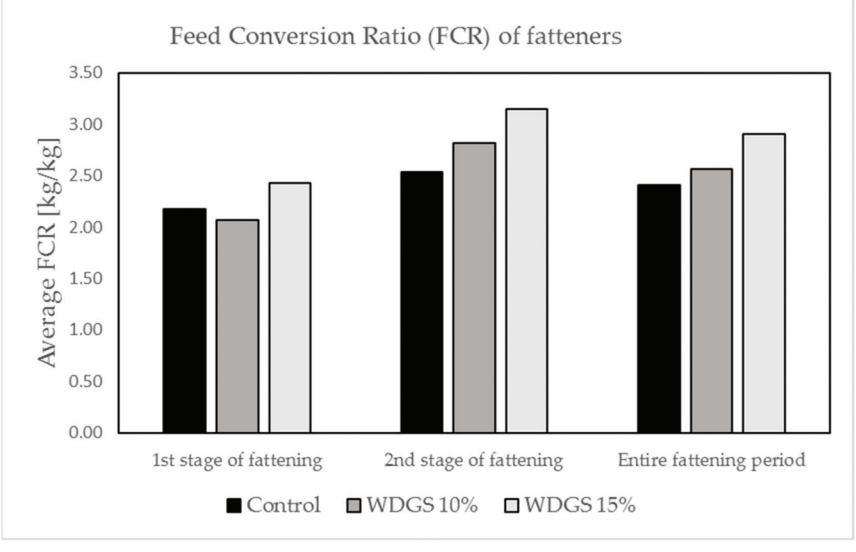

Figure 1. Average feed intake (kg) per weight gained by pigs (kg) throughout the fattening period. Schemes follow the same formatting (FCR).

Table 3 presents the final body weight of fatteners and the parameters of the carcass traits. The final body weight and the carcass traits were overall similar between each of the groups. No statistical differences between any of the groups were detected for the carcass traits ($p > 0.05$).

Table 3. Carcass traits of pigs in the experiment.

Item	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
Final body weight [kg]	135.1 ± 8.2	138.5 ± 12.9	132.0 ± 12.1	2.1283	0.5897
Hot carcass weight [kg]	102.6 ± 6.6	104.8 ± 11.1	99.6 ± 10.1	1.7861	0.6358
Dressing percentage [%]	75.9 ± 0.9	75.6 ± 1.6	75.4 ± 1.7	0.2738	0.6653
Meatiness [%]	60.5 ± 1.6	60.8 ± 1.8	60.7 ± 1.5	0.3030	0.9593
Length of *Musculus longissimus dorsi* [mm]	66.0 ± 4.7	63.8 ± 7.9	64.9 ± 6.0	1.1843	0.7604
Backfat thickness [mm]	12.0 ± 2.1	11.5 ± 2.7	11.5 ± 2.7	0.4721	0.8562

3.2. Chemical Analyses

Nutritional Value

The data on the chemical composition of meat are shown in Table 4. No effect or statistical differences were detected for the chemical composition of MLD ($p > 0.05$).

Table 4. Chemical composition of *Musculus longissimus dorsi*.

Item	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
	% in Meat				
Dry Matter	27.7 ± 0.9	27.4 ± 1.2	27.6 ± 0.5	0.1812	0.3289
Crude Protein	22.9 ± 0.6	23.3 ± 0.5	23.0 ± 0.4	0.0968	0.2711
Crude Fat	2.2 ± 0.4	2.2 ± 0.6	2.2 ± 0.1	0.0732	0.9582
Crude Ash	1.1 ± 0.07	1.1 ± 0.08	1.1 ± 0.01	0.0122	0.7709

3.3. Fatty Acid Profile

The data in Table 5 present the fatty acid composition of the intramuscular fat (IMF) of MLD. No statistical differences were detected for the concentrations of SFAs, MUFAs or PUFAs ($p > 0.05$). Out of all of the examined fatty acids only eicosenoic acid (C20:1n9) exhibited significant differences, where the highest concentration was determined for the control group and the lowest for the WDGS 15% group ($p < 0.05$). AI was the only one of the health-promoting indexes that was determined to be statistically different via the Kruskal–Wallis and the Dunn–Bonferroni tests.

Table 5. Fatty acid profile of intramuscular fat extracted from *Musculus longissimus dorsi* (g/100 g of fatty acids) and values of health-promoting indexes.

Specification	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
C10:0	0.12 ± 0.04	0.10 ± 0.00	0.10 ± 0.01	0.005	0.3487
C12:0	0.18 ± 0.09	0.14 ± 0.10	0.12 ± 0.03	0.018	0.3009
C14:0	1.31 ± 0.12	1.45 ± 0.18	1.19 ± 0.10	0.040	0.2160
C16:0	23.81 ± 1.08	24.36 ± 0.85	23.57 ± 1.02	0.233	0.4903
C16:1	3.04 ± 0.24	3.17 ± 0.25	3.29 ± 0.13	0.053	0.1083
C17:0	0.21 ± 0.02	0.19 ± 0.07	0.23 ± 0.04	0.012	0.3038
C17:1	0.21 ± 0.03	0.20 ± 0.08	0.24 ± 0.05	0.013	0.3313
C18:0	12.19 ± 0.83	11.89 ± 0.69	11.35 ± 0.89	0.198	0.4843

Table 5. *Cont.*

Specification	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
C18:1n9c	42.86 ± 3.05	43.02 ± 1.03	43.65 ± 1.57	0.465	0.4308
C18:1n7c	3.69 ± 0.32	3.78 ± 0.22	3.83 ± 0.23	0.059	0.4233
C18:2n6c	7.63 ± 1.03	8.29 ± 0.78	8.81 ± 1.04	0.242	0.1475
C18:3n3	0.30 ± 0.05	0.29 ± 0.04	0.30 ± 0.04	0.010	0.8355
C20:0	0.17 ± 0.01	0.15 ± 0.04	0.16 ± 0.02	0.006	0.3039
C20:1n9	0.73 ^{c} ± 0.01	0.43 ^{b} ± 0.05	0.64 ^{a} ± 0.03	0.032	0.0005
C20:2n6	0.27 ± 0.02	0.27 ± 0.03	0.29 ± 0.05	0.008	0.7099
C23:0	0.51 ± 0.12	0.52 ± 0.19	0.58 ± 0.25	0.043	0.7943
SFAs	38.49 ± 1.78	38.8 ± 1.36	37.29 ± 1.94	0.410	0.2714
MUFAs	50.54 ± 3.57	50.59 ± 1.12	51.65 ± 1.95	0.553	0.2636
PUFAs	8.20 ± 1.09	8.85 ± 0.82	9.40 ± 1.08	0.253	0.1477
PUFAs n-3	0.30 ± 0.05	0.29 ± 0.04	0.30 ± 0.04	0.010	0.8355
PUFAs n-6	7.90 ± 1.04	8.56 ± 0.80	9.10 ± 1.06	0.247	0.1475
AI	0.50 ^{ab} ± 0.04	0.51 ^{b} ± 0.01	0.46 ^{a} ± 0.02	0.007	0.0277
TI	1.24 ± 0.09	1.24 ± 0.04	1.15 ± 0.05	0.017	0.0408
S/P	0.67 ± 0.05	0.67 ± 0.02	0.62 ± 0.03	0.009	0.0408
DFA/OFA	2.17 ± 0.15	2.15 ± 0.05	2.29 ± 0.08	0.027	0.0759

SFAs (Saturated fatty acids) = C10:0 + C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C23:0. MUFAs (Monounsaturated fatty acids) = C16:1 + C17:1 + C18:1n9c + C18:1n7c + C20:1n9. PUFAs (Polyunsaturated fatty acids) = C18:3n3 + C18:2n6c + C20:2n6. PUFAs n-3 (OMEGA-3) = C18:3n3. PUFAs n-6 (OMEGA-6) = C18:2n6c + C20:2n6. Numerical values in the same row marked in pairs with letters abc differ at $p \leq 0.05$.

3.4. Oxidative Status of Meat

The ability to limit lipid peroxidation (TBARS) of MLD varied significantly between groups ($p < 0.05$), and is presented in Figure 2. The lowest concentration of lipid oxidation was observed in the WDGS 10% group, and it was significantly lower compared to the control and WDGS 15% groups. There were no statistical differences between the control and WDGS 15% groups.

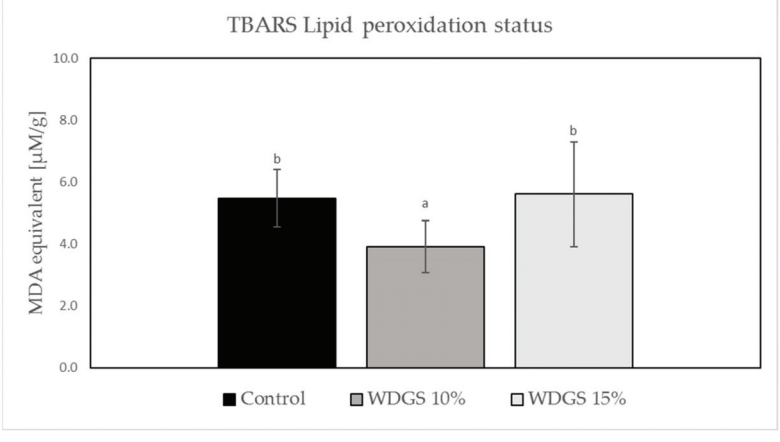

Figure 2. Lipid peroxidation status of *Musculus longissimus dorsi* samples ($p < 0.05$). Numerical values in the same row marked in pairs with letters ab differ at $p \leq 0.05$.

3.5. Meat Quality

As presented in Table 6, meat quality analyses included measurements such as: drip loss, WHC, thermal drip loss, and colorimetry measurements (luminosity; red–green intensity; yellow–blue intensity; hue; chroma). Samples of MLD displayed significant differences for WHC. The highest effluent exhibited samples were from the WDGS 15% group while the lowest were WDGS 10%. The control group did not differ between the former or the latter group ($p < 0.05$). The colorimetry measurement displayed statistical differences for red–green intensity (a*), yellow–blue intensity (b*) and chroma (C*), which were always the highest for the WDGS 10% group and the lowest for the WDGS 15% group ($p < 0.05$).

Table 6. Quality of meat from *Musculus longissimus dorsi*.

Item	Experimental Groups			SEM	*p*-Value
	Control	WDGS 10%	WDGS 15%		
Drip loss (%)	4.88 ± 0.97	5.42 ± 1.29	4.86 ± 0.72	0.1961	0.4028
Water holding capacity (cm^2/g)	22.26 ^{ab} ± 1.73	20.08 ^{a} ± 2.34	23.42 ^{b} ± 2.28	0.4787	0.0096
Thermal drip loss (%)	22.2 ± 3.65	21.63 ± 1.41	20.01 ± 4.40	0.6545	0.3796
L*	51.21 ± 2.22	51.38 ± 1.75	51.96 ± 1.91	0.3791	0.3445
a*	5.54 ^{ab} ± 0.94	5.86 ^{b} ± 0.74	5.03 ^{a} ± 1.09	0.1901	0.0066
b*	4.04 ^{ab} ± 1.10	4.62 ^{b} ± 7.49	3.94 ^{a} ± 6.41	0.1860	0.0180
h*	0.74 ± 0.21	0.80 ± 0.12	0.79 ± 0.14	0.0180	0.3839
C*	6.86 ^{ab} ± 1.12	7.47 ^{b} ± 0.86	6.39 ^{a} ± 1.34	0.2300	0.0033

Numerical values in the same row marked in pairs with letters ab differ at $p \leq 0.05$.

4. Discussion

WDGS has been actively utilized as a feedstuff source for ruminants, in particular cattle, which may be of practical interest for farms located in the vicinity of ethanol plants [4,6,11,35]. However, currently, with liquid feeding becoming more and more widespread in pig farming, WDGS seems as a viable component for pigs' diets. This study further reinforces the assertion that pigs can indeed be fed ensilaged WDGS under a wet lot feeding system, which can partially substitute protein feedstuff and cereal grains. In the WDGS 10% and WDGS 15% groups, the average combined share of soybean and rapeseed meals in the diets was lowered by 25% and 37%, respectively, throughout the entire fattening period. However, according to our study, nothing over about 15% WDGS should be used in the diets of fatteners as it may lead to feed refusals, stunted growth, and subsequently decreased average daily gains. This phenomenon was observed for fatteners which were initially fed 20% WDGS in their diet during the introductory period. This prompted us to decrease the concentration of WDGS by 5% so that the final feed mixture contained 15% WDGS. Similar findings were reported for fatteners being fed concentrations of above 15% DDGS [17]. Interestingly, some authors reported that apparently feeding fatteners with 20% [36] or 30% WDGS [7] did not affect weight gains, feed intake or feed conversion for the former, while for the latter it only affected feed intake and FCR. Additionally, fatteners fed 15% WDGS during the first stage of the fattening period displayed the lowest ADG which might have been explained by the initially lowered feed intake in the introductory period. While not statistically significant, during the second stage of the fattening period there was a tendency toward a slight increase in ADG for the WDGS 15% group, which can be explained by the compensatory growth phenomenon. Moreover, the addition of both concentrations of WDGS did not affect ADG during the entire fattening period. In general, the WDGS 15% group utilized its feed worse than the control group or pigs fed 10% WDGS. Despite the fact that the concentration of crude protein in the

diets was similar overall, and that the amino acid composition of replaced soybean meal is considered to be generally better, fatteners achieved fairly similar ADGs and final body weights. Therefore, it can be deduced that the inclusion of WDGS may in fact enhance the utilization of proteins. The dosage of distillers' grains did not affect the carcass traits: final body weight, hot carcass weight, *Longissimus dorsi* muscle, dressing percentage, depth and backfat thickness, or the chemical composition of the meat: dry matter and crude fat. Similar findings were obtained and described by other authors [18,37,38]. This phenomenon may be explained by an enhanced utilization of nutrients and the well-balanced energy and protein requirements of the diets. The composition of fatty acids is one of the most important measurements of the dietary value of meat. The addition of WDGS did not affect the total composition of the sum of SFA, MUFA and PUFA, including PUFA n-3 and n-6, except for C20:1n9. Moreover, it is noteworthy that WDGS is characterized by a rather high concentration of fat of around 12% DM [39]. WDGS's profile of unsaturated fatty acids is similar to that of corn oil's, where the fat consists of mainly oleic (21.9%) and linoleic acids (45.1%) [40]. In this study no statistical increase or decrease in the fatty acid composition of IMF was found, except for C20:1n9. This contradicts the findings of several authors. Contrary to our findings, Świątkiewicz et al. (2021) [38] state that the SFAs were affected by the addition of distillers' grains, while MUFAs or PUFAs were unaffected. However, according to Harris et al. (2018) [13], SFAs, MUFAs and PUFAs of IMF are affected by distillers' grains. The fatty acid profile is crucial for the determination of health-promoting indexes such as AI, TI, S/P and DFA/OFA [41]. The addition of ensilaged WDGS did not cause any of the health-promoting indexes to worsen. Higher concentrations of WDGS in the diets tended to slightly lower the AI values of IMF, which is beneficial for human consumption, however, the values of TI, S/P, DFA/OFA remained unaffected. According to Alagón et al. (2015) [42], replacing feed concentrate with 20% DDGS does not affect health-promoting indexes. Moreover, the fatty acids composition is responsible for lipid oxidation. One of the factors that may affect lipid peroxidation is the higher composition of unsaturated fatty acids such as PUFA and MUFA [43,44]. Lipid oxidation in creates secondary metabolites such as malonaldehyde (MDA), which are then used in TBARS assay to assess peroxidation [45,46]. The data show that the addition of WDGS into fatteners' diets did not negatively impact the oxidative status of pork meat. In comparison to the control feed, feed containing 15% WDGS did not significantly influence or increase the lipid peroxidation of pork loin. Moreover, pigs fed diets with the addition of 10% WDGS exhibited a significant decrease in lipid peroxidation, which points to the lowest oxidation of all the groups. Although statistically unproven in this study, the higher concentrations of MUFAs and PUFAs in the WDGS 15% group lead to higher TBARS than the WDGS 10% group. The received TBARS values for raw pork were considerably higher than those reported by Papastergiadis et al. (2012) [47]. This study demonstrates that the experimental diets affected only some of the parameters of meat quality. While meat quality is largely unaffected by the addition of WDGS there were statistical differences for WHC. WHC is a meat parameter that can be altered through pH level changes, yet pH was not measured during this experiment. In general the higher meat pH, the higher WHC of meat [48–50]. WHC is a parameter describing the capacity of meat to retain water, which is an important factor since water loss reduces sellable meat/carcass weight, reduces overall meat quality and creates exudative for microorganisms to proliferate [51]. The detected values of WHC (20.08–23.42 cm^2/g) of MLD were higher than the ones (15.2–17.1 cm^2/g) reported by Sońta et al. (2021) [41]. Sońta et al. (2021) [41] utilized the dry lot feeding of fatteners with the implementation of legume seeds into their diets. Meat coloration parameters are one of the most important factors influencing the consumption of meat as the consumer relies heavily on their sensory evaluation before the purchase. In this study, pigs fed 10% WDGS exhibited higher color saturation (C*), and saturation in red (a*) and yellow (b*) than pigs fed 15%. As a general rule, the redness (a*) is influenced mainly by the myoglobin content, while yellowness (b*) is heavily affected by the concentration of IMF [52]. Similarly to a*, saturation (C*) is a parameter responsible for illustrating the formation of oxymyoglobin,

which is an oxidated form of myoglobin [52,53]. Unlike WDGS, DDGS does not seem to affect color parameters [38].

5. Conclusions

The use of WDGS in pig feeding allows for the partial replacement of protein components. In conclusion, the results of this experiment showcased that the addition of ensilaged WDGS from 10% to 15% DM to the fatteners' feed did not cause any adverse effects on pig performance. Even as evidenced by the WDGS 10% group, WDGS may positively affect pork production. During the introductory period pigs fed diets above 15% WDGS decreased their feed intake. Moreover, the addition of ensilaged WDGS does not affect the carcass traits, meat quality, chemical composition of meat or the physiochemical characteristics of the pork. It is worth mentioning that a concentration above 15% WDGS may negatively affect pigs performances such as animal growth and feed intake. Furthermore, the substitution of ensilaged WDGS in fatteners' feed may become a viable and beneficial alternative to partially replace some of the concentrates such as cereal grains or protein feedstuff for liquid-fed pigs.

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Effects of the Dietary Replacement of Soybean Oil with Rubber Seed Oil on the Growth Performance, Carcass Trait, and Status of Lipid Metabolism in Pekin Ducks

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Abstract: The objective of this study is to determine the effects of the dietary replacement of soybean oil (SO) with rubber seed oil (RSO) on the growth performance, carcass trait, and lipid metabolism in Pekin ducks. A total of 160 1-day-old Pekin ducks were randomly allocated to four experimental treatments and fed diets with different ratios of SO to RSO as follows: 3:0 (control), 2:1, 1:2, and 0:3. Dietary RSO supplementation had no effect on their growth performance; however, it significantly decreased the yield of abdominal fat ($p < 0.05$). As the dietary RSO increased, the plasma TG, CHO, LDL-C, and HDL-C contents of ducks decreased ($p < 0.05$). Additionally, the contents of total fat, triglycerides, and cholesterol in the liver and breast reduced in the ducks fed RSO diets ($p < 0.05$). Liver n-3 PUFA levels linearly increased ($p < 0.05$), while the n-6/n-3 PUFA ratios reduced with increasing RSO levels ($p < 0.05$). Moreover, dietary RSO supplementation resulted in decreased gene expressions of *FABP1*, *ME1*, *SREBP1c*, *FASN*, *DGAT2*, and *HMGCR* ($p < 0.05$), while there was an increased expression of the *ABCA1* gene ($p < 0.05$) in the liver of the ducks. In conclusion, dietary RSO supplementation reduced fat deposition and enhanced n-3 PUFA levels without affecting the growth performance of Pekin ducks.

Keywords: alternative oil sources; plasma biochemical profile; ducks; fatty acid profile; liver gene expression

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1. Introduction

In China, the shortage of feed ingredients has become an urgent problem, especially the scarcity of soybean resources, resulting in an inadequate supply of energy feeds. Consequently, this has become an important factor limiting livestock development [1]. Therefore, there is an urgent search for alternative resources to relieve the pressure of feed shortages. China is the largest producer and consumer of ducks in the world, and the duck industry has become an important part of the national economy. The characteristics of roughage tolerance and disease resistance in ducks make them ideal for consuming unconventional feed.

Rubber trees are widely distributed in tropical and subtropical areas, and their seeds are often discarded and not effectively exploited. Rubber seed oil (RSO) contains 26% unsaturated fatty acids and 57% polyunsaturated fatty acids (PUFAs), with the ratio of n-6/n-3 PUFAs being close to 2:1, and it is rich in flavonoid and phenolic compounds [2]. RSO has been used as a biofuel in previous studies; however, its potential as a functional plant oil for feed has been ignored [3]. In recent years, there have been some studies that showed that RSO as a feed lipid source was added to animal diets. For example, dietary RSO supplementation increased the content of n-3 PUFAs and had no negative effect on milk production in cows [4]. In our previous study, dietary RSO added to the feed of laying hens

increased the DHA content and decreased the cholesterol content in eggs [5]. In addition, we also found that RSO exhibited anti-inflammatory and antioxidant properties, and it could alleviate the LPS-induced inflammatory response of RAW 267.4 macrophages [2], which were verified in laying hens [6]. Thus, we speculate that RSO has the potential to be an alternative resource to soybean oil for feeding ducks.

Recently, the increasing consumer demands for good food quality, driven by improved living standards, have led to the rising popularity of functional foods, such as those abundant in n-3 PUFAs. Studies have demonstrated that n-3 PUFAs can prevent cardiovascular diseases, such as hypertension and hyperlipidemia [7], promote nervous system development [8], and improve anti-inflammatory and antioxidant responses [9,10]. DHA, especially, plays a vital role in maintaining normal brain development and function [11]. In modern dietary structures, the intake of n-3 PUFAs by humans is limited [12]. The traditional way of obtaining n-3 PUFAs is mainly through the consumption of fish oil; however, it is limited because of its low yield and high price [5]. Previous studies showed that adding n-3 PUFA-enriched resources to animal diets resulted in an increase in n-3 PUFA content in animal products, such as linseed oil [13] and microalgae [14,15]. Since n-3 PUFA-enriched feed ingredients are scarce and expensive, searching for more suitable resources has become a hot topic. RSO contains more than 20% of n-3 PUFAs, which can not only be used as an energy feed but also as a potential resource for the production of n-3 PUFA foods. Therefore, our study evaluates the effects of the dietary replacement of soybean oil with RSO on the growth performance, carcass trait, and lipid metabolism status of Pekin ducks.

2. Materials and Methods

The Animal Ethics Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences approved all the procedures for this experiment (FRI-CAAS-20220706). Crude RSO in this experiment was obtained from rubber seeds by cold pressing.

2.1. Animals, Diet, and Management

A total of 160 1-day-old Pekin ducks were randomly allocated into 4 groups, each including 4 replicates with 10 Pekin ducks per replicate. No significant difference in the initial weight was observed among the groups ($p > 0.05$) and all replicates were equally distributed in different spatial directions. The four experimental diets with different ratios of soybean oil (SO) to RSO were as follow: 3:0 (control), 2:1, 1:2, and 0:3. The experimental diets were formulated according to the NRC (1994) recommendations for starter (1 to 21 days) and grower ducks (22 to 42 days). The nutrient levels and fatty acid compositions of the experimental diets are shown in Tables 1 and 2. The ducks were housed in single-layer cages with 10 birds per cage (170 cm × 70 cm × 50 cm) and were allowed to eat and drink freely. The temperature was kept at 32 °C from 1 to 7 days of age, and then gradually reduced to 25 °C at 21 days of age, after which it was kept constant and ambient humidity was maintained between 60% to 65%. Natural and artificial light were used in the duck house to provide 24 h of continuous light.

Table 1. Composition and nutrient levels of experimental diets for Pekin ducks (air-dry basis).

Items	Starter Phase (1 to 21 d)	Grower Phase (22 to 42 d)
Ingredients (%)		
Corn	56.90	64.05
Soybean meal	33.00	28.00
Wheat bran	3.00	
Oil	3.00	4.00
$CaHPO_4$	1.70	1.65
Limestone	0.85	0.80

Table 1. *Cont.*

Items	Starter Phase (1 to 21 d)	Grower Phase (22 to 42 d)
Salt	0.30	0.30
DL-Methionine	0.15	0.15
L-Lysine	0.10	0.05
Premix [1]	1.00	1.00
Total	100.00	100.00
Calculated values		
ME (kcal/kg) [2]	2925	3080
Crude protein (%) [3]	20.21	18.10
Calcium (%) [3]	0.93	0.88
Total phosphorus (%)	0.77	0.72
Available phosphorus (%)	0.45	0.42
Methionine (%)	0.45	0.43
Lysine (%)	1.14	0.95
Methionine + Cysteine (%)	0.79	0.74
Threonine (%)	0.75	0.67
Tryptophan (%)	0.23	0.20
Valine (%)	0.92	0.83
Isoleucine (%)	0.81	0.72

[1] Supplied per kilogram of total diet: Cu, 10 mg; Fe, 60 mg; Zn, 60 mg; Mn, 80 mg; Se, 0.3 mg; I, 0.2 mg; choline chloride, 1000 mg; vitamin A, 10,000 IU; vitamin B_1, 2 mg; vitamin B_2, 10 mg; vitamin B_5, 11 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.02 mg; vitamin D_3, 3000 IU; vitamin E, 20 IU; vitamin K_3, 2 mg; nicotinic acid, 50 mg; folic acid, 1 mg; biotin, 0.2 mg. [2] Values are calculated according to ME of feedstuffs for poultry provided by NRC (1994). [3] The numbers are analyzed values.

Table 2. Fatty acid composition (% of total fatty acids) of experimental diets for Pekin ducks (measured value).

Items	Starter Phase (1 to 21 d)				Grower Phase (22 to 42 d)			
	3:0	2:1	1:2	0:3	3:0	2:1	1:2	0:3
C16:0	14.35	13.86	13.37	12.67	14.06	13.62	13.12	12.43
C18:0	5.51	6.08	6.64	6.98	5.35	6.52	6.54	7.47
C18:1	25.35	25.21	25.06	24.94	25.34	24.93	25.36	24.98
C18:2N6(LA)	44.60	42.56	40.37	39.17	45.25	41.52	40.00	38.04
C18:3N3(ALA)	6.45	9.19	12.03	14.34	7.29	10.60	12.83	14.96
SFA	21.36	21.14	21.20	20.54	20.85	21.62	20.66	20.96
MUFA	26,03	25.87	25.70	25.55	25.98	25.64	26.04	25.65
PUFA	52.61	52.99	53.10	53.90	53.18	52.75	53.29	53.39
n-3 PUFA	6.79	9.50	12.19	14.52	7.55	10.87	13.03	15.14
n-6 PUFA	45.82	43.49	40.91	39.39	45.63	41.88	40.26	38.26
n-6/n-3	6.75	4.58	3.35	2.71	6.04	3.85	3.09	2.53

LA, linoleic acid; ALA, α-linolenic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

2.2. Data Collection and Sample Preparation

At 1, 21, and 42 days of age, after overnight fasting, the ducks were weighed in each replicate and the average body weight (BW) was calculated. The average daily feed intake (ADFI), average daily weight gain (ADG), and feed/gain (F/G) at 1 to 21, 22 to 42, and 1 to 42 days of age were corrected for mortality. At 42 days of age, two ducks from each replicate were randomly selected and blood was collected from a wing vein into heparin sodium-containing tubes, centrifuged at 3500 rpm for 10 min, then kept at −20 °C for further analysis. Thereafter, these ducks were euthanized by CO_2 inhalation; then, the breast muscle, thigh muscle, abdominal fat, heart, liver, pancreas, spleen, thymus, and bursa were removed and weighed to calculate the carcass traits and relative organ weight (%, organ weight as a percentage of body weight). Subsequently, liver and breast samples were collected and frozen in liquid nitrogen, and then stored at −80 °C for further analysis.

2.3. Plasma Biochemical Parameters

The plasma biochemical parameters were determined with an automatic biochemical analyzer (Hitachi 7080, Tokyo, Japan), including total protein (TP), albumin (ALB), globulin (GLB), triglyceride (TG), total cholesterol (CHO), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), and alanine transaminase (ALT).

2.4. Liver and Breast Chemical Compositions

The dry matter of liver and breast samples was calculated by freeze-drying; then, the freeze-dried samples were added to chloroform–methanol (2:1, v/v) for total fat extraction [16]. About 1 g of tissue was homogenized in 9 mL of saline on ice, then the mixture was centrifuged at 3500 rpm for 10 min at 4 °C, and we extracted the supernatant for biochemical parameter analysis; the TG and CHO contents of the liver and breast were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) the and results were expressed as mmol per mg total protein.

2.5. Liver Fatty Acid Profile

We used gas chromatography–mass spectrometry (GC-MS) to determine the liver fatty acid profile [5]. In brief, GC-MS was performed using Agilent 7890/5975C (Agilent, CA, USA) to detect the hepatic fatty acid profile. The sample was injected into the Agilent DB-WAX (30 m × 0.25 mm × 0.25 μm) column at a flow rate of 1.0 mL/min, and the carrier gas was helium. This was programmed for an initial temperature of 50 °C (3 min) to 220 °C (5 min) with a 10 °C/min increase. The standard curves were based on fatty acid standards from Sigma (St. Louis, MO, USA). The results are shown as the percentage (%) of each fatty acid in the total fatty acid.

2.6. Liver Gene Expression

Total RNA from the liver samples was extracted by an RNAiso Plus kit (Takara, Dalian, China), according to the manufacturer's instructions, and the purity and concentration of the total RNA were determined by NanoDrop ND-2000 (Thermo Scientific, Madison, MA, USA) with an OD260/280 ratio of 1.8–2.0. The first strand cDNA was synthesized using equal amounts of total RNA and a Reverse Transcription cDNA Kit, and then stored at −80 °C for further analysis. The real-time PCR was performed in a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) using an SYBR Green Quantitative PCR kit, and the cycling conditions referred to previous reports [17]; the relative expression results of the target gene mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method [18]. The primer sequences for target and reference genes are shown in Table 3.

Table 3. Primer sequences for real-time PCR.

Gene	GeneBank Accession No.	Primer Sequence (5′-3′)	Product Size (bp)
FABP1	XM_005023289.5	F: AGAGAAAGCCAAGACCGTTG R: GGTGATGGTGTCTCCGTTGA	103
ME1	XM_038177167.1	F: ATTTCGGAGGCCAAGAGGAC R: GCCAGTTTACCAACCGGGAT	177
SREBP1C	JQ080310.1	F: AGCAGAGCAACCAGAAGCTG R: AGGGACTTGCTCTTCTGCAC	70
FASN	XM_027459847.2	F: TCTGCACTGACTTCAAGCGT R: GTTGCATGACTGGGTCTGGA	153
DGAT2	XM_027466264.2	F: TTGGGTACCATCCACATGGC R: CAGGGTAGCAAGGTACGGTC	161
HMGCR	XM_027446071.2	F: GGCGTAGCAGGACCATTGTA R: TTGCTCCTCCACCGAGACAT	124
ABCA1	XM_038169977.1	F: TGAGGACATGCGCTACGTTT R: TTGCACCCTGATGATTGCCT	172

Table 3. *Cont.*

Gene	GeneBank Accession No.	Primer Sequence (5′-3′)	Product Size (bp)
apoA-I	XM_027444321.2	F: AGCCCCTCCTGCATAAATAGC R: CGACTCTCATCTTCGGGCAG	174
β-actin	NM_001310421.1	F: GGTATCGGCAGCAGTCTTA R: TTCACAGAGGCGAGTAACTT	158

FABP1 = fatty acid-binding protein 1; *ME1* = malic enzyme 1; *SREBP1c* = sterol regulatory element-binding protein 1c; *FASN* = fatty acid synthase; *DGAT2* = diacylglycerol acyltransferase 2; *HMGCR* = 3-Hydroxy-3-Methylglutaryl-CoA reductase; *ABCA1* = ATP-binding cassette transporter A1; *apoA-I* = apolipoprotein A-I.

2.7. Statistical Analysis

The data were analyzed by one-way ANOVA and Duncan's test using SAS9.4 software (SAS Institute Inc., Cary, NC, USA), and the results are expressed as means and SEM. The probability level of $p < 0.05$ for the multiple comparison results was considered to be statistically significant.

3. Results

3.1. Growth Performance and Carcass Traits

The growth performances (BW, ADFI, ADG, and F/G) of ducks were unaffected by the partial or complete substitution of SO with RSO ($p > 0.05$; Table 4). Compared to the control group, the abdominal fat percentage decreased in the 0:3 groups ($p < 0.05$; Table 5).

Table 4. Effects of dietary replacement of soybean oil with rubber seed oil on growth performance of Pekin ducks [1].

Items	Age (Days)	Dietary SO:RSO Ratio				SEM	*p*-Value
		3:0	2:1	1:2	0:3		
BW	1	53.75	54.00	53.59	53.53	0.15	0.1729
	21	1324.68	1301.56	1256.87	1287.81	16.44	0.0744
	42	2934.15	2950.62	2921.77	2872.20	45.28	0.6535
ADFI	1–21	84.00	83.48	79.95	82.04	1.52	0.2836
	22–42	169.20	169.81	174.46	170.60	3.71	0.7510
	1–42	126.60	126.65	127.21	126.32	2.12	0.9925
ADG	1–21	59.94	59.41	57.77	58.78	0.78	0.0762
	22–42	76.64	78.85	79.3	76.70	1.90	0.4960
	1–42	68.58	68.96	68.29	67.11	1.08	0.6557
F/G	1–21	1.39	1.41	1.40	1.40	0.02	0.9354
	22–42	2.21	2.16	2.21	2.26	0.04	0.4441
	1–42	1.85	1.83	1.86	188	0.02	0.4980

BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; F/G = feed/gain. [1] Values are the means of 4 cages (n = 4).

Table 5. Effects of dietary replacement of soybean oil with rubber seed oil on carcass traits in Pekin ducks [1].

Items	Dietary SO:RSO Ratio				SEM	*p*-Value
	3:0	2:1	1:2	0:3		
Breast yield (%)	13.15	13.14	12.79	12.89	0.50	0.9389
Thigh yield (%)	8.49	8.82	8.53	8.16	0.36	0.6310
Abdominal fat (%)	0.63 ^{a}	0.55 ^{ab}	0.54 ^{ab}	0.44 ^{b}	0.04	0.0327
Heart (%)	0.51	0.50	0.54	0.54	0.02	0.3591
Liver (%)	2.35	2.34	2.37	2.02	0.12	0.1158
Gizzard (%)	2.18	2.21	2.20	2.29	0.09	0.8693
Spleen (%)	0.06	0.06	0.08	0.07	0.01	0.0655

Table 5. *Cont.*

Items	Dietary SO:RSO Ratio				SEM	*p*-Value
	3:0	2:1	1:2	0:3		
Pancreas (%)	0.27	0.28	0.31	0.30	0.01	0.0840
Bursal (%)	0.10	0.09	0.10	0.08	0.01	0.6036
Caecum (%)	0.27	0.31	0.31	0.23	0.03	0.1950

[a,b] Values within a column with different letter superscripts mean significant differences ($p < 0.05$). [1] Values are the means of 8 ducks (n = 8).

3.2. Plasma Biochemical Parameters

Compared to the control group, dietary supplementation with RSO decreased ALT activity and the contents of TG, CHO, HDL-C, and LDL-C in the plasma ($p < 0.05$; Table 6). However, there were no significant differences for TP, ALB, GLB, and AST levels ($p > 0.05$; Table 6).

Table 6. Effects of dietary replacement of soybean oil with rubber seed oil on plasma biochemical parameters in Pekin ducks [1].

Items	Dietary SO:RSO Ratio				SEM	*p*-Value
	3:0	2:1	1:2	0:3		
TP (g/L)	29.05	27.43	28.45	28.58	0.96	0.6896
ALB (g/L)	12.34	12.77	12.22	11.67	0.59	0.6182
GLB (g/L)	17.15	14.64	15.84	16.49	0.86	0.2111
TG (mmol/L)	1.17 [a]	0.86 [b]	0.90 [b]	0.75 [b]	0.07	0.0087
CHO (mmol/L)	5.32 [a]	4.18 [b]	4.28 [b]	4.10 [b]	0.29	0.0189
HDL-C (mmol/L)	3.20 [a]	1.90 [b]	2.21 [b]	1.90 [b]	0.12	<0.0001
LDL-C (mmol/L)	1.75 [a]	1.45 [b]	1.49 [b]	1.40 [b]	0.07	0.0073
AST (U/L)	4.51	3.68	3.89	3.90	0.54	0.7054
ALT (U/L)	19.80 [a]	12.99 [b]	9.13 [c]	9.08 [c]	0.96	<0.0001

TP = total protein; ALB = albumin; GLB = globulin; TG = triglyceride; CHO = cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; AST = aspartate aminotransferase; ALT = alkaline aminotransferase. [a–c] Values within a column with different letter superscripts mean significant differences ($p < 0.05$). [1] Values are the means of 8 ducks (n = 8).

3.3. Liver and Breast Chemical Composition

In this experiment, we found that RSO supplementation significantly decreased ($p < 0.05$; Table 7) the total fat level and CHO content in the liver and breast, and TG content decreased in the 0:3 group ($p < 0.05$; Table 7).

Table 7. Effects of dietary replacement of soybean oil with rubber seed oil on liver and breast chemical composition in Pekin ducks [1].

Items	Dietary SO:RSO Ratio				SEM	*p*-Value
	3:0	2:1	1:2	0:3		
Liver						
Moisture (%)	69.09 [c]	69.94 [bc]	70.58 [ab]	71.74 [a]	0.42	0.0012
Total fat (%)	3.33 [a]	2.52 [b]	2.34 [b]	2.27 [b]	0.20	0.0149
TG (μmol/g prot)	286.54 [a]	268.16 [ab]	219.18 [bc]	191.86 [c]	17.54	0.0038
CHO (μmol/g prot)	62.22 [a]	47.53 [b]	49.17 [b]	40.77 [c]	3.97	0.0079
Breast						
Moisture (%)	77.54	78.04	77.83	78.34	0.32	0.3464
Total fat (%)	1.42 [a]	1.17 [b]	1.22 [b]	1.10 [b]	0.07	0.0167
TG (μmol/g prot)	70.65 [a]	59.10 [ab]	65.89 [a]	46.37 [b]	6.64	0.0323
CHO (μmol/g prot)	55.81 [a]	43.70 [b]	44.03 [b]	37.82 [b]	3.81	0.0200

TG = triglyceride; CHO = cholesterol. [a–c] Values within a column with different letter superscripts mean significant differences ($p < 0.05$). [1] Values are the means of 8 ducks (n = 8).

3.4. Liver Fatty Acid Profile

Compared with the control group, dietary RSO supplementation significantly increased n-3 PUFA levels ($p < 0.05$), especially a-linolenic (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic (DHA) contents, whereas it decreased n-6 PUFA levels and the n-6/n-3 PUFA ratio in the liver ($p < 0.05$). No statistical differences among the groups were found in C14:0, C16:0, C16:1, C18:0, C18:1, C20:2 N6, C20:3 N6, arachidonic acid (AA), saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) levels ($p > 0.05$; Table 8).

Table 8. Effects of dietary replacement of soybean oil with rubber seed oil on hepatic fat acid profile in Pekin ducks (%) [1].

Items	Dietary SO:RSO Ratio				SEM	*p*-Value
	3:0	2:1	1:2	0:3		
C14:0	0.46	0.37	0.32	0.34	0.07	0.5159
C16:0	16.78	16.35	17.03	16.04	0.84	0.8425
C16:1	0.97	0.98	0.85	0.90	0.19	0.9505
C18:0	14.19	13.75	15.91	14.90	1.23	0.6380
C18:1	19.01	18.01	17.82	18.20	1.72	0.9626
C18:2 N6(LA)	20.35 ^{a}	17.95 ^{b}	16.83 ^{bc}	16.59 ^{c}	0.34	0.0010
C18:3 N3(ALA)	1.38 ^{c}	1.96 ^{bc}	2.25 ^{b}	3.13 ^{a}	0.17	0.0018
C20:2 N6	1.04	0.96	0.98	0.84	0.06	0.1526
C20:3 N6	1.96	1.55	1.84	1.55	0.13	0.1287
C20:4 N6(AA)	15.87	15.52	15.99	15.73	1.59	0.9970
C20:5 N3(EPA)	0.29 ^{c}	0.45 ^{bc}	0.67 ^{ab}	0.83 ^{a}	0.09	0.0141
C22:4 N6	2.65 ^{a}	1.57 ^{b}	2.09 ^{ab}	1.54 ^{b}	0.21	0.0373
C22:5 N6	1.96 ^{a}	1.19 ^{b}	1.21 ^{b}	0.95 ^{b}	0.18	0.0229
C22:5 N3(DPA)	1.41 ^{b}	2.09 ^{ab}	2.47 ^{ab}	2.97 ^{a}	0.32	0.0441
C22:6 N3(DHA)	0.98 ^{c}	1.55 ^{bc}	1.88 ^{b}	3.35 ^{a}	0.23	0.0005
SFA	31.83	30.94	33.69	31.70	1.86	0.7609
MUFA	20.75	19.86	19.61	20.33	1.91	0.9745
PUFA	47.43	49.20	46.70	47.96	1.83	0.8039
n-3 PUFA	4.41 ^{c}	6.77 ^{b}	7.45 ^{b}	10.47 ^{a}	0.47	0.0001
n-6 PUFA	43.01 ^{a}	40.79 ^{ab}	37.89 ^{b}	37.49 ^{b}	1.17	0.0360
n-6/n-3	9.75 ^{a}	6.37 ^{b}	5.28 ^{c}	3.58 ^{d}	0.30	<0.0001

LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-6 PUFA = C18:2 N6 + C20:4 N6 + C22:4 N6 + C22:5 N6; n-3PUFA = C18:3 N3 + C22:5 N3 + C22:5 N3 + C22:6 N3. $^{a–d}$ Values within a column with different letter superscripts mean significant differences ($p < 0.05$). [1] Values are the means of 3 ducks (n = 3).

3.5. Liver Gene Expression

Figure 1 presents the mRNA expression of genes in the liver. The results show that RSO supplementation significantly decreases the expressions of liver *ME1*, *FASN*, *DGAT2*, *HMGCR*, and *apoA-I* genes ($p < 0.05$), while it increases *ABCA1* mRNA expression ($p < 0.05$). Meanwhile, the mRNA expressions of *FABP1* and *SREBP1c* genes decreased in the 1:2 and 0:3 groups.

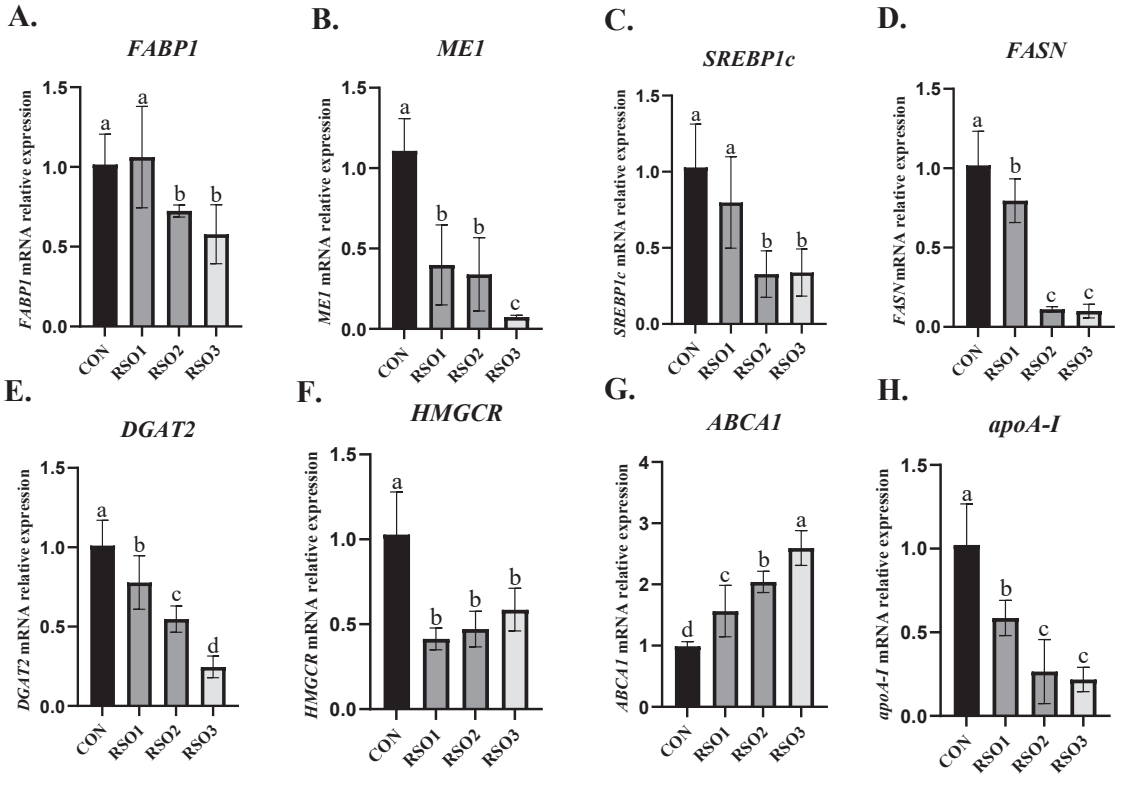

Figure 1. Effects of dietary replacement of soybean oil with rubber seed oil on hepatic lipid metabolism-related genes expression in Pekin ducks. Relative mRNA expressions of *FABP1* (**A**), *ME1* (**B**), *SREBP1c* (**C**), *FASN* (**D**), *DGAT2* (**E**), *HMGCR* (**F**), *ABCA1* (**G**), and *apoA-I* (**H**) genes normalized to *β-actin*, and expressed as a fold change in the control group. Different letters of a–d above bars mean statistically significant differences ($p < 0.05$) in different groups. Results are presented as means with plus error bars (n = 8).

4. Discussion

The shortage of conventional feed resources is a serious problem that China's livestock farming is facing; therefore, searching for alternative resources has become an important research direction [1]. Some studies show that RSO as a non-conventional feed oil can be used for livestock and poultry, such as dairy cows [4] and laying hens [5,6]. Therefore, we attempted to evaluate the feasibility of the dietary replacement of soybean oil with RSO to feed Pekin ducks. In this study, dietary RSO supplementation had no negative effect on the growth performance of ducks. Carcass traits were considered to be the key indexes for evaluating animal performance. From the results of the carcass traits of Pekin ducks, it was observed that there were no significant differences in breast and thigh yields among the groups. However, abdominal fat percentage exhibited a significant decrease in the 0:3 group. Several studies also demonstrated similar results. Feeding broilers with high n-3 PUFA plant oils, such as linseed oil, led to a reduction in abdominal fat deposition and a decrease in abdominal fat weight [13,19]. For the experimental diets, as the level of RSO increased, the level of n-3 PUFA in the diet gradually increased. Thus, we speculated that the reduction in abdominal fat percentage may have been due to n-3 PUFA in RSO.

High triglyceride and high cholesterol contents in plasma are potentially dangerous to human health and are major risk factors for cardiovascular disease [9,20]. The effect of n-3 PUFA on blood lipid levels has been extensively studied in human clinical practice [20],

and the hypolipidemic effect of n-3 PUFA has also been observed in broilers [14,19] and ducks [21,22]. In this study, TG and CHO contents in plasma were gradually decreased in the RSO group. Similar results were observed in the liver and breast. Dietary RSO supplementation significantly reduced the total fat, TG, and CHO contents. Li et al. [22] also reported a similar phenomenon where an increase in dietary n-3 PUFA levels led to a decrease in plasma TG and CHO contents, as well as reduced total fat in the liver and breast of ducks. Superfluous cholesterol at the intracellular level can be transferred to apolipoproteins by transporter proteins to form HDL-C, which is subsequently transported to the liver for metabolism [23]. However, the level of LDL-C in the blood is positively correlated with cardiovascular disease, and its increase is believed to increase the risk of atherosclerosis [21,24]. Ibrahim et al. [19] found that increasing n-3 PUFA levels in diets increased the HDL-C content in broiler plasma and decreased the LDL-C content. However, Huo et al. [25] reported that there were no obvious effects on the contents of HDL-C and LDL in the serum of broilers with increasing n-3 PUFA levels in their diets. In this study, dietary RSO supplementation reduced the LDL-C content in plasma; however, it also reduced the HDL-C content. Some reports also observed that increasing n-3 PUFA levels in the diets decreased the HDL-C content of plasma in ducks [21,22]. HDL-C works to transport cholesterol from the outside of the tissue and cells to the liver for further metabolism [26]. As shown in Tables 6 and 7, dietary RSO supplementation significantly reduces total cholesterol in the plasma, breast, and liver; thus, we speculated that HDL-C reduction was associated with total cholesterol reduction. The levels of AST and ALT in the liver are crucial for maintaining normal metabolism levels, and high AST and ALT activities in the plasma indicate liver injury [27]. Previous studies found that RSO was also rich in flavonoids and phenols in addition to n-3 PUFAs, which can all help alleviate the inflammatory response of RAW 267.4 macrophages induced by LPS [2], and a decrease in AST activity in plasma was observed in the LPS-induced inflammatory response of laying hens [6]. In this experiment, the dietary supplementation of RSO reduced ALT activity in the plasma, suggesting that RSO may alleviate liver injury induced by the vigorous metabolism of ducks, leading to oxidative stress and inflammatory reactions.

Normally, the fatty acid profile of animals is influenced by the type of fatty acid in their diets [28]. Li et al. [22] found that the n-3 PUFA level and n-6/n-3 PUFA ratio were significantly correlated with the dietary n-6/n-3 PUFA ratio in the liver of ducks. Wen et al. [5] revealed that dietary RSO supplementation promoted the deposition of n-3 PUFAs in eggs and reduced the n-6/n-3 PUFA ratio. In this experiment, as RSO supplementation increased, dietary n-3 PUFA level (mainly ALA) increased and the n-6/n-3 PUFA ratio decreased in the liver (Table 8). Similarly, the level of n-3 PUFAs in liver significantly increased, while the n-6 PUFA level and n-6/n-3 PUFA ratio significantly decreased in the RSO groups, which was consistent with the previous reports. Animals can convert ALA to n-3 long-chain PUFAs (EPA and DHA) in vivo with the involvement of fatty acid desaturases and elongases, and this conversion is closely related to the ALA substrate concentration [29,30]. In this study, liver EPA, DPA, and DHA contents gradually increased with increasing dietary RSO supplementation. Overall, the results show that RSO is an effective plant oil to change the fatty acid profile, and dietary RSO supplementation can increase n-3 PUFA levels, such as ALA, EPA, DPA, and DHA, in Pekin ducks.

In the present study, we found that dietary RSO supplementation reduced triglyceride and cholesterol levels in the liver, plasma, and breast, whereas lipid synthesis and metabolism primarily occurred in the liver, from which lipids were transported to tissues and cells via the blood; therefore, we examined the expression of lipid metabolism genes in the liver. *FABP1* is specifically expressed in the liver to transfer fatty acids to the endoplasmic reticulum to synthesize triglycerides [31]. *ME1* encodes a cytosolic enzyme to produce NADPH for fatty acid biosynthesis, and its reduced expression inhibited NADPH production, thereby reducing fatty acid production [32]. *SREBP1c* is a crucial postprandial lipogenic transcription factor that regulates fatty acid synthesis and is increased in the liver of obese animals [33]. *FASN* is a key enzyme in fat synthesis, regulates the *Novo*

synthesis of palmitic acid, and is the basis of long-chain fatty acid synthesis [34]. *DGAT2* is a rate-limiting enzyme that catalyzes the process of diacylglycerols transforming into triglycerides [35]. As shown in Figure 1, we can observe that the gene expressions of *FABP1*, *ME1*, *SREPB1c*, *FASN*, and *DGAT2* decrease with increasing dietary RSO levels. Therefore, we speculated that the low TG levels in the RSO group may have occurred due to reduced fatty acid synthesis in the liver. *HMGCR* is a rate-limiting enzyme in the endogenous cholesterol synthesis pathway of acetyl-CoA in the liver [36]. HDL-C is deemed to promote reverse cholesterol transport, which removes excess cholesterol from the cells outside the liver to transport it to the liver for metabolism [26]. *ABCA1* is a crucial regulator of cholesterol export to lipid-free apolipoprotein A-I resulting in the formation of nascent HDL-C, and also shows positive effects on immunity and inflammatory responses [37]. In this study, we found that *HMGCR* expression decreased, while *ABCA1* increased as a dietary RSO level. However, *apoA-I* relative expression decreased with the addition of dietary RSO. The expression of these genes indicated that dietary RSO reduced cholesterol synthesis in the liver and promoted reverse cholesterol transport to the liver. The possible reason for the reduction in *apoA-I* expression can be attributed to a decrease in extracellular cholesterol levels in the liver, thereby reducing the requirement for additional *apoA-I* to facilitate cholesterol transfer. In brief, the data indicate that dietary RSO supplementation reduces triglyceride and cholesterol levels in ducks by reducing liver lipid synthesis and improving reverse cholesterol transport to the liver, finally reducing abdominal fat deposits. In this study, we did not consider the differences in the energy values and metabolizable energy between SO and RSO in the ducks. However, the metabolizable energy of plant seed oil for poultry was stable range in 35.02~36.83 MJ/kg according to CHINESE FEED DATABASE. Therefore, we speculated that the metabolizable energy of RSO and SO with similar. Further research is required to determine the optimal replacement of RSO and the lipid-lowering pathway of RSO in ducks.

5. Conclusions

In conclusion, the dietary replacement of soybean oil with RSO had no negative effect on the ducks' growth performance; however, it reduced triglyceride and cholesterol levels by decreasing liver lipid synthesis and improving reverse cholesterol transport, and reduced the abdominal fat percentage. Meanwhile, dietary RSO could also enrich n-3 PUFAs in the Pekin ducks. These results contribute to a better understanding of the application of RSO in duck feed, as well as its value as n-3 PUFA-enriched feed ingredient.

Author Contributions: Z.Z. and Y.G. performed the experimental work and created the experimental design, data analysis, and writing—original draft. Z.Z., Y.G., L.Z., J.L. and Z.W. (Zhanyue Wu) performed the feeding experiment on ducks and determined the main experiments. Y.W., J.C. and Z.W. (Zhiguo Wen) performed, reviewed, and edited the manuscript. Z.W. (Zhiguo Wen) had primary responsibility for the final content. All authors have read and agreed to the published version of the manuscript.

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Effectiveness of Information Acquisition via the Internet in Standardizing the Use of Antimicrobials by Hog Farmers: Insights from China

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Abstract: Antimicrobial residues and resistance caused by farmers' overuse of veterinary antimicrobials have seriously threatened food safety, the ecological environment, and public health. With the popularization of the Internet in rural areas, especially in developing countries, the constraints of obtaining agricultural technical information provided by governments or organizations are greatly eased, farmers' knowledge and skills are significantly improved, and the agricultural standardized production system is effectively constructed. However, there is still a research gap on whether information acquisition via the Internet (IAI) can induce farmers to standardize the use of antimicrobials. Using the data of 675 hog farmers in the Hebei, Shandong, Henan, and Hubei provinces, China, the IV-Heckman and mediating effect models were used to analyze the phenomenon empirically. The main findings revealed that the IAI had exerted a significant influence on the standardized use of veterinary antimicrobials by hog farmers, i.e., the IAI not only helped farmers to decide to standardize the use of antimicrobials but also reduced the amount of investment in the standardized use of antibiotics. Moreover, information-sharing and feedback mechanisms partially mediated the relationship between the IAI and farmers' standardized use of antimicrobials. Finally, considering the heterogeneity of individual endowments, the study further revealed that the IAI significantly impacted the standardized use of antimicrobials for farmers below the age of 36 years. However, the IAI was found to positively and significantly promote farmers' standardized-use decisions only if they had less than five years of breeding time.

Keywords: IAI; veterinary antimicrobials; standardized use; IV-Heckman model; China

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1. Introduction

Since the 1950s, veterinary antimicrobials have been playing an irreplaceable role in reducing livestock morbidity and mortality, promoting livestock growth, and improving the quality of meat products. As people's consumption of meat increases, it is predicted that the use of antimicrobials will also increase globally by 67% in 2030 [1]. However, the overuse and residue of antimicrobials make bacterial microorganisms gradually express resistance characteristics [2]. There are complex transmission routes of antimicrobial resistance between livestock and humans, especially antimicrobials that can re-enter agroecosystems through animal manure, biosolids, and groundwater [3]. Moreover, pathogenic and symbiotic microorganisms in the ecological environment can also transfer to humans directly through contact and food intake [4–6].

Farmers are users of antimicrobials in livestock production and are directly responsible for the standardized use of antimicrobials. The standardized use of antimicrobials is closely related to disease type, breeding techniques, experience, and veterinary services and is

a fluctuating veterinary technical standard [7]. Some studies have demonstrated that the standardized use of antimicrobials is characterized primarily by recommended doses prescribed by veterinarians (prescription antimicrobials) or doses specified in package inserts (over-the-counter antimicrobials) [8]. Specifically, there are significant differences in veterinary experience or skill level. A veterinarian with a high degree of expertise may also be more effective in administering a prescription of antimicrobial for the same disease. In contrast, a veterinarian with poor technologies may need multiple trials and errors to achieve the same effect. Therefore, in many developing countries where veterinary technology lags, although the dosage of antibiotics is still in line with the standardized use of antimicrobials, the standardized dosage of antibiotics is generally at a high level. In addition, farmers have greater operability in the selection and use of over-the-counter antimicrobials, and they are always at greater risk of non-standardized use of over-the-counter antimicrobials under disease stress or loss [9].

The final decision-makers in the value chain of the animal health system, breeders' production decisions are largely influenced by the practices and demands of other actors in the system [10,11]. The main core strategy of guiding the standardized use of antimicrobials and promoting its development towards "no-antimicrobials" is to enhance the cooperation between breeders and other actors such as the government, butchers, processors, and consumers and to realize the circulation of antimicrobials use information in the whole industrial chain [12,13]. Previous studies have found that the government, as the most powerful external stakeholder, can define illegal behaviors through coercive measures to force farmers to standardize the use of antibiotics, or it can use non-coercive means to change the external environment of farmers' safety production, encouraging and guiding them towards standardized use of antimicrobials [11,14–16]. However, information asymmetry may increase the gap between the intended objectives and implementation outcomes of mandatory and non-mandatory policy instruments and even promote the emergence of illicit markets in the non-standardized use of antimicrobials [13]. In addition, many kinds of literature also discussed the factors affecting the use of antibiotics by farmers, including individual characteristics such as gender, age, and education level [17,18]; cognitive characteristics such as risk perception, risk cognition, and risk preference [19]; operational characteristics such as breeding scale, breeding years, breeding mode, organizational participation, and biosafety measures [20,21]; social factors such as relationship network, individual norms, and contractual governance; as well as various policy measures [22–24].

According to the Statistical Report on China's Internet Development, as of June 2022, the Internet penetration rate in China's rural areas reached 58.8%, an increase of 1.2 percentage points over December 2021. Of course, due to many kinds of factors such as terrain characteristics, population density, and service quality, the Internet broadband speed in some rural areas is very slow. Moreover, with the implementation of China's modern agricultural strategy, the Internet has become an essential learning medium for the government or organizations to promote agricultural technology and farmers to obtain the latest agricultural information. Therefore, the information acquisition via the Internet (IAI) in this article mainly refers to farmers' access to information on standardized use of antimicrobials provided by governments or organizations through the Internet. Specifically, the government or organization carries out online technical training, online video exhibition, and solving difficult problems through Internet means, with the purpose of improving farmers' knowledge or skills concerning the standardized use of antimicrobials. Thus, IAI requires farmers to access the standardized use of antibiotics through the Internet. Unfortunately, previous research has not paid much attention to the role of the IAI in standardizing the use of antimicrobials by farmers.

The main objective of the research is to empirically analyze the influence of the IAI on the standardized use of antimicrobials by farmers and its induction mechanism by using the survey data of 675 hog farmers in the Hebei, Henan, Shandong, and Hubei provinces, China. The current study contributes to the literature in the following ways: firstly, the study innovatively defines the concept and measurement criteria for farmers' standardized

use of antimicrobials. Secondly, considering the sample selection bias caused by farmers' standardized use decisions and endogeneity caused by missing variables and reverse causality, the IV-Heckman and the mediating effect models are employed to empirically analyze the promoting effect of the IAI on the standardized use of antimicrobials by farmers and its induction mechanism. Finally, considering the heterogeneity of individual endowments, the study further explores the phenomenon by considering the group differences in the IAI's effects. The overall findings may provide an essential reference for the government to promote farmers' standardized use of veterinary antimicrobials accurately.

The rest of the paper is organized as follows. The second provides the theoretical analysis and research hypothesis. The third part presents data sources and research methods. The fourth part reports the model results and discussion. Finally, conclusions are presented in the last section.

2. Theoretical Analysis and Research Hypothesis

Firstly, the IAI affects farmers' standardized use of antimicrobials through the information supply mechanism. Information constraint is a bottleneck for farmers' safe production in developing countries [25]. Previous studies have confirmed that the farmers' main reason for the overuse of antimicrobials is the lack of medication skills or knowledge [8]. Additionally, the situation is exacerbated by the inadequate supply of veterinary services in rural areas [26]. Through the timely transmission and acquisition of information, the IAI has changed the original channels of information transmission and significantly reduced the cost of government information supply and farmers' information acquisition through the reconfiguration of technology, labor, and capital production factors [27]. Furthermore, the IAI has also changed the supply mode of government information services, updated the original medication knowledge of farmers, improved their cognition of standardized use, and actively guided them to engage in the standardized use of antimicrobials. Hence, hypothesis H1 is proposed.

H1. *The IAI is beneficial to standardize the use of antimicrobials by farmers through the information supply mechanism.*

Secondly, the Internet promotes farmers' standardized use of antimicrobials through the information-sharing mechanism. Through low communication costs, the IAI has changed farmers' original social capital accumulation [28]. Most farmers choose the decentralized breeding mode to reduce the infection rate of livestock disease. However, due to the popularization of the Internet, geographical distance has not changed the strength of the relationship network among farmers [29]. Government training in the practical skills of antimicrobial use is often aimed at targeting specific groups, such as companies, cooperatives, or large farmers. The IAI can realize information sharing between the initial information recipients, such as cooperative organization and other small farmers, and accelerate the rapid dissemination of information or skills [30,31]; reduce the government's cost of agricultural technology promotion; and improve the standardized use of antimicrobials. In addition, the IAI is also likely to accelerate the spread of standardized antimicrobial use technology among farmers through the peer effect, finally forming the imitation and learning effect of standardized antimicrobial use technology. So, hypothesis H2 is given.

H2. *The IAI significantly influences farmers' standardized use of antimicrobials through the information-sharing mechanism.*

Lastly, the IAI guides the farmers to standardize antimicrobials through the information feedback mechanism. The industrial chain system and the food traceability system built by the Internet are the two pillars for constructing a modern food safety system. Especially on the one hand, integrating the industrial chain is an essential part of constructing modern agricultural and industrial systems [32,33]. The Internet can strengthen the links among the stakeholders in the industrial chain, such as producers, butchers, processors,

and sellers, who timely communicate market information such as market price, product quality, and consumer demand [34,35]. Effective market information reduces information asymmetry in the industrial chain, and stakeholders adjust their production decisions in time [36,37]. On the other hand, many countries have set up food traceability systems via the Internet. The antimicrobial residue is key to meat-derived food safety detection [38,39]. Once veterinary antimicrobials' residues exceed the slaughter, processing, or distribution limit, the traceability system pushes the related information to the target farmers. Meanwhile, liability traceability can also restrain farmers from overusing antimicrobials. Therefore, hypothesis H3 is proposed in this paper. The theoretical framework used in the current study is shown in Figure 1.

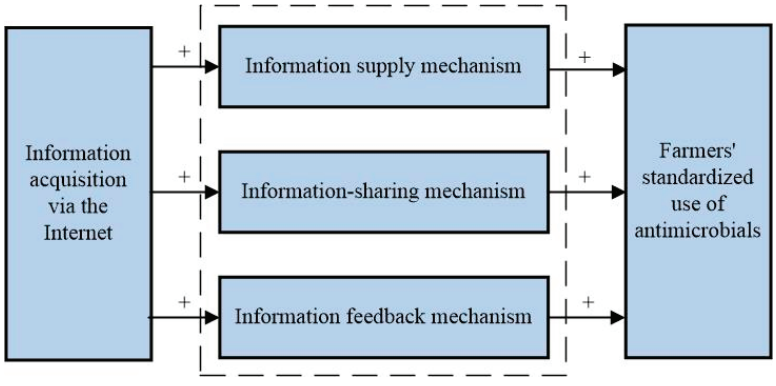

Figure 1. The theoretical framework used in the current study.

H3. *The IAI significantly influences farmers' standardized use of antimicrobials through the information feedback mechanism.*

3. Data and Methods

3.1. Data Source

The data used in this study were obtained from a field survey of hog farmers from the Hebei, Shandong, Henan, and Hubei provinces, China, from July to September 2021. The main reason for selecting sample areas was that the breeding scales in Hebei, Shandong, Henan, and Hubei were 18.101 million, 31.51 million, 43.92 million, and 25.301 million hogs, respectively, in 2020. Meanwhile, the breeding density was large, the incidence of bacterial infectious diseases was high, and the use of antimicrobials was large. The main contents of the questionnaire survey also included individual characteristics, family characteristics, operation and management, antimicrobials use, Internet use, policy measures, etc. A combination of stratified and random samplings was employed for a questionnaire survey. Specifically, five counties were chosen from each province, two–four towns were randomly selected, and 12–16 farmers were randomly selected from each village. About 750 questionnaires were distributed, 75 invalid samples were excluded, and 675 valid samples were selected for empirical analysis, with an effective sample rate of 96.70%. The sampled households from Hebei, Henan, Hubei, and Shandong were 168, 182, 177, and 148, accounting for 24.8%, 26.9%, 26.2%, and 21.9%, respectively.

Table 1 shows the basic characteristics of sampled households' heads. The households' heads were mainly male, accounting for 73.4%, and female farmers occupied a secondary position in family breeding decisions. Male laborers accounted for more than 60% of the total family laborers. The farmers were mainly middle-aged people, 36–60 years old, accounting for 62.52%, and the family laborers' age structure was reasonable. The education time was short, among which primary school (0–6 years) accounted for 41.93%, and middle school accounted for 33.93%. The breeding time was mainly 5–15 years, accounting for

62.37%, indicating that the surveyed farmers had good breeding experience. In addition, most of the family's breeding scale was less than 150 heads, accounting for 60.59%, showing that the sampled area held mainly medium- and small-scale breeding.

Table 1. Basic characteristics of sample farmers.

Variables	Classification	Ratio %
Gender	Male	73.4%
	Female	26.5%
Age	<36 years	3.85%
	36–60	62.52%
	>60	33.63%
Educational time	0–6 years	41.93%
	7–9	33.93%
	10–12	10.22%
	>12	0.59%
Breeding time	<5 years	25.63%
	5–15	62.37%
	>15	12.00%
Breeding scale	<150 heads	60.59%
	150–300	24.30%
	>300	15.11%
Male laborers	<0.3	1.33%
	0.3–0.6	22.67%
	>0.6	76.00%

3.2. Variable Selection

3.2.1. Dependent Variable

The dependent variable in the current study was farmers' standardized use of antimicrobials, which was measured by the decisions and the degree of standardized use by farmers. On the one hand, questions in the questionnaire included "Did you use the recommended dosage of the veterinary prescription (prescription antimicrobials) or the package Insert (over-the-counter antimicrobials)?" to measure the decisions of standardized use by farmers. If the farmer chose the standardized use, the value was 1; otherwise, the value was 0. On the other hand, the degree of antimicrobial use by farmers was expressed by the amount of investment in the standardized use of antibiotics. Due to the different types, preparations, concentrations, and packages of antimicrobials, the number of antimicrobials used could not be directly summed up in milliliters or grams. Sun and Zhou's [40] studies used the payment amount of antimicrobials to calculate the number of antimicrobials. In addition, to eliminate individual differences brought by the breeding scale, the "degree of the standardized use" was measured by the "ratio of the payment amount of standardized use of antimicrobials to the number of hogs", and it was a continuous variable.

3.2.2. Independent Variable

The core explanatory variable was the IAI. The development of modern communication technology, represented by the Internet, could broaden farmers' access to information, reduce the cost of information acquisition, and reduce the asymmetry of market information, thus affecting farmers' production safety decisions [41,42]. Meanwhile, the Internet was an important medium or platform for animal husbandry, cooperative organizations, or veterinarians to provide disease diagnosis and antimicrobial use services. Therefore, "Had you obtained the knowledge of veterinary antimicrobial use through the Internet?" in the questionnaire was set to determine whether farmers used the Internet as a primary channel for searching and acquiring antimicrobial knowledge or skills. The IAI was a discrete binary variable. Those who used the Internet to acquire antimicrobial use knowledge or skills were assigned a value of 1; otherwise, 0 was assigned. About 41.77% of farmers in the sample area searched for antimicrobial knowledge or skills through mobile the Internet.

3.2.3. Control Variables

Referring to the studies of Liu et al. [43], Si et al. [44], and Xu et al. [45], this paper also selected gender, age, education level, organizational participation, peer effect, breeding time, family laborers, transaction mode, and breeding mode as control variables. Meanwhile, regional dummy variables "Were you in Henan?", "Were you in Shandong?", and "Were you in Hebei?" were added, and, according to the principle of random extraction, Hubei was regarded as the control group. According to the descriptive statistical analysis and independent sample *T*-test in Table 2, it was found that there was a significant mean difference between the control variables of the IAI group and the non-IAI group. The differences of the decision and degree of standardized use between the IAI group and the non-IAI group were statistically significant at 1%, with a mean difference of −0.113 and −0.411, respectively. Meanwhile, the differences of age, family laborers, and transaction mode between the IAI group and the non-IAI group was statistically significant, with mean differences of 1.830, 0.283, and 0.183, respectively. In addition, the mean value of different groups in Hebei province had a significant difference of −0.084 at the statistical level of 5%.

Table 2. Descriptive statistical analysis of variables.

Variables	Variable Assignment	Sample Mean		Mean Difference (A − B)
		IAI Group (A)	Non-IAI Group (B)	
The decision of standardized use	Standardized use = 1, non-standardized use = 0	0.326	0.213	−0.113 ***
Degree of standardized use	The ratio of the payment amount for standardized use of antimicrobials to the number of hogs	12.536	12.125	−0.411 ***
Internet	Have you obtained knowledge of veterinary antimicrobial use through a mobile phone or computer? (Yes = 1, no = 0)	——	——	——
Gender	Male = 1, female = 0	0.716	0.748	0.032
Age	Actual age (year)	54.819	56.649	1.830 **
Educational time	Actual educational time (year)	5.965	5.941	−0.023
Organizational participation	Joining = 1, non-joining = 0	0.564	0.601	0.037
Peer effect	Is your standardized use of antimicrobials influenced by the behavior of other farmers? (No effect at all = 1—very significant effect = 5)	3.486	3.422	−0.063
Breeding time	Time spent breeding hogs (year)	8.535	8.608	0.073
Family laborers	Actual family laborers (people)	2.972	3.254	0.283 *
Transaction mode	Vertical order transaction = 1, loose market transaction = 0	0.227	0.410	0.183 ***
Breeding mode	Cooperative, family farm or company breeding = 1, small family breeding = 0	0.439	0.401	0.038
Were you in Henan?	Yes = 1, no = 0	0.280	0.255	0.025
Were you in Shandong?	Yes = 1, no = 0	0.234	0.199	0.036
Were you in Hebei?	Yes = 1, no = 0	0.214	0.298	−0.084 **

Note: *, **, and *** represent the significance levels of 10%, 5%, and 1%, respectively; robust standard error is in parentheses.

3.3. Model Setting

3.3.1. IV-Heckman Model

The issues of sample selection caused by farmers' standardized use decisions and the endogeneity caused by the missing variables and reverse causality should be dealt with well to analyze the influence of the IAI on farmers' standardized use of veterinary antimicrobials. The standardized use of antimicrobials could be divided into two independent and interrelated stages: the decision and the degree of veterinary antimicrobials use. Due to some factors such as risk perception, disease pressure, and expected loss, some farmers did not choose the standardized use of antimicrobials, so the degree of standardized use could not be observed. Thus, the sample of farmers' standardized use degree was the sample selected. Moreover, in addition to control variables, some missing variables might affect both dependent and independent variables, thus generating endogeneity issues. The farmers' need for standardized antimicrobial use techniques would also increase their options for the IAI. Hence, there was a reverse causal relationship between the IAI and farmers' standardized use of antimicrobials. By referring to Guo et al.'s [43] studies, the IV-Heckman model was adopted to overcome both sample selection and endogeneity issues. This study selected the "number of mobile phone contacts" as the tool variable. On the one hand, farmers with more mobile phone contacts were likely to access the Internet more often, thus obtaining more antimicrobial use knowledge through the Internet. On the other hand, the number of mobile phone contacts did not directly correlate with farmers' standardized use of antimicrobials, which satisfied the condition of the exogeneity of an instrumental variable.

Thus, the IV-Heckman model was divided into two stages: in the first stage, the endogenous explanatory variable "IAI" was linear regression to instrumental variables and all exogenous explanatory variables, and the potential variable fitting value of the IAI was obtained. In the second stage, the Heckman model estimated the decision and the degree of standardized use. Specifically, the first step was to establish an equation to analyze the decision of farmers' standardized use (selection equation). The equation was expressed as follows:

$$\text{Probit}(decision_i) = \alpha_1 + \beta_1 IAI^* + \gamma X + \varepsilon_i \quad (1)$$

where IAI^* was the latent variable of the Internet, and $decision_i$ signified farmers' standardized use decisions. If farmers decided to standardize the use of antimicrobials, the value was 1; otherwise, the value was 0. X was control variables, ε_i was the random error term, and α_1, β_1 and γ were the estimated values of the parameter.

Meanwhile, the IV-Heckman model required that at least one variable should be included in the first stage selection equation but not in the result equation of the second stage. Therefore, "the distance between the farmers and the veterinary service station" was the identification variable. The closer the farmers were to the veterinary service station, the higher the probability of the decision to use standardized. Still, there was no direct causal relationship between the identification variable and the degree of standardized use by farmers. In addition, to correct the sample selection issue caused by the farmer's decision, the Inverse Mills Ratio (IMR) of the farmer's sample should be estimated.

So, the second step was constructed to analyze the degree of standardized use by farmers (the result equation). OLS regression was performed for the fitting value of the latent variables "IAI", residual, inverse mills-ratio, and control variables against the degree of standardized use by farmers, and the equation was expressed as follows:

$$Degree = \alpha_2 + \beta_2 IAI^* + \gamma Z + \varepsilon_i \quad (2)$$

where *Degree* signified the degree of standardized use by farmers, Z indicated the vector of the control variables, γ was the coefficient to be estimated, ε_i represented the random error term, and β_2 was the estimator after overcoming the issues of sample selection and endogeneity. In addition, the significance of IMR values could be used to determine whether there was a sample selection issue.

To compare the estimation results that only considered endogeneity or sample selection, the following estimates were also made in the current study: (1) IV-probit model and two-stage least square method (2SLS) were used separately to estimate the decision and the degree of farmers' standardized use of antimicrobials, and only the endogeneity issue was fixed. The findings were illustrated in regression 1 and regression 2 (see Table 3). (2) The Heckman two-stage model was also used to estimate the decision and the degree of antimicrobials standardized use, and the issue of sample selection was considered. The regression results were illustrated in regressions 3 and 4. (3) The IV-Heckman model was also employed to analyze the effectiveness of the IAI in standardizing the use of antimicrobials by farmers, and sample selection and endogeneity issues were considered simultaneously. The results were shown in regression 5 and 6.

Table 3. Estimates of the impact of the Internet on the standardized use of antimicrobials by farmers.

Variables	Regression 1	Regression 2	Regression 3	Regression 4	Regression 5	Regression 6
	Decision	Degree	Decision	Degree	Decision	Degree
	IV-Probit	2SLS	Heckman		IV-Heckman	
IAI	0.272 *** (0.092)	−0.104 * (0.060)	0.332 *** (0.113)	−0.301 * (0.171)	0.335 *** (0.114)	−0.372 ** (0.169)
Gender	−0.054 (0.094)	−0.081 (0.186)	0.024 (0.122)	−0.036 (0.165)	0.027 (0.139)	−0.012 (0.163)
Age	−0.009 ** (0.003)	−0.013 * (0.007)	−0.003 (0.004)	0.000 (0.007)	−0.003 (0.005)	−0.001 (0.007)
Educational time	0.009 (0.010)	0.017 (0.020)	0.013 (0.014)	−0.005 (0.026)	0.013 (0.055)	−0.001 (0.022)
Organizational participation	−0.013 (0.09)	0.378 ** (0.019)	−0.022 ** (0.011)	0.002 (0.036)	−0.023 ** (0.011)	−0.012 (0.020)
Peer effect	−0.026 (0.024)	−0.066 (0.051)	−0.022 (0.028)	−0.050 (0.052)	0.021 (0.031)	−0.046 (0.042)
Breeding time	0.089 (0.085)	−0. 110 (0.165)	0.078 (0.108)	−0.063 (0.187)	0.076 (0.115)	−0.002 *** (0.159)
Family laborers	0.011 (0.033)	0.002 (0.067)	0.004 (0.041)	−0.013 (0.057)	0.005 (0.045)	−0.029 (0.056)
Transaction mode	0.018 * (0.108)	−0.139 (0.234)	0.435 *** (0.112)	−0.229 (0.629)	0.433 *** (0.125)	−0.130 (0.337)
Breeding mode	0.119 *** (0.031)	−0.265 *** (0.055)	0.227 *** (0.037)	−0.068 (0.326)	0.228 *** (0.037)	−0.074 (0.178)
Were you in Henan?	−0.057 (0.121)	−0.327 (0.259)	−0.154 (0.154)	−0.131 (0.317)	−0.155 (0.166)	−0.117 (0.078)
Were you in Shandong?	−0.032 (0.128)	−0.527 ** (0.260)	0.045 (0.158)	−0.537 ** (0.239)	0.051 (0.169)	−0.587 *** (0.175)
Were you in Hebei?	0.027 ** (0.121)	0.104 (0.252)	0.136 (0.156)	−0.352 (0.029)	0.135 (0.161)	0.000 (0.000)
Distance between the enclosure and the veterinary service station	— —	— —	0.120 *** (0.043)	— —	0.121 *** (0.043)	— —
IMR value	— —	— —	8.25 ***		8.12 ***	
DWH test value	23.14 **	20.69 **	— —		18.25 **	
The T value of the tool variable	5.12 ***	5.29 ***	— —		5.25 ***	
F value in stage one	121.56	102.56	— —		229.5	

Note: *, **, and *** represent the significance levels of 10%, 5%, and 1%, respectively; robust standard error is in parentheses.

3.3.2. Mediating Method

The mediating model was used to verify the induction mechanisms of information supply, sharing, and feedback mechanisms on the IAI influencing farmers' standardized use of antimicrobials. The specific test was carried out in two stages. In the first stage, the influence of the IAI on mediating variables was tested, and the endogeneity of the IAI also needed to be overcome. Therefore, this study constructed model estimation based on the two-stage least square method (2SLS), and the equation was expressed as follows:

$$Media_i = \alpha_3 + \beta_3 IAI^* + \gamma X + \varepsilon_i \quad (3)$$

where $Media_i$ was the mediating variables, namely, information supply, sharing, and feedback mechanisms, which were, respectively, used as "quality evaluation of antimicrobials use information or technology provided by animal husbandry department (very poor = 1—very good = 5)", "quality evaluation of antimicrobials use information or technology obtained by other farmers? (Very poor = 1—very good = 5)", and "the influence of stakeholder's information feedback on farmers' antimicrobial use (very weak = 1—very strong = 5)". Z represented the vector of control variables, ε_i was the random error term, α_3, β_3, and γ was the estimated values of the parameters, respectively.

$$\text{Probit}(decision_i = 1) = \alpha_4 + \beta_4 IAI^* + \beta_5 Media_i + \gamma X + \varepsilon_i \quad (4)$$

$$Degree_i = \alpha_5 + \beta_6 IAI^* + \beta_7 Media + \gamma Z + \varepsilon_i \quad (5)$$

where $\text{Probit}(decision_i = 1)$ was the probability of farmers' standardized use decision, $Degree_i$ was the degree of farmers standardizing the use of antimicrobials, and α_4, α_5, $\beta_4 \sim \beta_7$, and γ were the estimated values of the parameters, respectively. The specific testing process of mediating effect was drawn from the study of Wen et al. [46] and Sun et al. [47].

4. Results and Discussion

4.1. Influence of the Internet on the Standardized Use of Antimicrobials by Farmers

Considering the multicollinearity issue that might exist between multiple variables, a multicollinearity test was conducted on the respective variables, and the test results showed that the maximum value of variance inflation factor (VIF) was 1.77, less than the critical value of 10. Thus, it was considered that there was no multicollinearity issue. Additionally, the Wald value for the IV-Heckman model's overall goodness of fit was 54.98, statistically significant at the 1% level. Therefore, the overall fitting degree of the IV-Heckman model was good, indicating that the model selection was reasonable and feasible. According to regression 1 and 2, DWH test values were found statistically significant at the 5% level, indicating an endogeneity issue caused by missing variables and reverse causation. The F values of the first stage were 121.56 and 102.56, respectively, larger than the critical value 10, indicating that the instrumental variables passed the weak instrumental variable test. The T values of the instrumental variables were all statistically significant at 1%, indicating that the variable "number of mobile phones contacts" could be used as the instrumental variable, and the model estimation results were valid. According to regression 3 and regression 4, the IMR value was statistically significant at the 1% level, indicating that the degree of standardized use of antimicrobials by farmers resulted in sample selection bias. According to regression 5 and regression 6, the T value of the instrumental variable and IMR value of sample selection bias was statistically significant at 1%, and the identification variable "distance between farmers and veterinary service station" was also statistically significant at 1%, indicating that the estimation results of IV-Heckman model were effective.

The results of regressions 1, 3, and 5 showed that the coefficients of the IAI were all positive, which had a positive and significant influence on farmers' decisions of standardized use of antimicrobials. According to regression 5, after overcoming the endogeneity and sample selection issues, the coefficient values of the IAI were greater than the estimated

results of regressions 1 and 3, suggesting that missing variables and reverse causation did affect farmers' decisions of standardized uses of antimicrobials, i.e.,, the IV-Heckman model was more appropriate. Regressions 5 and 6 results showed that the IAI significantly and positively influenced farmers' decisions to standardize antimicrobials at the 1% statistical level. In comparison, the IAI significantly and negatively impacted farmers' degree of standardized use of antimicrobials at a 5% significance level. This suggested that the IAI could improve the probability of farmers' decisions to standardize antimicrobials and reduce the payment amount for the standardized use of antimicrobials, which was consistent with Talanow et al.'s study [48]. Especially, farmers could continuously improve their skills in the standardized use of antimicrobials through the IAI to obtain skills training on the use of antimicrobials, video lectures on Internet platforms, and timely responses on challenging issues by animal husbandry departments or cooperative organizations [49]. However, other scholars had also pointed out that the IAI was an essential channel for farmers to obtain veterinary antibiotics illegally, and information on antibiotic use obtained through the IAI was unofficial or false sometimes [50–52]. Therefore, the IAI was beneficial to encourage farmers' standardized use of antimicrobials but needed to be strongly supervised by the government.

Some control variables were also found to influence the farmers' decisions and the degree of standardized use of antimicrobials. Specially, breeding time had a significant negative influence on farmers' decisions on the standardized use of antimicrobials, which supported the research conclusion of Lekagul et al. [53] and Zhong [54]. The longer the breeding time, the stronger the empirical dependence. Farmers often used antimicrobials based on experience and neglected to adopt standardized antibiotic techniques [55]. Meanwhile, the empirical results were consistent with those of Zheng et al. [56] and Nie et al. [57], arguing that breeding and transaction modes significantly impacted farmers' standardized use of antimicrobials. On the one hand, in the vertical trading mode, the regular trading partners had enough market incentives, such as possible epidemic risk, price risk, and market premium, to strengthen farmers' supervision and strictly control pork quality. On the other hand, compared with small-family breeding, cooperative, family farming or company farming had a higher degree of scale and standardization, better biosafety measures, lower incidences of disease, and were more inclined to standardized use of antimicrobials. In addition, our study confirmed that organizational participation had a significant negative effect on the degree of standardized use of antimicrobials by farmers. Cooperative organizations influenced farmers' safe production behaviors through technical service supply, safe production management, and unified product sales [58,59].

4.2. Empirical Analysis of Induction Mechanism

4.2.1. Test Results of the Information Supply Mechanism

The test results of the information supply mechanism's mediating effect are shown in Table 4 (regression 7–9). According to regression 5 in Table 3, the IAI had a positive and significant influence on farmers' decisions of standardized the use of antimicrobials at the significance level of 1%, with a coefficient of 0.335, which was the total effect of the IAI. According to regression 7 in Table 4, after adding the mediating variable "information supply mechanism," the IAI was found to have a positive and significant influence on farmers' decisions to standardize the use of antimicrobials, but the influence coefficient decreased. Moreover, according to regression 8, the information supply mechanism positively and insignificantly influenced farmers' standardized use decisions. Hence, it was necessary to conduct the Sobel test to verify the significance of the information supply mechanism. The test results showed that the Z-value of the Sobel test was 0.317 (lower than the critical value of 0.97), indicating that the information supply mechanism's mediating effect was insignificant. Additionally, according to regression 9, the information supply mechanism had a positive and insignificant effect on the degree of standardized use of antimicrobials by farmers, and the Sobel test value was 0.328 (lower than the critical value of 0.97). Therefore, the mediating effect of the information supply mechanism concerning

the influence of the IAI on farmers' standardized use decisions and the degree were not significant, which was contrary to Si et al.'s [60] study, and hence H1 was falsified. Possible explanations were that the information supply of the animal husbandry sector was an important source for farmers to obtain new technology or information on standardized the use of antimicrobials. However, their information supply also had many real issues, such as low technical training frequency, low service quality, and weak timeliness [61]. Thus, the livestock sector's information supply had not improved the information or skill constraints experienced by farmers in standardizing the use of antimicrobials.

4.2.2. Test Results of the Information-Sharing Mechanism

The mediating effect test results of the information-sharing mechanism were shown in regression 10–12. According to regression 10, the IAI positively promoted the information-sharing mechanism at the significance level of 10%, with a coefficient of 1.190. According to regression 11, after adding the variable "information-sharing mechanism", the IAI positively and significantly influenced farmers' standardized use decisions. Meanwhile, the information-sharing mechanism positively affected farmers' standardized use decisions at the significance level of 5%, and the coefficient was 0.123. Thus, the information-sharing mechanism had a partial mediating effect on the influence of the IAI on farmers' standardized use decisions of antimicrobials, and the mediating effect was 0.146 (1.119×0.123), accounting for 43.69% (0.146/0.335) of the total effect.

Similarly, the IAI had a significant negative influence on the degree of standardized use by farmers at the statistical level of 5%, and the coefficient was -0.372, i.e., the total effect was -0.372. After adding the variable "information-sharing mechanism", the IAI significantly and negatively impacted the degree of standardized use at the significance level of 1%. Meanwhile, the information-sharing mechanism negatively affected the degree of standardized use by farmers at the significance level of 1%, and the coefficient was -0.089. According to regression 10, the IAI positively impacted the information-sharing mechanism at the statistical level of 10%, with a coefficient of 0.190. The information-sharing mechanism had a partial mediating effect on the influence of the IAI on the degree of the standardized use of antimicrobials by farmers, and the mediating effect was -0.106 ($0.190\times$ 0.089), accounting for 28.47% ($-0.016/0.372$) of the total effect. To sum up, the IAI positively and significantly affected farmers' decisions and degrees of standardized use of antimicrobials through the information-sharing mechanism, which was consistent with Si et al. [44], and thus H2 was confirmed. It might have to be explained as follows. The IAI reshaped the network structure of farmers through employment channel selection and labor transfer [62]. Suppose the information on antimicrobial use skills provided by the livestock sector was transmitted to farmers. In that case, the information would spread to other farmers and form a peer effect through the new relationship network formed by the IAI.

4.2.3. Test Results of the Information Feedback Mechanism

The mediating effect test results of the information feedback mechanism were shown in regression 13–15. According to regression 13, the IAI had a positive promoting effect on the information feedback mechanism at the significance level of 5%, and the influence coefficient was 1.170. According to regression 14, after adding the variable "information feedback mechanism", the IAI positively and significantly impacted farmers' standardized use decisions at the statistical level of 1%. Meanwhile, the information feedback mechanism positively impacted farmers' standardized use decisions at the significance level of 1%, with a coefficient of 0.062. The information feedback mechanism showed a partial mediating effect in the influence of the Internet on farmers' decisions on standardized use of antimicrobials, and the mediating effect was 0.073 (1.170×0.062), accounting for 21.65% (0.073/0.335) of the total effect.

Table 4. Results of mediating effect test.

Variables	Regression 7	Regression 8	Regression 9	Regression 10	Regression 11	Regression 12	Regression 13	Regression 14	Regression 15
	Information Supply Mechanism	Decision	Degree	Information-Sharing Mechanism	Decision	Degree	Information Feedback Mechanism	Decision	Degree
IAI	1.287 ** (0.570)	0.334 *** (0.114)	−0.201 *** (0.021)	1.190 * (0.661)	0.331 *** (0.114)	−0.201 *** (0.032)	1.170 ** (0.303)	0.322 *** (0.115)	−0.160 *** (0.023)
Information supply mechanism	——	0.017 (0.020)	−0.014 (0.030)	——	——	——	——	——	——
Information-sharing mechanism	——	——	——	——	0.123 ** (0.058)	−0.089 *** (0.019)	——	——	——
Information feedback mechanism	——	——	——	——	——	——	——	0.062 *** (0.021)	−0.069 *** (0.019)
Control variables	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled
Sample size	675	675	188	675	675	188	675	675	188

Note: *, **, and *** represent the significance levels of 10%, 5%, and 1%, respectively; robust standard error is in parentheses.

Similarly, the IAI had a significant negative impact on the degree of standardized use by farmers at the statistical level of 5%, and the coefficient was −0.372, i.e., the total effect was −0.372. After adding the information feedback mechanism, the IAI had a significant negative influence on the degree of standardized use by farmers at the statistical level of 1%. Meanwhile, the information feedback mechanism significantly and negatively affected the degree of standardized use by farmers at the statistical level of 5%, and the coefficient was −0.069. The information-sharing mechanism had a partial mediating effect on the influence of the IAI on the standardized use of antimicrobials by farmers, and the mediating effect was −0.081 (1.170 × −0.069), accounting for 21.77% (−0.081/−0.372) of the total effect. Consequently, the IAI affected farmers' decisions and the degree of standardized use of antimicrobials through the information feedback mechanism, and thus H3 was assumed. The possible explanation was that farmers' standardized use of antimicrobials rather than overuse was often detected by testing for antimicrobial residues in the slaughtering or marketing process [63,64]. An Internet-based food traceability system could feed information on antimicrobial use back to farmers and stakeholders. Liability traceability could constrain farmers to standardize the use of antimicrobials [43].

4.3. Heterogeneity Analysis Based on the Age and Breeding Time

According to the model estimation results in Table 4, the households' age had no significant influence on farmers' decisions and the degree of standardized use of antimicrobials. However, elderly farmers had cognitive or technical barriers to Internet use, and the "digital divide" issue was more prominent in the elderly group. Therefore, the surveyed farmers were divided into under 36 years old (27 households), 36–60 years old (421 households), and over 60 years old (227 households) groups. The IV-Heckman model was adopted to explore the influence of the IAI on the standardized use of antimicrobials by farmers of different ages. The results in Table 5 showed that the IAI could significantly influence the standardized use of antimicrobials by farmers under 36 years old, motivate them to improve the decision probability of standardized use of antimicrobials, and reduce the payment amount of standardized use. However, the IAI has had little influence on the standardized use of antimicrobials by farmers over 60. Thus, if the age differences were not considered, the impact of the IAI on the standardized use of antimicrobials by farmers under 36 years of age might be overlooked. The possible explanation was with the transfer of rural young and middle-aged laborers and non-agricultural employment: elderly farmers had become the main force of agricultural production [65]. Just as Si et al. [8,60] held, due to limited educational level and experience, alleviating the "digital divide" of elderly farmers should be the government's focus in implementing the standardization of antimicrobials.

Table 5. Analysis results based on the heterogeneity of the age.

Variables	Under 36 Years Old		36–60 Years Old		Over 60 Years Old	
	Regression 16	Regression 17	Regression 18	Regression 19	Regression 20	Regression 21
	Decision	Degree	Decision	Degree	Decision	Degree
IAI	0.378 *** (0.145)	−0.579 ** (0.239)	0.374 * (0.192)	−0.276 (0.386)	0.129 (0.211)	−0.240 (0.367)
Control variables	controlled	controlled	controlled	controlled	Controlled	controlled
Sample size	27	5	421	125	227	58

Note: *, **, and *** represent the significance levels of 10%, 5%, and 1%, respectively; robust standard error is in parentheses.

In addition, to verify the nonlinear relationship between breeding time and farmers' standardized use of antimicrobials, according to the heterogeneity of breeding time, we divided the surveyed farmers into less than five years (173 households), 5–15 years

(421 households), and more than 15 years (81 households) groups. The IV-Heckman model was used to explore the impact of the IAI on the standardized use of antimicrobials by farmers with different breeding times. The results in Table 6 showed that the IAI only positively and significantly influenced the standardized use decisions of farmers whose breeding years were less than five years. However, the IAI harmed the standardized use decisions of farmers with more than 15 years of breeding time but did not pass the significance test. However, according to the results in Table 4, the breeding time negatively and significantly influenced farmers' decisions on standardized use at the significance level of 5%. This suggested that the impact of the IAI on standardized use decisions of farmers with less than five years of breeding time would be misestimated without heterogeneity analysis, which was contrary to Lekagul et al.'s [53] study. For a long time, small farmers in developing countries had been engaged in agricultural production mainly based on experience and traditional knowledge [66]. Dependence on experience also inhibited their willingness to adopt standardized techniques for using antimicrobials.

Table 6. Analysis results based on the heterogeneity of the breeding time.

Variables	Less than 5 Years		5–15 Years		More than 15 Years	
	Regression 22	Regression 23	Regression 24	Regression 25	Regression 26	Regression 27
	Decision	Degree	Decision	Degree	Decision	Degree
IAI	0.036 *** (0.004)	−0.099 (0.078)	0.049 (0.101)	−0.005 (0.016)	−0.038 (0.276)	−0.028 (0.093)
Control variables	controlled	controlled	controlled	controlled	Controlled	controlled
Sample size	173	39	421	119	81	30

Note: *** represents the significance level of 1%, respectively; Robust standard error is in parentheses.

5. Conclusions

Information or skill constraints are the main factors in standardizing the use of antimicrobials by farmers. The Internet has become the main source of farmers' information acquisition. Using data from 675 hog farmers from Hebei, Shandong, Henan, and Hubei provinces of China, the IV-Heckman model is used to analyze the influence of the IAI on farmers' standardized use of veterinary antimicrobials. The main conclusions are as follows: first, the IAI helps standardize the use of veterinary antimicrobials. Second, information-sharing and feedback mechanisms partially mediate the relationship between the IAI and farmers' standardized use of antimicrobials. Finally, based on the heterogeneity of households' age and breeding time, our study confirms that the IAI only incentivizes farmers' standardized use of antimicrobials under 36. Meanwhile, the IAI has a positive and significant influence on the standardized use decisions by farmers with less than five years of breeding time.

Of course, the study still has some flaws. Disease degree, risk preference, and expected loss may also affect farmers' standardized use of antimicrobials. Still, these variables are not included due to the limitations of data acquisition. Additionally, due to the difficulty in selecting instrumental variables, we only selected one without an over-identification test. These research deficiencies will become the focus of the research group in the future.

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Effect of Dietary Calcium Propionate Inclusion Period on the Growth Performance, Carcass Characteristics, and Meat Quality of Feedlot Ram Lambs

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Abstract: The objective was to determine the effect of calcium propionate (CaPr) inclusion in the diet, at different periods on the growth performance, carcass characteristics, and meat quality of finishing lambs. Thirty-six Dorper × Katahdin crossbred male lambs (39.1 ± 0.44 kg BW) were housed in individual pens during a 42 d feeding period and assigned to four treatments (n = 9) consisting of CaPr administered at a dose of 10 g/lamb/d for 0 (control), 14, 28, or 42 d before slaughter. Final BW (FBW), average daily gain (ADG), dry matter intake (DMI), and ADG:DMI ratio increased quadratically ($p < 0.05$) by CaPr supplementation, being optimal at an estimated inclusion period of 25 d for FBW and ADG, 15 d for DMI, and 28 d for ADG:DMI ratio. Hot carcass weight (HCW), cold carcass weight (CCW), and dressing were quadratically improved ($p < 0.05$) at an estimated inclusion period of 24 d for HCW and CCW, and 20 d for dressing. The increased inclusion period (42 d) augmented fat thickness (linear effect, $p < 0.05$). At 28 d of CaPr supplementation, maximal response (quadratic effect, $p < 0.05$) was estimated in the empty body weight at 28 d, forequarter at 26 d, and neck at 24 d, but a longer inclusion period (42 d) increases the weight of leg and rack and reduced the proportion of loin as a percentage of CCW (linear effect, $p < 0.05$). In conclusion, dietary CaPr can be included for a period of 24 to 28 d to improve growth performance and carcass weight, without affecting organ mass or meat quality.

Keywords: crossbred lambs; gluconeogenic precursor's; dietary energy; feedlot

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1. Introduction

Feedlot finishing of lambs is more frequent nowadays. These feeding systems require high energy density provided diets mainly by large amounts of cereal grains [1–3]. However, cereals are expensive, and when included in high dietary levels (greater than 60%), they precipitate ruminal acidosis [4–6] leads to liver abscesses, which are related to reductions of 6% dry matter intake (DMI) and 25% the average daily gain (ADG) [7], and the increase in the morbidity rate and/or the severity of morbidity (days in medical treatment) [8].

As a result, research on alternative non-conventional energy sources that pose a lower risk of acidosis, such as gluconeogenic precursors [9,10] like calcium propionate (CaPr) [11], glycerol [12], propylene glycol [13], and sodium propionate [14], is gaining popularity. The energy contribution of CaPr is similar to that of propionic acid [15], and promising results have been reported [9,10]. In addition, it is approved by the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) for use in food or feed additives [16].

Previous reports have consistently shown that increasing dietary energy improves the growth performance of finishing lambs [2,17–19]. It has been found that CaPr dissociates

in the rumen, releasing calcium ions and propionate, leading to an enhanced energy status through increased glucose synthesis in the liver [20].

In finishing lambs supplemented with CaPr for 42 d, Carrillo-Muro et al. [11] found that the dose of 10 g of CaPr per lamb per day increased DMI by 13%, ADG by 28%, ADG:DMI ratio by 17%, final body weight (FBW) by 7%, and empty body weight (EBW) by 4%. Additionally, the cooling loss was reduced by 13%. However, they did not observe any effects on carcass characteristics and meat quality. Martinez-Aispuro et al. [9] reported that lambs fed CaPr for 42 d at a daily dose of 13.9 g/lamb/d increased the FBW, ADG, ADG:DMI ratio, and without effects in DMI, longissimus muscle area (LMA), or fat thickness (FT). Furthermore, Cifuentes-López et al. [21] fed CaPr in the diet of growing lambs for 42 d, observed an improvement in dressing, carcass conformation, and LMA, with a reduction in adipose tissue, perirenal fat, and FT; however, they did not observe differences in the meat pH, DMI and ADG. In contrast, Lee-Rangel et al. [22] did not observe differences in DMI, ADG, ADG:DMI ratio, LMA or hot carcass weight (HCW) in growing lambs fed CaPr for 42 d. Based on the previous results, we determined that the inclusion of CaPr in the diet of feedlot ram lambs, to increase dietary energy, has always been supplied for 42 d before slaughter, ignoring whether that period is the right optimum.

We hypothesized that increasing dietary energy with CaPr in finishing lambs during the appropriate inclusion period improves growth performance, carcass characteristics, and meat quality in finishing lambs. Therefore, the present study proposed to determine the optimal inclusion period (0, 14, 28, or 42 d) of CaPr at a dose of 10 g/lamb/d on growth performance, carcass characteristics, and meat quality of finishing lambs.

2. Materials and Methods

The experiment was carried out in the Small Ruminant Experimental Center, the slaughter in an abattoir and the carcass quality measurements in the Meat Science and Technology Laboratory, all in the Unidad Académica de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Zacatecas (UAMVZ-UAZ), located in state Zacatecas, Mexico (22° N and 102° W). The experiment was carried out during the months of August to September 2022, with a maximum temperature of 28 °C and a minimum of 8 °C.

Protocols, management, and animal care procedures were in agreement with the Bioethics and Animal Welfare Committee of UAMVZ-UAZ and adhered to the Official Mexican Standards NOM-024-ZOO-1995 [23]; NOM-033-SAG/ZOO-2014 [24]; NOM-051-ZOO-1995 [25], and NOM-062-ZOO (1999) [26].

2.1. Animals, Housing, and Management

Thirty-six Dorper × Katahdin crossbred ram lambs, with an average body weight of 39.1 ± 0.44 kg and 5.5 months of age, were individually housed in 1.5 m × 1.5 m pens equipped with individual feeders and provided with fresh water using the free choice method. Before entry to the feedlot, all animals were treated for internal (Closantel 5%) and external parasites (Doramectina 1%) and received an intramuscular injection of bacterin-toxoid (for *Clostridium* spp.). During the next two weeks, the ram lambs adapted to the finishing basal diet (Table 1), and we also monitored their health status.

Table 1. Ingredients and nutritional composition of the basal diet (dry matter basis).

Ingredients	g kg^{-1} DM
Alfalfa hay	100.0
Oats hay	102.0
Dry-rolled corn	460.0
Dried distillers grains	129.0
Soybean meal 44% CP	127.0
Molasses cane	43.0
Calcium carbonate	11.0

Table 1. *Cont.*

Ingredients	g kg^{-1} DM
Sodium bentonite	10.0
Sesquicarbonate	17.0
Microminerals [a]	0.5
Vitamins [b]	0.5
Chemical composition, g kg^{-1} DM	
Dry matter	842.8
Crude protein	163.0
Ether extract	24.0
Neutral detergent fiber	217.0
Calcium [c]	8.3
Phosphorus [c]	2.7
Ca:P ratio	3.1
Calculated net energy, Mcal/kg	
Maintenance	2.0
Gain	1.3

[a] Microminerals: Co (0.5 g), Fe (50 g), I (2.5 g), Mn (50 g), Zn (50 g), Se (0.2 g) y Cu (15 g). Excipient q.s.1000 g. [b] Vitamins A (5,000,000 IU), D (2,000,000 IU) y E (10,000 IU). Excipient q.s.1000 g. [c] Calculated values from NRC (2007) feed composition tables [27].

2.2. Treatments and Experimental Design

Lambs were randomly assigned to one of the following treatments (n = 9 lambs per treatment): (1) Basal diet without CaPr before slaughter, (2) Basal diet + 10 g/lamb/d 14 d before slaughter, (3) Basal diet + 10 g/lamb/d 28 d before slaughter, and (4) Basal diet + 10 g/lamb/d 42 d before slaughter. The CaPr used in the study was Nuprocal ™ (Nutryplus, Mexico), which provides an energy contribution similar to that of propionic acid, with an estimated gross energy of 3.965 Mcal/kg and a metabolizable energy (ME) of 3.766 Mcal/kg [15]. Individual CaPr doses were weighed on a certified scale (Pioneer-PX523, Ohaus Corp., Parsippany, NJ, USA) and mixed with 100 g of a basal diet. The treatments were offered individually during the morning feeding with careful observation to ensure their total intake. The remainder of the basal diet was administered immediately afterward.

Samples of the basal diet were taken daily and analyzed in a triplicate proportion for the following: dry matter (DM, dried for 24 h at 100 °C in a forced-air oven), crude protein (CP, FP-528 LECO nitrogen analyzer) [28], neutral detergent fiber (fiber Ankom analyzer), and ether extract (extractor of Ankom^{xt15}). Net energy for maintenance (NE_m) and net energy for gain (NE_g) of diets were calculated according to the equations [27]: NE_m (Mcal/kg of DM) = 1.37 ME − 0.138ME^2 + 0.0105ME^3 − 1.12, and NE_m (Mcal/kg of DM) = 1.42 ME − 0.174ME^2 + 0.0122ME^3 − 1.65.

2.3. Growth Performance and Ultrasound Measurements

Individual body weight was recorded at 0700 h before the morning feeding on d 0 (initial body weight, IBW), 14, 28, and 42 (FBW) d during the experiment. The diet offered and refusals were weighed and recorded daily. DM was used to estimate DMI (feed intake multiplied by the percentage of DM). Ad libitum feeding was offered with two daily meals (8:00 and 16:00 h). The diet amount offered was adjusted daily (as-feed basis), increasing by 5% from the consumption recorded the previous day. The ADG:DMI ratio was calculated as the ADG divided by the corresponding DMI. Fat thickness (FT) and longissimus muscle area (LMA) measurements were obtained from the longissimus muscle (LM) at 5 cm lateral to the middle line and between the 12th and 13th ribs using a real-time ultrasound (Aloka, Prosound 2, Tokyo, Japan) equipped with a 3.5 MHz linear transducer.

2.4. Slaughter Procedure

At the end of the 42 d feeding period, lambs underwent solid fasting for 18 h before being transported to the UAMVZ-UAZ abattoir at 0700 h. Immediately before slaughter,

lambs were weighed (pre-slaughter BW). Slaughter procedures were under approved human methods by Mexican Official Standard NOM-033-SAG/ZOO-2014 [24].

2.5. Organ Mass

Non-carcass components, skin, complete gastrointestinal tract and digesta-free, heart, lungs, liver, spleen, kidney, and perirenal fat, were removed immediately after slaughter and weighed. The weights were expressed as grams per kilogram of empty body weight (EBW). The EBW was calculated by deducting the total non-carcass component weight from the pre-slaughter BW.

2.6. Carcass Characteristics

After evisceration, the carcasses (including kidneys and internal fat) were weighed to obtain the hot carcass weight (HCW) and chilled at 4 °C for 24 h. After the chilling period, the carcasses were reweighed to obtain cold carcass weight (CCW). In addition, carcass dressing ([CCW/EBW] × 100), and cooling loss ([HCW-CCW]/HCW) × 100 were calculated. After chilling, the carcasses were measured with a flexible tape measure to obtain carcass length, leg length, and chest circumference.

2.7. Whole Cuts and Tissue Composition

The right sides of the carcasses were cut to obtain the following whole cuts: forequarter, hindquarter, shoulder, leg, loin, rack, short rib, flank, breast, and neck, following the guidelines of the North American Meat Processors Association [29]. The yield of each cut was calculated by expressing its respective weight or as a percentage of the CCW. Carcasses were split in half, and the left side was dissected. The tissue composition of the shoulder was determined through physical dissection to calculate the percentage of muscle, fat, and bone.

2.8. Meat Characteristics

Muscle samples were collected from the cold carcass longissimus muscle (LM, approximately 500 g) and frozen at 20 °C for later analysis of meat quality. The meat color was measured by triplicate (and averaged) on the surface of the LM cut between rib 12th and 13th rib using a spectrophotometer Minolta CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan; measuring aperture 8 mm, D65 illuminant, 2° observer angle, SCE mode). With the values of L* = lightness (0 = black, 100 = white); a* = red to green (positive values = red, negative values = green); b* = yellow to blue (positive values = yellow, negative values = blue). The pH obtained after chilling the carcass (24 h at 4 °C) was measured between the first and second lumbar vertebrae (LM), using a portable pH meter (Hanna Instruments, HI–9025).

The water-holding capacity (WHC) was quantified following the methodology described by Grau and Hamm, as proposed by Tsai and Ockerman [30]. Briefly, 300 mg of LM were covered with filter papers (Whatman #1), placed between glass plates (15 cm × 15 cm), and pressed under 10 kg at constant pressure for 20 min. LM steaks (2 cm-thick) obtained between the 12th rib and L2 vertebrae were vacuum-packed in plastic bags and frozen at −20 °C. After 14 d of storage, steaks were tempered for 24 h at 4 °C, blotted dry, and weighed. The weights taken before and after opening the vacuum packages were used to calculate purge loss. Steak samples (15 to 20 g) were used to determine cook loss (weight loss after cooking). Sealed plastic bag samples were individually placed and plunged in a water bath at 75 °C until reaching an internal temperature of 70 °C. Once cooked, the samples were cooled under running tap water, removed from the packaging, blotted, and weighed. WHC, purge loss, and cook loss were expressed as a percentage of weight loss relative to the initial weight, calculated as [(initial weight − final weight)/initial weight] × 100.

Warner–Bratzler shear force (WBSF) was measured by cooking steak samples in electric grills (model GR2120B, George Foreman Electronics) until they reached an internal temperature of 70 °C [31]. Once cooked, the steaks were cooled at room temperature (20–25 °C). Three samples from each steak were obtained (parallel to the muscle fiber) and sheared

perpendicularly to the muscle fiber using a slice shear force blade mounted in a Warner-Bratzler shear machine (G-R Manufacturing, New York, NY, USA) at the crosshead speed of 200 mm/minute. Shear force measurements were averaged and expressed in kg/cm^2.

2.9. Statistical Analyses

Statistical analysis was carried out with SAS University software. Normality assumptions were confirmed using the UNIVARIATE procedure. Data were analyzed using a completely randomized design with the GLM procedure, except for ADG, DMI, and ADG:DMI ratio which were analyzed using the MIXED procedure for repeated measurements. Lamb and carcass were the experimental units for growth performance and meat characteristics. CCW was included as covariates for the analysis of carcass characteristics. Levels of CaPr supplementation were partitioned into linear and quadratic orthogonal polynomials, considering four equally spaced levels with the LSMEANS and ESTIMATE statements. When the quadratic polynomials were significant, the quadratic equations were calculated using the REG procedure. Significance was stated when the p-value was $\leq$0.05 and tendency when the p-value was >0.05 and $\leq$0.10.

3. Results and Discussion

3.1. Growth Performance and Ultrasound Measurements

Compared to Controls, lambs supplemented with CaPr showed greater ($p < 0.01$) FBW, ADG, and ADG:DMI ratio (Table 2) without effects on DMI ($p = 0.73$). The growth performance augmented quadratically ($p < 0.05$), being maximal at an estimated (from calculated equations) inclusion period of 25 d for FBW and ADG, 15 d for DMI, and 28 d for ADG:DMI ratio, with increments of 4.7, 26.8, 1.1, and 25.8% for the FBW, ADG, DMI, and ADG:DMI ratio, respectively.

Table 2. Growth performance of finishing lambs fed calcium propionate (CaPr) at different periods.

Item [b]	Days in CaPr [a]				SEM [c]	Effects (p-Value)		CaPr vs. Control
	0	14	28	42		Linear	Quadratic	
IBW, kg	39.5	39.1	39.4	38.6	0.44	0.27	0.71	0.39
FBW, kg	49.2	50.4	51.5	49.5	0.55	0.35	0.01	0.05
ADG, g/d	226.87	269.8	287.7	259.5	13.28	0.06	0.01	0.01
DMI, g/d	1419.1	1437.5	1435.0	1354.5	25.37	0.10	0.05	0.73
ADG:DMI ratio	0.16	0.19	0.20	0.19	0.01	0.09	0.05	0.01

[a] Treatments consisted of oral administration of CaPr (Nuprocal^{TM}, Nutryplus, Mexico) at a dose of 10 g/lamb/d at four feeding periods of 0, 14, 28, or 42 d before slaughter. [b] IBW = initial body weight, FBW = final body weight, ADG = average daily gain, DMI = dry matter intake. [c] SEM = standard error of the mean. FBW $y = -0.0043x^2 + 0.2164x + 48.28$ ($R^2 = 0.998$), maximal value at 25 d of inclusion. ADG $y = -0.0907x^2 + 4.6376x + 225.82$ ($R^2 = 0.988$), maximal value at 26 d of inclusion. DMI $y = -0.1261x^2 + 3.8961x + 1416.2$ ($R^2 = 0.9641$), maximal value at 15 d of inclusion. ADG:DMI ratio $y = -0.00006x^2 + 0.0034x + 0.1577$ ($R^2 = 0.995$), maximal value at 28 d of inclusion.

Previous reports demonstrate that increasing dietary energy consistently improves the growth performance of finishing lambs [2,17–19]. In that respect, it has been shown that CaPr dissociates in the rumen into propionate and calcium ions, raising energy status through a greater glucose synthesis in the liver [20]. Moreover, this compound alters the pattern of ruminal volatile fatty acid (VFA), reduces methane production, improves DM digestibility, and promotes fermentative efficiency [32–34].

Similar to our results, Martinez-Aispuro et al. [9] provided CaPr in diet to finishing Hampshire × Suffolk lambs (IBW 23.8 kg) and reported increments ($p < 0.05$) in FBW, ADG, ADG:DMI ratio, serum glucose, and ruminal propionate, but they observed no significant effects ($p > 0.05$) on DMI. The basal diet contained 66% grains and 2.5 Mcal/kg ME. Furthermore, Carrillo-Muro et al. [11] observed that the FBW, ADG, DMI, and ADG:DMI ratio were improved ($p < 0.05$) in Dorper × Katahdin (IBW 36.6 kg) lambs supplemented with CaPr (basal diet contained 59% grains and 2.8 Mcal/kg ME). In both studies, the CaPr

was administered for a fixed time of 42 d before harvest and demonstrated the usefulness of CaPr in improving growth performance.

In contrast, other studies did not report significant differences ($p > 0.05$) in growth performance. Lee-Rangel et al. [22] did not observe differences in DMI, ADG, or ADG:DMI ratio of growing Criollo lambs (IBW 28.1 kg) fed CaPr mixed in the diet for 42 d (basal diet contained 55 or 65% grains) when compared to Control lambs. The authors assume that the amount of CaPr added to the diet in their study was insufficient to affect ADG. In addition, Mendoza-Martínez et al. [15] supplemented CaPr in the diet (57% concentrate and 2.5 Mcal/kg ME) of finishing Criollo lambs (IBW 25.3 kg) for 42 d. The authors did not observe differences in growth performance among treatments and attributed this phenomenon to the lack of effects on the ruminal VFA pattern (total and proportional VFA) of the lambs.

The reasons for inconsistencies among studies are not clear, given the various experimental conditions (dose, breed, initial weight, previous experimental conditions, diet quality, and energy content), since previous research showed that animal response to CaPr administration is affected by diverse factors such as roughage quality [35], dose [32], stage of growth period [36], grain content [22], age [37] and physiological status [16].

Previous studies in lambs have not thoroughly investigated the optimal length of CaPr supplementation. Nevertheless, the changes observed through periods in this study suggest a modification of the growth response over time ($p < 0.05$) with the use of CaPr being optimized when the supplementation period approaches 28 d and a slight reduction at 42 d of supplementation.

One possible explanation for the observed quadratic ($p < 0.05$) growth performance in the present study could be the slight reduction in DMI observed when feeding CaPr for 42 d, which may have affected other growth performance variables. However, the overall effect of CaPr inclusion on DMI (hypophagia) was not significant (CaPr vs. Control, $p = 0.74$), which is consistent with further reported studies in finishing ram lambs fed CaPr [9,11,21,22,38].

This reduction effect on DMI is known as hepatic oxidation theory (HOT) and was described by Allen [39] to explain the role of the ruminant liver in signaling and controlling satiety in ruminants through temporal patterns of oxidative fuels such as lactate, propionate, and non-esterified fatty acids (NEFAs). Signals are carried from the liver to the brain via afferents in the vagus nerve and are affected by hepatic oxidation and the generation of ATP. The effect was previously reported in ruminants due to constant and rapid VFA production with increased rumen propionate levels and raised available energy related to plasma glucose concentration [40,41]. However, a significant reduction in DMI due to the HOT effect was observed only when propionate was directly added directly to the rumen or infused into the portal vein [42–44].

3.2. Organ Mass, Ultrasound Measurements and Carcass Characteristics

CaPr supplementation significantly improved EBW compared to the control group ($p < 0.05$). Additionally, the EBW increased quadratically ($p < 0.05$) with the maximum value estimated at 28 d of inclusion based on the quadratic equations. However, the mass of the different organs was not affected ($p \geq 0.5$) (Table 3).

The ultrasound FT increased linearly ($p < 0.05$) with the inclusion period, while the LMA remained unaffected ($p > 0.05$) by the dietary CaPr administration. Additionally, lambs supplemented with CaPr showed significantly greater HCW, CCW, and dressing compared to the Control group ($p < 0.05$) (Table 4). The greatest responses (quadratic effect, $p < 0.05$) were reached within the inclusion period of 24 d for HCW and CCW and 20 d for dressing, with increments of 8.3, 8.5, and 4.2% for the HCW, CCW, and dressing, respectively. However, cooling loss, carcass measurements (carcass length, leg circumference, chest circumference) and shoulder composition (muscle, fat, and bone) were unaffected ($p > 0.05$) by the dietary CaPr administration.

Table 3. Organ mass of finishing lambs fed calcium propionate (CaPr) at different periods.

Item [b]	Days in CaPr [a]				SEM [c]	Effects (*p*-Value)		CaPr vs. Control
	0	14	28	42		Linear	Quadratic	
Empty BW, kg	40.9	42.7	42.9	42.7	0.71	0.1	0.04	0.05
Skin	158	142	159	170	8.61	0.2	0.16	0.89
Limbs	26.5	25.3	27.6	24.4	1.42	0.56	0.54	0.7
Head	41.9	42.9	39.1	42.4	0.98	0.62	0.31	0.75
Heart	5.4	5.6	4.8	5.7	0.38	0.9	0.5	0.96
Lungs	23.3	22.2	22.1	23.7	1.73	0.9	0.5	0.77
Liver	22.5	21.6	21	19.4	2.43	0.39	0.91	0.56
Spleen	2.8	2.6	2.3	2.7	0.38	0.65	0.52	0.54
Kidney	3.1	3	2.8	3.1	0.15	0.68	0.2	0.37
Testicles	20.3	17.8	15.8	18	1.32	0.16	0.13	0.08
Visceral fat	36.5	49.5	37.5	32.6	5.5	0.36	0.16	0.62
Perirenal fat	13.8	17.5	14.5	12.6	3.1	0.64	0.41	0.78
Stomach [d]	31.6	27.8	29.9	31.9	1.7	0.68	0.13	0.42
Large intestine	11.2	9.7	10.2	10.4	1.1	0.7	0.47	0.42
Small intestine	19.1	17.6	19.6	20.3	1.1	0.26	0.38	0.92

[a] Treatments consisted of oral administration of CaPr (Nuprocal™, Nutryplus, Mexico) at a dose of 10 g/lamb/d at four feeding periods of 0, 14, 28, or 42 d before slaughter. [b] Non carcass components are expressed in g/kg of empty body weight. [c] SEM = standard error of the mean. [d] Includes the rumen-reticulum, omasum, and abomasum. Empty BW $y = -0.0026x^2 + 0.1471x + 40.96$ ($R^2 = 0.9727$), maximal value at 28 d of inclusion.

Table 4. Ultrasound measurements, carcass characteristics, and shoulder composition of finishing lambs fed calcium propionate (CaPr) at different periods.

Item [b]	Days in CaPr [a]				SEM [c]	Effects (*p*-Value)		CaPr vs. Control
	0	14	28	42		Linear	Quadratic	
Ultrasound measurements								
Fat thickness, mm	3.0	3.5	3.6	3.9	0.23	0.01	0.78	0.02
LMA, cm^2	12.5	12.6	13.2	12.0	0.47	0.68	0.2	0.88
Carcass characteristics								
HCW, kg	23.3	25.4	24.9	24.4	0.4	0.2	0.01	0.01
CCW, kg	22.5	24.6	24.1	23.6	0.5	0.18	0.02	0.01
Dressing, %	55.2	57.6	56.2	55.4	0.5	0.77	0.01	0.05
Cooling loss, %	3.40	2.71	3.04	2.95	0.237	0.44	0.22	0.08
Carcass length, cm	70.09	67.32	70.79	67.52	1.60	0.74	0.88	0.42
Leg circumference, cm	45.90	45.24	43.97	43.89	1.55	0.29	0.86	0.40
Chest circumference, cm	76.8	79.3	76.3	78.3	1.23	0.88	0.53	0.61
Shoulder composition								
Muscle, %	66.47	66.51	65.22	64.71	1.10	0.18	0.81	0.44
Fat, %	14.17	15.18	16.05	16.33	1.25	0.19	0.77	0.25
Bone, %	19.36	18.32	18.73	18.95	0.504	0.81	0.23	0.25

[a] Treatments consisted of oral administration of CaPr (Nuprocal™, Nutryplus, Mexico) at a dose of 10 g/lamb/d at four feeding periods of 0, 14, 28, or 42 d before slaughter. [b] LMA = longissimus muscle area, HCW = hot carcass weight, CCW = cold carcass weight. [c] SEM = standard error of the mean. HCW $y = -0.0033x^2 + 0.1593x + 23.43$ ($R^2 = 0.8603$), maximal value at 24 d of inclusion. CCW $y = -0.0033x^2 + 0.1593x + 22.63$ ($R^2 = 0.8603$), maximal value at 24 d of inclusion. Dressing $y = -0.0041x^2 + 0.1657x + 55.42$ ($R^2 = 0.7281$), maximal value at 20 d of inclusion.

Previous studies using CaPr as a gluconeogenic precursor in fattening lambs reported an inclusion period of 42 d before slaughter as a common rule and generally at higher doses than those used in this study. Martínez-Aispuro et al. [9] observed an improvement ($p < 0.05$) in the FBW of wool lambs at a CaPr daily dose of 13.9 g/lamb/d (average of 0.46 g/kg BW) for 42 d but observed no effect ($p > 0.05$) on LMA or FT. Furthermore, Lee-Rangel et al. [22] fed CaPr in the diet at 12 g/lamb/d (average of 0.36 g/kg BW), while Mendoza-Martinez et al. [15] fed CaPr at 11.7 and 25.9 g/lamb/d (average of 0.40 and 0.87 g/kg BW), in both studies lambs were fed CaPr for 42 d; however, neither study

reported differences ($p > 0.05$) in HCW or LMA (CaPr vs. Control) of male Criollo lambs. Conversely, Cifuentes-López et al. [21] fed CaPr at high doses of 30, 35, and 40 g/kg DM (equivalent to 1.33, 1.65, 1.83 g/kg BW) in the diet of growing Rambouillet lambs (28.1 kg of IBW) for 42 d. They observed improvements ($p < 0.05$) in dressing, carcass conformation, and LMA, along with reductions ($p < 0.05$) in adipose tissue, perirenal fat, and FT, but no differences ($p > 0.05$) in organ weights (reported as rumen and intestines, and viscera and integuments). Additionally, Carrillo-Muro et al. [11] fed CaPr to crossbreed (Dorper x Katahdin) finishing lambs (35 kg BW) to test doses of 10, 20, and 30 g/lamb/d, with the greatest response observed at a dose of 20 g/lamb/d (0.49 g/kg BW). They reported that EBW, heart weight, and small intestine weight increased ($p < 0.03$) in lambs fed CaPr, with a tendency ($p < 0.07$) to augment liver mass, but no effects ($p \geq 0.43$) on other organ mass. Moreover, the authors reported increases in HCW ($p = 0.09$) and CCW ($p = 0.08$) and a lower cooling loss ($p < 0.05$), but they found no significant effects ($p < 0.05$) on dressing percentage, pH, carcass measurements, or the tissue composition. The authors of both studies related their results to the energy enhancement promoted by CaPr supplementation.

The positive effects of increased energy levels in the diet on carcass characteristics of finishing lambs have been demonstrated previously by Piola-Junior et al. [45] who reported a linear relationship between dietary ME density (2.0, 2.3, 2.5, 2.8 Mcal/kg) and the slaughter BW ($R^2 = 0.95$), HCW ($R^2 = 0.96$), CCW ($R^2 = 0.95$), and dressing ($R^2 = 0.91$) in crossbred Ile de France ram lambs (7.9 months of age, IBW 26.6 kg) fed with iso-protein diets. In addition, Moloney conducted a study to determine if isoenergetic (2.9 Mcal/kg ME) and isonitrogenous (16.5% CP) rations with different ruminal fermentation patterns (inclusion of sodium propionate at 0 or 40 g/kg) altered the growth and carcass composition in ram lambs. The authors observed a decrease in fat deposition, an increase in skeletal muscle growth, and an altered ratio of acetate to propionate in ruminal fluid from the effect of adding sodium propionate to the diet. They attributed the increase in protein accumulation to absorbed propionate, which is used for gluconeogenesis, thus sparing amino acids to increase protein synthesis.

The increase in energy availability offered by gluconeogenic precursors explains the observed increments in EBW, FT, and carcass weight. However, the lack of differences in the rest of the evaluated characteristics reflects that the level of CP and energy provided in the diet used in this study (16.3% CP, 2.8 Mcal/kg ME and 1.3 NE_g, Mcal/kg) was adequate for finishing Dorper crossbred lambs. The results of the present study agree with Deng et al. [46] since they estimated that Dorper crossbred lambs need a range between 0.267 and 1.27 Mcal/d NE_g for ADG of 100 to 400 g.

Under the conditions of this study, the EBW, carcass weight, and dressing were optimized (maximal values calculated from regression equations) when the supplementation period was approximately 28, 24, and 20 d, respectively (quadratic effect, $p < 0.05$). In addition, a longer inclusion period favored an increase in ultrasound FT (linear effect, $p < 0.05$) which is expected because of the greater energy available. Therefore, to improve carcass weight without increasing TF, it is recommended to administer CaPr for a maximum of 28 d and not for 42 d, which is the inclusion period commonly reported in this literature.

3.3. Whole Cuts

As shown in Table 5, the CaPr administration improves (CaPr vs. Control, $p < 0.03$) the forequarter, leg, rack, and neck cuts (g/kg of EBW). At 28 d of CaPr inclusion, greater weight (quadratic effect, $p < 0.04$) in the forequarter (g/kg of EBW) and the neck (expressed as both g/kg of EBW and as a percentage of CCW) was observed. However, for 14 to 42 d of CaPr inclusion, greater rack weight (linear effect, $p < 0.04$) was appreciated, in addition, the more extended inclusion period reduced the loin as a percentage of CCW (linear effect, $p < 0.03$).

Table 5. Whole cuts of finishing lambs fed calcium propionate (CaPr) at different periods.

Item [b]	Days in CaPr [a]				SEM [c]	Effects (p-Value)		CaPr vs. Control
	0	14	28	42		Linear	Quadratic	
Whole cuts, g/kg of EBW								
Forequarter	5.7	6.3	6.4	6.1	0.18	0.14	0.04	0.03
Hindquarter	5.3	5.6	5.6	5.4	0.19	0.71	0.17	0.28
Shoulder	2	2	2.1	2	0.06	0.78	0.25	0.48
Shoulder IMPS206	1.1	1	1	1.1	0.11	0.94	0.51	0.65
Leg IMPS233	2.9	3.2	3.2	3.2	0.09	0.02	0.37	0.02
Loin IMPS231	1.5	1.4	1.5	1.3	0.07	0.12	0.43	0.31
Rack IMPS204	0.7	0.8	0.8	0.8	0.02	0.02	0.02	0.001
Short rib	0.7	0.7	0.7	0.6	0.05	0.7	0.21	0.6
Flank IMPS232	0.8	0.9	0.9	0.9	0.04	0.21	0.3	0.09
Breast	0.8	0.9	1	0.9	0.06	0.23	0.22	0.12
Neck	0.7	0.9	1	0.8	0.07	0.26	0.01	0.03
Whole cuts, as percentage of cold carcass weight (CCW)								
Forequarter	51.2	51.2	53.4	51.8	0.66	0.44	0.57	0.51
Hindquarter	46.6	45.4	46.4	45.2	0.75	0.68	0.98	0.65
Shoulder	17.4	16.4	17.2	16.6	0.25	0.54	0.7	0.3
Shoulder IMPS206	9.8	8.4	8.4	9.2	0.4	0.67	0.2	0.28
Leg IMPS233	25.6	25.6	26.2	27.4	0.4	0.1	0.5	0.4
Loin IMPS231	13.4	11.6	12.4	11	0.28	0.03	0.83	0.04
Rack IMPS204	6.2	6.6	6.4	6.6	0.05	0.09	0.6	0.07
Short rib	5.8	6	5.8	5.4	0.18	0.39	0.46	0.81
Flank IMPS232	7	7.4	7.2	7.6	0.19	0.43	0.9	0.44
Breast	7.2	7.4	8	7.8	0.25	0.39	0.63	0.41
Neck	5.8	7.2	8.2	6.4	0.32	0.4	0.04	0.1

[a] Treatments consisted of oral administration of CaPr (Nuprocal^{TM}, Nutryplus, Mexico) at a dose of 10 g/lamb/d at four feeding periods of 0, 14, 28, or 42 d before slaughter. [b] Whole cuts are expressed in g/kg of empty body weight and percentage of cold carcass weight. [c] SEM = standard error of the mean. Forequarter g/kg of EBW $y = -0.0011x^2 + 0.0575x + 5.705$ ($R^2 = 0.9983$), maximal value at 26 d of inclusion. Rack IMPS204, g/kg of EBW $y = -0.0001x^2 + 0.0075x + 0.705$ ($R^2 = 0.9333$), maximal value at 36 d of inclusion. Neck, g/kg of EBW $y = -0.0005x^2 + 0.0243x + 0.69$ ($R^2 = 0.96$), maximal value at 24 d of inclusion. Neck as percentage of CCW $y = -0.0041x^2 + 0.1914x + 5.68$ ($R^2 = 0.9111$), maximal value at 24 d of inclusion.

No previous information was found about the effect of CaPr on the whole cuts of lambs since the authors did not report these measurements in the available literature. In addition, information on other gluconeogenic precursors fed to ruminants on whole cuts is scarce. A study conducted by Gomes et al. [47] evaluated the influence of diets supplemented with glycerin, as an alternative ingredient to corn on Santa Inês confined lambs (IBW 26.33 kg) with diets (40% roughage and 60% concentrate) containing 0, 15 or 30% glycerin in the total feed, and slaughtered with an average live weight of 34 to 36 kg. The authors did not report effects ($p > 0.05$) of the gluconeogenic precursors on final live weight and carcass weight and neither observed any effect on the percentage of whole cuts.

Otherwise, Shadnoush et al. [48] evaluated the effect of three slaughter weights (45, 52.5, and 60 kg) and two energy levels in the diet (2.64 and 2.4 Mcal/kg ME) on carcass characteristics of the Lori-Bakhtiari ram lambs (IBW 35.7 kg). They reported that high-energy diets increased ($p < 0.05$) shoulder weight but did not affect ($p > 0.05$) neck, leg, back, or tail fat weight. Furthermore, as slaughter weight increased, all cuts except the neck showed a greater weight and denoted a faster maturation of the head than the rest of the whole cuts.

Lamb carcass tissue growth is influenced by multiple factors such as breed, sex, carcass weight, type of delivery, and rearing [49,50]. In this regard, Bradford and Spurlock [51] reported that ram lambs (as the animals used in the present study) had a higher percentage of carcass weight in the forequarters than whether lambs. However, whole cuts are mainly affected by the plane of nutrition.

Furthermore, Owens et al. [52] stated that organs and tissues mature at different relative growth rates, with an apparent general gradient in organ/muscle formation from head to tail and from extremities to the core. Relative growth or tissue maturation is affected by age and growth rate (gain in weight per unit of time). However, the general sequence of body maturation is head, metatarsus, and kidney fat first; followed by the neck, bone, tibia-fibula, and intramuscular fat; later the thorax, muscle, femur, and subcutaneous fat and finally, the loin, pelvis, and intramuscular fat. Therefore, animals with similar ages but different growth rates influenced by diet possibly show different tissue maturation and relative growth.

Based on the above, we can infer that the changes observed in the values of the whole cuts result from an increase in general body weight and differential tissue growth promoted by the rise in the growth rate due to CaPr supplementation in lambs of the same breed and age. The results could imply the possibility of increasing the weight of the highest value cuts by providing CaPr in a different inclusion period; for example, providing CaPr for 28 d to enlarge the weight of forequarter, or for only 14 d if we are interested in maximizing the weight of the Leg IMPS233 or Rack IMPS204 cuts; however, this possibility could be the subject of future research.

3.4. Meat Characteristics

Regardless of the inclusion period, dietary CaPr supplementation did not affect ($p > 0.05$) purge loss, cook loss, WHC, WBFS, or color (L*, a*, and b* values) (Table 6). However, CaPr administration increased (CaPr vs. Control, $p < 0.01$) overall muscle pH values, which also increased linearly as the inclusion period became longer ($p < 0.02$).

Table 6. Meat characteristics and meat color of finishing lambs fed calcium propionate (CaPr) at different periods.

Item [b]	Days in CaPr [a]				SEM [c]	Effects (*p*-Value)		CaPr vs. Control
	0	14	28	42		Linear	Quadratic	
Meat characteristics								
pH_{24h}	5.2	5.6	5.7	5.7	0.15	0.02	0.24	0.01
Purge $loss_{24h}$, %	5	3.9	6	5	0.73	0.66	0.7	0.67
Cook loss, %	26.4	22.3	22.6	24.4	2.6	0.62	0.28	0.29
WHC, %	23.3	23	24.9	21.1	2.3	0.65	0.47	0.9
WBSF, kg/cm^2	5	4.4	4.9	4.7	0.19	0.74	0.38	0.2
Color								
L*	44.1	43.7	46.3	43.5	1.75	0.91	0.5	0.83
a*	17.8	17.2	17	16.9	0.89	0.48	0.84	0.49
b*	6.1	6.5	6.2	5.1	0.61	0.23	0.26	0.76

[a] Treatments consisted of oral administration of CaPr (Nuprocal™, Nutryplus, Mexico) at a dose of 10 g/lamb/d at four feeding periods of 0, 14, 28, or 42 d before slaughter. [b] Meat characteristics were measured on the longissimus muscle between the 12th rib and the 2nd lumbar vertebrae. WHC = water-holding capacity, WBSF = Warner-Bratzler shear force. [c] SEM = standard error of the mean.

Meat quality is principally affected by temperature cooling and pH in the post-mortem period being involved in tenderness, WHC and color [53]. The ideal pH value has been established between 5.5 and 5.8 since adequate proteolysis of myofibrillar proteins occurs in this range, in addition to the reversal of rigor mortis and improvement of meat tenderness [54]. pH values higher than 5.8 significantly increase shear force values [55] and decrease acceptance scores of meat by consumers such as aroma, flavor, juiciness, texture, and tenderness [56].

An explanation for the greater pH values observed in meat from lambs fed CaPr than the Control lambs is uncertain. However, the CaPr treatment values remained within the ideal pH values (5.5 and 5.8), contrary to the Control treatment that obtained lower values than the ideal. In this regard, Stewart et al. [57] carried out a study to test the associations between the plasma stress indicators and lamb ultimate pH, observing a significant positive

association between the plasma glucose concentration and the pH at 24 h post-mortem ($p < 0.01$), and as plasma glucose concentration increased from 2 mmol/L to 10 mmol/L, the pH increased by 0.16 pH units from 5.60 to 5.76.

In the present study, the blood glucose level was not measured. However, it is well known that dietary CaPr is dissociated in the rumen increasing the ruminal propionate proportion that promotes glucose synthesis in the liver [20,58]. Therefore, it is plausible that the higher pH observed in meat could be attributed to an increase in blood glucose levels resulting from CaPr supplementation [9,40,59].

Similar to our results, other authors neither reported any effect of CaPr supplementation on purge loss, cook loss, WHC, or WBFS [11,60]. In addition, the WBSF values obtained in the present study were less than the 5.0 kg/cm^2 value established by Hopkins et al. [61] as the upper limit value to consider lamb meat tenderness by consumers.

A fresh appearance and light color are preferred attributes by traditional lamb meat consumers [62]; however, these preferences depend on cultural issues and regionalism [63]. Meat color is affected by animal nutrition, carcass cooling rate, muscle pH, storage time, oxygen exposure, and myoglobin content [64,65].

In agreement with our results, Piao et al. [66] did not observe changes ($p < 0.05$) in the instrumental color of steers supplemented with glycerol in replacement of dietary grains. However, contrary to the results of the present study, Carrillo-Muro et al. [11] observed that in lambs fed CaPr, the L* (lightness) value was reduced ($p < 0.001$), but a* and b* values increased ($p < 0.001$) at 30 g/lamb/d.

Based on the previous results, we can suggest that including CaPr in the diet of finishing lambs for 14 d at a dose of 10 g/lamb/d is sufficient to improve the meat pH value, approaching the ideal values that enhance quality attributes. However, it is noteworthy that the improvement in pH values did not lead to alterations in meat quality characteristics such as purge loss, cook loss, WHC, WBFS, or color.

4. Conclusions

We concluded that CaPr is a useful feed additive that provides an additional energy source for finishing lambs. Under the current experimental conditions, the optimal inclusion period was 24 to 28 d because maximized most growth performance (FBW, ADG, and ADG:DMI ratio), carcass characteristics (HCW, CCW, carcass dressing, EBW), and some whole cuts (forequarters and neck), without affecting organ mass or meat quality.

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Data Availability Statement: If required, the corresponding author can provide the database.

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Productive, Qualitative, and In Vitro Fermentation Traits of Amaranthus Grains as Potential Ingredients for Pig Diet

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Abstract: The present work compared the agronomic traits, chemical composition, fatty acid profile, and in vitro fermentation characteristics of twelve accessions of *Amaranthus* spp., belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor*, grown in a semiarid Mediterranean area. Among accessions, Benin and Arizona (*A. cruentus*) and Pennsylvania (*A. hypochondriacus*) showed the highest seed yield (on average, 322.1 g m^{-2}), while Taiwan (*A. tricolor*) and India and Iowa (*A. hypochondriacus*) the highest thousand seed weight (on average, 0.81 g). Among the species, *A. hypochondriacus* showed the highest crude protein (16 g $100g^{-1}$), starch (51.5 g $100g^{-1}$), and soluble detergent fiber (2.03 g $100g^{-1}$) contents and the most favorable in vitro fermentation characteristics with the highest short-chain fatty acid (SCFA 52.6 mmol g^{-1}) and butyric acid (20.7% SCFA) production together with the lowest crude fiber (4.93 g $100g^{-1}$) and insoluble dietary fiber (12.5 g $100g^{-1}$) content. Arizona (*A. cruentus*) showed the highest level of monounsaturated fatty acids (32.67 g $100g^{-1}$), Ohio (*A. hybridus*) had the highest levels of polyunsaturated fatty acids (44.62 g $100g^{-1}$) and n6-PUFA (44.21 g $100g^{-1}$), and India (*A. hypochondriacus*) had the highest level of n3-PUFA (0.63 g $100g^{-1}$). *A. hypochondriacus* exhibited not only desirable nutritive characteristics, agronomic traits, and suitability to Mediterranean growing conditions, but also a potential beneficial effect. Nonetheless, it is recommended to run longer-term field trials to confirm these findings and to assess the genotype by environment interaction either with current accessions or others from the wide Amaranth germplasm available.

Keywords: pseudo-cereal; accessions; monogastric; quality traits; in vitro degradability; fatty acids profile

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1. Introduction

Amaranth (*Amaranthus* spp.) is a grain crop similar to cereals with desirable agronomic peculiarities and elevated nutritional value suitable for animal nutrition [1]. The genus *Amaranthus* consists of several species, from annual to short-lived perennial plants, and can be grouped into grain and vegetable types. The most important species are *A. cruentus*, *A. hypochondriacus*, *A. hybridus*, and *A. caudatus*, which are mainly used for grain production, while *A. tricolor*, *A. dubius*, and *A. lividus* are used for vegetable production [2]. Recently, grain amaranth has gained popularity as a feed and food crop [3] due to its excellent nutritional value [4]. The protein content is higher (13.0–19.85%) than other cereal grains, with a favorable amino acid profile [5], more lysine than soybean, and relatively abundant sulfur-containing amino acids, which are usually limited in pulse crops [6,7]. Moreover, unlike other cereals, amaranth proteins consist of albumins (about 40%), glutenins (25–30%), and globulins (20%) and contain very small amounts of prolamins (2–3%). Amaranth prolamins

are richer in glutamic acid and essential amino acids than albumins and globulins, which makes amaranth a good protein source closer to the composition of animal proteins [8]. The lipid content (5.5–16.7%) is also higher than conventional cereals [9], and it is characterized by high levels of unsaturated fatty acids [10], with a saturated/unsaturated fatty acid ratio ranging from 0.12 to 0.50 [2,11,12]. Starch is the main component of amaranth grain, although it is slightly lower (48–69%) than in common cereals [2,3]. The total dietary fiber in amaranth grains is slightly lower (7.1–16.4%) than in wheat [2]; nonetheless, dietary fiber has been reported to be comparable to quinoa and other cereals [5,8,13]. Furthermore, the high crude fiber content in amaranth grains has been associated with monogastric animals' gut health [14].

Currently, limited literature exists on the use of amaranth grains in pig nutrition. Zralý et al. [15], tested diets containing 10% amaranth grain on fattening pigs and obtained some remarkable results in growth efficiency, live body weight gains, and health status of pigs during the entire experiment (pre-fattening, fattening stages I and II). Shilov and Zharkovskii [16] reported an increase in digestibility when amaranth was added to pig diets, and Manyelo et al. [3] demonstrated positive results in pig growth performance. Unlike conventional cereals, the proteins and dietary fiber contents in amaranth grains meet the pigs' nutritional requirements [4]. Furthermore, it can be expected that the high content of essential fatty acids could positively modify the lipid composition of the tissues producing healthy meat [17].

A comparison between eight accessions of *Amaranthus cruentus* grown in the Mediterranean area highlighted large genotypic variability of agronomic traits [18], with yields comparable to barley and oat [11]. Taking into account our previous study [19], where *A. cruentus* grains were evaluated as a component of the feeding plan for dairy cows for productive, qualitative, and in vitro fermentation traits, the present study addresses the agronomic traits, proximate composition, in vitro degradability, and fatty acid content of twelve accessions of amaranth, belonging to *A. cruentus*, *A. hypochondriacus*, *A. hybridus*, and *A. tricolor* species, grown in a field trial in a semiarid Mediterranean area. The hypothesis of the investigation is that the nutritional characteristics of amaranth (energy density, protein, starch, and fat content) are useful for pig diets; in fact, the main objective was to explore the quantitative and qualitative profile of amaranth grains as a feed ingredient in swine nutrition.

2. Materials and Methods

2.1. Plant Materials and Field Experiment

The twelve accessions of *Amaranthus* spp. were obtained from the USDA seed bank (Washington, DC, USA), and their main information is reported in Table 1. Accessions were sown in expanded polystyrene trays in a nursery at 26 °C and 85 ± 5% relative humidity (RH) on 21 March 2014 and kept until the fourth true leaf stage.

Meanwhile, a randomized block design with three replications was arranged in a previously plowed and fertilized (40 kg N ha^{-1}, 80 kg P_2O_5 ha^{-1}, and 60 kg K_2O ha^{-1}) sandy–loamy textured soil in Bovalino (20 m a.s.l. 38°08′ N, 16°10′ E, Calabria, Southern Italy). Plants were transplanted at a density of 10 plants m^{-2} in single plots of 9 m^2 (3 × 3 m). Throughout the growing season, weed control was carried out by hand and 320 mm of water was supplied by a drip irrigation system. Before anthesis, a further 80 kg N ha^{-1} was broadcasted. Seed weight was obtained by removing edge plants and by harvesting a sample area of 4 m^2 inside each plot. Due to uneven seed ripening among accessions, grains were harvested at physiological maturity from 29 June to 11 July. Seeds were separated by inflorescences with a laboratory thresher and allowed to dry at room temperature. The mean thousand seed weight was then calculated by three sets of one hundred seeds in each accession and replication. Rainfall amount and distribution and air temperature during the growing season were typical of the area [11].

Table 1. Amaranth (*Amaranthus* spp.) species and origin.

Species	Origin	Accession	Acronym
A. cruentus	Benin (Republic of Benin)	PI 618962	Benin
	Shaba (Kenya)	PI 628793	Shaba
	Arizona (USA)	PI 566896	Arizona
A.hybridus	Delaware (USA)	PI 636181	Delaware
	Ohio (USA)	PI 603891	Ohio
	Goias (Brazil)	PI 652417	Goias
A.hypochondriacus	India	PI 477915	India
	Iowa (USA)	PI 568125	Iowa
	Pennsylvania (USA)	PI 572256	Pennsylvania
A.tricolor	Beijing (China)	PI 419057	Beijing 1
	Taiwan	PI 604669	Taiwan
	Beijing (China)	AMES 26210	Beijing 2

2.2. Sample Preparation

Amaranth seed samples were finely ground with a 1.1 mm sieve by a grounding machine (Pulverisette 19, Fritsch, Idar-Oberstein, Germany).

2.3. Proximate Chemical Analysis

Chemical analyses of the amaranth grain samples were carried out using the standard procedures of the Association of Official Analytical Chemists [20]; method no. 930.15, 942.05, 2001.11, 978.10, and 920.39 for the content of moisture, ash, crude protein, crude fiber, and ether extract were applied.

Total starch content was determined by means of a Megazyme Total Starch Assay Kit (Megazyme©, NEOGEN, Lansing, MI, USA) for enzymatic extraction and using AOAC method no. 996.11 [20]. A UV–visible spectrophotometer setting the wavelength at 510 nm was applied for the quantification of starch content [19].

A Total Dietary Fiber Assay Kit (Megazyme©, NEOGEN, Lansing, MI, USA) for the analysis of total dietary fiber was used.

Each chemical analysis was performed in triplicate for each amaranth accession and the results were expressed as g $100g^{-1}$, as fed.

2.4. Analysis of Fatty Acids, Fatty Acid Classes, and Peroxidation Index Calculation

Lipid extracts underwent direct transesterification for fatty acid (FA) determination [21]. FA methyl esters (FAMEs) were analyzed by a gas chromatograph system (GC-FID, TRACE 1310, Thermo Fisher Scientific, Milan, Italy). For FAME separation, an Omegawax 250 (Supelco, Bellefonte, PA, USA) was used as reported by Oteri et al. [12]. Chromeleon^{TM} Data System Software (Version 7.2.9, Thermo Fisher Scientific, Milan, Italy) was employed for GC-FID data collection. The identification of FA in amaranth seeds was performed by comparing the relative retention times of FAMEs identified in the sample with retention times of the certified standard mixture (mix 37 FAMEs, Supelco, Inc., Bellefonte, PA, USA) analyzed using the same chromatographic method. The single FA concentration was expressed as g $100g^{-1}$ where 100 g was the sum of all areas of FAMEs that were identified. The equation proposed by Luciano et al. [22] was applied to calculate the peroxidation index (PI).

2.5. In Vitro Gas Production

To study the fermentation kinetics in pig's large intestine, the in vitro gas production technique, according to Theodorou et al. [23], was utilized. The twelve amaranth samples were weighed (0.5049 ± 0.0024 g) into 120 mL serum flasks. For each sample, three replications were made and three flasks were incubated without substrate to correct in vitro parameters. Three healthy adult male castrated Large White pigs (mean age:

350 ± 10 days; mean live weight: 159.8 ± 5.2 kg) bred for meat were used as inoculum donor animals. The animals were fed with a commercial diet used for the finishing period (CP: 14.8%; CF: 4.0%). The fecal samples were collected per rectum, pooled and filtered through a double layer of cheesecloth, diluted (1:6) in NaCl solution, homogenized, and added to each flask (5 mL) containing medium (79 mL) under CO_2 flow. The flasks were incubated at 39 °C for 72 h. During the incubation, gas pressure and volume were manually measured (14 times) using a pressure transducer (Cole and Parmer Instrument Co., Vernon Hills, IL, USA); the final gas production was related to incubated organic matter (OMCV, mL g^{-1}). The organic matter degradability (OMD, %) was determined by filtering flask content through #2 porosity glass crucibles and burning at 550 °C [19].

Fermentation liquor was collected from each flask after 72 h to determine pH and short-chain fatty acid (SCFA, mmol g^{-1}) production by gas chromatography (Thermo Fisher Scientific, Rodano, MI, Italy; model trace 1310) [24]. Branched-chain fatty acid proportion (BCFA) was also calculated as follows:

$$[(\text{iso-valerate} + \text{iso-butyrate})/\text{SCFA}] \quad (1)$$

2.6. Statistical Analysis

Agronomic (seed yield, thousand seed weight) and chemical (seed composition) in vitro fermentation parameters (OMCV, OMD, pH) and end-products (SCFA) were statistically analyzed by NESTED ANOVA (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989–2019) as follows:

$$y_{ijk} = \mu + acc_i + spec(acc)_{ij} + \varepsilon_{ijk} \quad (2)$$

where y_{ijk} is the observation k in level i of factor accession and level j of factor species; μ is the overall mean; acc_i is the effect of level i of factor accession; $spec(acc)_{ij}$ is the effect of level j of factor species within level i of factor accession; ε_{ijk} is the random error with mean 0 and variance σ^2.

When the main effects were significant, means were separated by the Tukey honest significance test at a 95% confidence level.

In vitro fermentation parameters and seed chemical composition, and the in vitro fermentation and fatty acid profile and peroxidation index were further assessed for significant correlations (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989–2019).

3. Results

3.1. Agronomic Traits

Seed yield was significantly different among amaranth accessions (Figure 1). The highest seed yield was registered in Pennsylvania (436.7 g m^{-2}) and the lowest in Taiwan (135.6 g m^{-2}). Benin did not differ from Pennsylvania and Arizona. This latter was similar to India, Iowa, and Goias. The remaining accessions, namely, Shaba, Delaware, Ohio, Beijing1, and Beijing2, did not differ from Goias. Among the four species, *A. cruentus* and *A. hypochondriacus* showed similar seed yields (on average, 322.1 g m^{-2}) that were greater than those of *A. hybridus* and *A. tricolor* (on average, 160.2 g m^{-2}).

Thousand seed weight (TSW) was significantly different among amaranth accessions (Figure 2). The highest TSW was found in Taiwan (0.90 g) and the lowest in Delaware and Goias (on average, 0.28 g). India and Iowa did not differ from Taiwan or Beijing1 and Beijing2. These two latter did not differ from Arizona. The remaining accessions, namely, Benin, Shaba, and Ohio, had significantly different TSW means. Among the four species, *A. tricolor* and *A. hypochondriacus* showed the highest TSW (on average, 0.81 g), followed by *A. cruentus* (0.56 g). The lowest TSW was observed in *A. hybridus* (0.29 g).

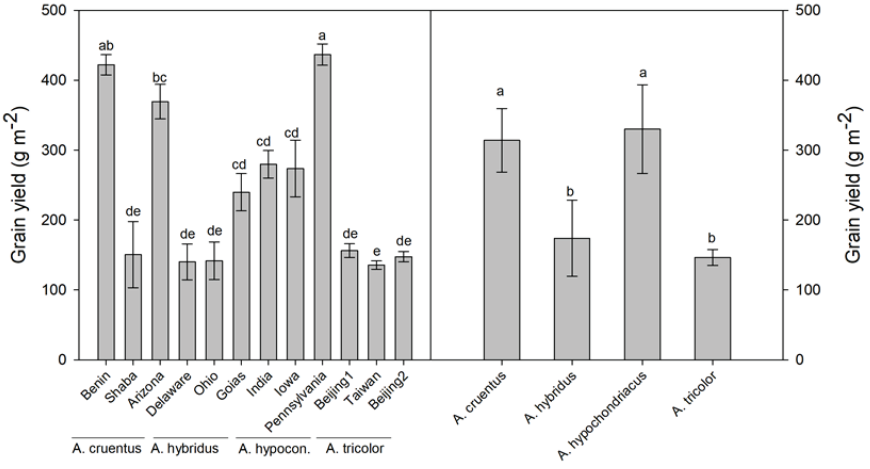

Figure 1. Seed yield (g m^{-2}) of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species. Means ± standard errors followed by different letters indicate significant differences ($p \leq 0.05$).

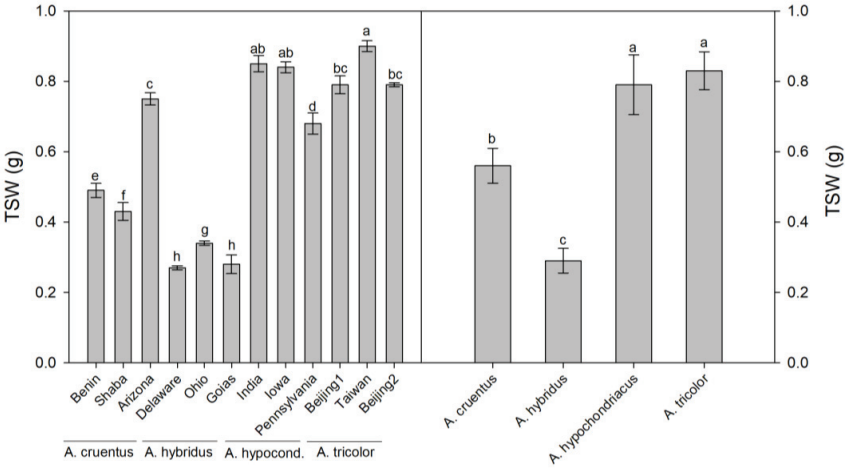

Figure 2. Thousand seed weight (TWS, g) of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species. Means ± standard errors followed by different letters indicate significant differences ($p \leq 0.05$).

3.2. Proximate Chemical Composition

The chemical compositions of the four amaranth seed species and accessions are reported in Table 2. Considering species effect, significant differences ($p \leq 0.001$) were shown. *A. cruentus* had the highest values of CP and EE and the lowest SDF. *A. hypochondriacus* showed the highest values of CP, starch, and SDF and the lowest values of EE, ash, CF, and IDF. *A. hybridus* had the lowest values of CP and starch and the highest values of CF, TDF, and IDF, while *A. tricolor* showed the lowest value of ash, intermediate values for all the other parameters, and the highest value of TDF.

Table 2. Chemical composition (g 100 g^{-1}, as fed) of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species.

Species		DM	Ash	CP	CF	EE	Starch	TDF	IDF	SDF
Species Effect										
A. cruentus		89.0 ^{C}	3.32 ^{B}	16.1 ^{A}	10.9 ^{B}	6.68 ^{A}	50.2 ^{C}	14.9 ^{B}	14.0 ^{C}	0.88 ^{D}
A.hybridus		89.0 ^{C}	3.22 ^{C}	14.4 ^{B}	16.2 ^{A}	5.91 ^{B}	47.6 ^{D}	19.3 ^{A}	18.0 ^{A}	1.26 ^{C}
A.hypochondriacus		89.7 ^{A}	3.04 ^{D}	16.0 ^{A}	4.93 ^{C}	5.56 ^{C}	51.5 ^{A}	14.5 ^{C}	12.5 ^{D}	2.03 ^{A}
A.tricolor		89.1 ^{B}	3.86 ^{A}	14.6 ^{B}	11.4 ^{B}	6.13 ^{B}	50.7 ^{B}	16.8 ^{A}	15.0 ^{B}	1.62 ^{B}
Origin		**Accession Effect**								
A. cruentus	Benin	88.6 ^{E}	3.29 ^{CD}	16.1 ^{b}	11.7 ^{CD}	5.79 ^{CD}	49.4 ^{DE}	15.4 ^{DE}	14.5 ^{CD}	0.90 ^{EF}
	Shaba	88.9 ^{D}	3.23 ^{D}	16.3 ^{b}	10.5 ^{E}	5.68 ^{CD}	49.3 ^{DE}	15.5 ^{CD}	14.3 ^{C}	1.26 ^{CDE}
	Arizona	89.6 ^{B}	3.44 ^{BC}	16.0 ^{b}	10.5 ^{E}	6.25 ^{BCD}	51.8 ^{AB}	13.6 ^{FG}	13.2 ^{DE}	0.47 ^{F}
A.hybridus	Delaware	88.9 ^{CD}	3.20 ^{DE}	13.9 ^{c}	16.6 ^{A}	6.63 ^{AB}	48.2 ^{E}	19.5 ^{A}	18.2 ^{AB}	1.35 ^{CDE}
	Ohio	89.0 ^{CD}	3.17 ^{DE}	14.1 ^{c}	14.7 ^{B}	6.13 ^{BCD}	49.4 ^{D}	18.0 ^{B}	17.1 ^{B}	0.89 ^{EF}
	Goias	89.1 ^{CD}	3.29 ^{CD}	15.4 ^{bc}	17.2 ^{A}	7.27 ^{A}	45.2 ^{F}	20.2 ^{A}	18.7 ^{A}	1.55 ^{BCD}
A.hypochondriacus	India	89.7 ^{AB}	3.01 ^{EF}	16.4 ^{ab}	4.34 ^{F}	4.92 ^{E}	51.5 ^{B}	14.3 ^{EFG}	12.0 ^{E}	2.33 ^{A}
	Iowa	89.7 ^{AB}	3.17 ^{DE}	15.5 ^{bc}	5.25 ^{F}	5.64 ^{D}	51.2 ^{BC}	14.8 ^{DE}	13.0 ^{DE}	1.79 ^{ABC}
	Pennsylvania	89.9 ^{A}	2.93 ^{F}	15.9 ^{b}	5.18 ^{F}	6.14 ^{BCD}	51.7 ^{AB}	14.5 ^{DEF}	12.5 ^{E}	1.98 ^{AB}
A.tricolor	Beijing1	89.2 ^{C}	4.76 ^{A}	18.1 ^{A}	10.9 ^{DE}	5.79 ^{CD}	49.3 ^{DE}	13.3 ^{F}	12.3 ^{E}	1.04 ^{DEF}
	Taiwan	89.2 ^{C}	3.32 ^{BCD}	14.1 ^{C}	11.1 ^{CDE}	6.32 ^{BC}	52.8 ^{A}	14.4 ^{DEFG}	12.4 ^{E}	2.03 ^{AB}
	Beijing2	89.1 ^{CD}	3.49 ^{B}	15.0 ^{BC}	12.1 ^{C}	6.31 ^{BC}	50.1 ^{CD}	16.8 ^{BC}	15.0 ^{C}	1.79 ^{ABC}
RMSE		0.01	0.002	0.17	0.08	0.05	0.01	0.07	0.07	0.02

DM = dry matter; CP = crude protein; CF = crude fiber; EE = ether extract; TDF = total dietary fiber; IDF = insoluble dietary fiber; SDF = soluble dietary fiber. RMSE = mean square error. Along the column, lowercase and uppercase letters indicate $p \leq 0.05$ and 0.001, respectively.

Regarding accession effects, Beijing1 showed the highest values of CP and ash and the lowest value of IDF; Delaware showed the highest values of CF and TDF and the lowest value of CP; Goias showed the highest values of CF, EE, IDF, and TDF and the lowest value of starch; Taiwan showed the highest value of starch and the lowest value of CP and IDF. India, Iowa, and Pennsylvania had the lowest CF (on average, 4.9 g 100g^{-1}, as fed); India also showed the lowest values of EE and IDF, the highest value of SDF, and a high level of CP. The lowest value of SDF was found in the Arizona accession.

3.3. Fatty Acids, Fatty Acid Classes, and Peroxidation Index

Regarding the individual fatty acids in the four species (Table 3), significant differences ($p \leq 0.05$) were observed. In particular, *A. cruentus* showed the significantly highest levels of palmitic (C16:0) and oleic (C18:1n9) acids and the lowest levels of myristic (C14:0) and linoleic (C18:2n6) acids. *A. hybridus* showed the significantly highest levels of myristic and stearic (C18:0) acids and the lowest levels of oleic and alpha-linolenic (C18:3n3) acids; *A. hypochondriacus* showed the highest levels of alpha-linolenic acids and the lowest level of stearic acid. Finally, *A. tricolor* exhibited the highest level of linoleic acid and the lowest levels of saturated fatty acids as myristic, palmitic, and stearic acids.

Regarding the effect of the accessions on the fatty acids of nutritional interest (Table 3), the significantly highest levels were observed for palmitic acid in Shaba, oleic acid in Arizona, myristic and oleic acids in Delaware, linoleic acid in Ohio, and alpha-linolenic acid in India. The significantly lowest values were observed for myristic acid in all the accessions of *A. tricolor* (Beijing1, Taiwan, Beijing2), with an average value of 0.34 g 100g^{-1}, for palmitic and oleic acids in Beijing1. Oleic acid was the lowest in Delaware and Ohio; stearic acid was lowest in Taiwan and Beijing2; linoleic acid was lowest in Shaba, Arizona, and Goias; and alpha-linolenic acid was lowest in Delaware.

Table 3. Fatty acid profiles (g $100g^{-1}$) of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species.

Species		C14:0	C16:0	C16:1	C17:0	C18:0	C18:1n9	C18:1n7	C18:2n6	C18:3n3	C20:0	C22:0	Others
							Species Effect						
A. cruentus		0.60 ^{c}	28.15 ^{a}	0.28 ^{ab}	0.60 ^{a}	6.52 ^{b}	29.38 ^{a}	1.42 ^{b}	29.38 ^{c}	0.38 ^{b}	1.02 ^{b}	0.46 ^{b}	1.82 ^{c}
A.hybridus		2.49 ^{a}	26.38 ^{c}	0.27 ^{ab}	0.50 ^{b}	8.10 ^{a}	25.25 ^{d}	1.40 ^{b}	32.77 ^{b}	0.31 ^{d}	0.96 ^{c}	0.40 ^{d}	1.19 ^{d}
A.hypochondriacus		0.72 ^{b}	27.41 ^{b}	0.32 ^{a}	0.43 ^{c}	5.89 ^{c}	27.15 ^{b}	1.43 ^{b}	32.83 ^{b}	0.54 ^{a}	0.90 ^{d}	0.44 ^{c}	1.96 ^{b}
A.tricolor		0.34 ^{d}	25.29 ^{d}	0.23 ^{b}	0.13 ^{d}	4.59 ^{d}	26.13 ^{c}	2.30 ^{a}	36.45 ^{a}	0.35 ^{c}	1.19 ^{a}	0.71 ^{a}	2.29 ^{a}
	Origin						**Accession Effect**						
A. cruentus	Benin	0.78 ^{d}	29.47 ^{b}	0.33 ^{ab}	0.65 ^{b}	7.13 ^{d}	28.58 ^{b}	1.43 ^{b}	28.04 ^{f}	0.36 ^{efg}	1.06 ^{cd}	0.48 ^{e}	1.72 ^{f}
	Shaba	0.64 ^{e}	30.79 ^{a}	0.28 ^{b}	0.70 ^{a}	7.44 ^{c}	28.53 ^{b}	1.41 ^{b}	26.08 ^{g}	0.33 ^{gh}	1.25 ^{b}	0.56 ^{d}	2.03 ^{de}
	Arizona	0.40 ^{g}	24.20 ^{g}	0.23 ^{b}	0.44 ^{de}	5.00 ^{h}	31.02 ^{a}	1.42 ^{b}	34.03 ^{d}	0.45 ^{c}	0.76 ^{f}	0.35 ^{h}	1.72 ^{f}
A.hybridus	Delaware	3.81 ^{a}	29.02 ^{c}	0.31 ^{ab}	0.53 ^{c}	10.35 ^{a}	24.17 ^{f}	1.44 ^{b}	27.68 ^{f}	0.20 ^{i}	0.99 ^{d}	0.41 ^{fg}	1.12 ^{h}
	Ohio	0.57 ^{f}	20.72 ^{i}	0.18 ^{b}	0.46 ^{d}	5.44 ^{g}	24.00 ^{f}	1.27 ^{c}	44.21 ^{a}	0.41 ^{cd}	0.85 ^{ef}	0.36 ^{h}	1.57 ^{g}
	Goias	3.10 ^{b}	29.42 ^{b}	0.31 ^{ab}	0.51 ^{c}	8.50 ^{b}	27.58 ^{c}	1.51 ^{b}	26.42 ^{g}	0.33 ^{fgh}	1.05 ^{cd}	0.43 ^{f}	0.88 ^{i}
A.hypochondriacus	India	0.75 ^{d}	27.04 ^{d}	0.24 ^{b}	0.42 ^{e}	5.90 ^{f}	26.45 ^{e}	1.43 ^{b}	33.96 ^{d}	0.63 ^{a}	0.85 ^{e}	0.42 ^{fg}	1.96 ^{e}
	Iowa	0.59 ^{f}	26.23 ^{e}	0.29 ^{b}	0.36 ^{f}	5.38 ^{g}	28.43 ^{b}	1.43 ^{b}	33.84 ^{d}	0.54 ^{b}	0.82 ^{ef}	0.40 ^{g}	1.71 ^{f}
	Pennsylvania	0.83 ^{c}	28.96 ^{c}	0.45 ^{a}	0.52 ^{c}	6.39 ^{e}	26.58 ^{de}	1.44 ^{b}	30.70 ^{e}	0.44 ^{c}	1.03 ^{d}	0.49 ^{e}	2.21 ^{c}
A.tricolor	Beijing1	0.34 ^{h}	23.78 ^{h}	0.25 ^{b}	0.12 ^{h}	5.13 ^{h}	23.96 ^{f}	2.24 ^{a}	39.14 ^{b}	0.38 ^{de}	1.36 ^{a}	0.86 ^{a}	2.47 ^{a}
	Taiwan	0.35 ^{h}	25.09 ^{f}	0.22 ^{b}	0.13 ^{gh}	4.25 ^{i}	27.14 ^{cd}	2.34 ^{a}	36.02 ^{c}	0.38 ^{def}	1.30 ^{c}	0.62 ^{c}	2.32 ^{b}
	Beijing2	0.34 ^{h}	27.02 ^{d}	0.21 ^{b}	0.15 ^{g}	4.40 ^{i}	27.30 ^{c}	2.31 ^{a}	34.20 ^{d}	0.31 ^{h}	1.08 ^{cd}	0.66 ^{b}	2.07 ^{d}
RMSE		0.014	0.132	0.051	0.009	0.051	0.231	0.038	0.204	0.015	0.030	0.011	0.011

The concentration of fatty acids was expressed as g $100g^{-1}$, considering 100 g as the sum of the areas of all identified FAMEs. C14:0 = myristic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C17:0 = heptadecanoic acid; C18:0 = stearic acid; C18:1n9 = oleic acid; C18:1n7 = cis-vaccenic acid; C18:2n6 = linoleic acid; C18:3n3 = α-linolenic acid; C20:0 = arachidic acid; C22:0 = behenic acid. RMSE: root mean square error; along the column, letters indicate $p \leq 0.05$. Regarding the fatty acid classes (Table 4) in the four species, *A. hybridus* had the significantly highest values of saturated fatty acids (SFA) and the SFA/UFA (unsaturated fatty acids) ratio. *A. cruentus* showed the highest monounsaturated fatty acids (MUFA), and *A. tricolor* the highest polyunsaturated fatty acids (PUFA) and the highest levels of the PUFA of the n6-series (n6-PUFA) and of the peroxidation index (PI). *A. hypochondriacus* showed the highest PUFA levels of the n3-series (n3-PUFA). The significantly lowest levels of fatty acid classes, ratios, and quality indexes were observed in *A. tricolor* (SFA and SFA/UFA ratio), in *A. hybridus* (MUFA and n3-PUFA), and in *A. cruentus* (PUFA, n6-PUFA, and PI).

Table 4. Fatty acid classes (g $100g^{-1}$), SFA/UFA ratios, and peroxidation indexes of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species.

Species		SFA	MUFA	PUFA	SFA/UFA	n3-PUFA	n6-PUFA	PI
				Species Effect				
A. cruentus		37.35 ^{b}	31.08 ^{a}	29.76 ^{c}	0.62 ^{b}	0.38 ^{b}	29.38 ^{c}	30.13 ^{d}
A.hybridus		38.82 ^{a}	26.92 ^{c}	33.08 ^{b}	0.67 ^{a}	0.31 ^{d}	32.77 ^{b}	33.39 ^{c}
A.hypochondriacus		35.78 ^{c}	28.90 ^{b}	33.36 ^{b}	0.58 ^{c}	0.54 ^{a}	32.83 ^{b}	33.89 ^{b}
A.tricolor		32.25 ^{d}	28.65 ^{b}	36.80 ^{a}	0.49 ^{d}	0.35 ^{c}	36.45 ^{a}	37.16 ^{a}
	Origin				**Accession Effect**			
A. cruentus	Benin	39.56 ^{d}	30.35 ^{b}	28.39 ^{f}	0.68 ^{d}	0.36 ^{efg}	28.04 ^{f}	28.74 ^{f}
	Shaba	41.37 ^{c}	30.21 ^{b}	26.40 ^{g}	0.73 ^{c}	0.33 ^{gh}	26.08 ^{g}	26.73 ^{g}
	Arizona	31.14 ^{h}	32.67 ^{a}	34.48 ^{d}	0.47 ^{i}	0.45 ^{c}	34.03 ^{d}	34.93 ^{d}
A.hybridus	Delaware	45.10 ^{a}	25.92 ^{ef}	27.88 ^{f}	0.84 ^{a}	0.20 ^{i}	27.68 ^{f}	28.05 ^{f}
	Ohio	28.39 ^{i}	25.44 ^{f}	44.62 ^{a}	0.41 ^{j}	0.41 ^{cd}	44.21 ^{a}	45.02 ^{a}
	Goias	42.99 ^{b}	29.40 ^{c}	26.75 ^{g}	0.77 ^{b}	0.33 ^{fgh}	26.42 ^{g}	27.07 ^{g}
A.hypochondriacus	India	35.37 ^{f}	28.11 ^{d}	34.58 ^{d}	0.57 ^{f}	0.63 ^{a}	33.96 ^{d}	35.20 ^{d}
	Iowa	33.78 ^{g}	30.15 ^{bc}	34.38 ^{d}	0.52 ^{g}	0.54 ^{b}	33.84 ^{d}	34.91 ^{d}
	Pennsylvania	38.21 ^{e}	28.46 ^{d}	31.13 ^{e}	0.64 ^{e}	0.44 ^{c}	30.70 ^{e}	31.57 ^{e}
A.tricolor	Beijing1	31.56 ^{h}	26.44 ^{e}	39.52 ^{b}	0.48 ^{hi}	0.38 ^{de}	39.14 ^{b}	39.91 ^{b}
	Taiwan	31.57 ^{h}	29.70 ^{bc}	36.40 ^{c}	0.48 ^{h}	0.38 ^{def}	36.02 ^{c}	36.78 ^{c}
	Beijing2	33.63 ^{g}	29.82 ^{bc}	34.50 ^{d}	0.53 ^{g}	0.31 ^{h}	34.20 ^{d}	34.80 ^{d}
RMSE		0.161	0.265	0.216	0.005	0.015	0.204	0.227

The concentration of fatty acids was expressed as g $100g^{-1}$, considering 100 g as the sum of the areas of all identified FAMEs. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; n3 = n3-polyunsaturated fatty acids; n6 = n6-polyunsaturated fatty acids; SFA/UFA = saturated/unsaturated fatty acid ratio; PI = peroxidation index; RMSE: root mean square error; along the column, letters indicate $p \leq 0.05$.

Among the accessions (Table 4), Shaba showed the lowest values of PUFA and PI; Arizona showed the highest MUFA; Delaware showed the highest SFA and SFA/UFA ratio; Ohio showed the highest PUFA, n6-PUFA, and PI and the lowest SFA, MUFA, and SFA/UFA ratio; India showed the highest n3-PUFA; and Beijing2 showed the lowest n3-PUFA.

3.4. In Vitro Fermentation Characteristics, Kinetics, and End-Products

In Table 5, the in vitro parameters results are described. *A. hypochondriacus* showed the highest percentage of OMD while *A. hybridus* showed the lowest (<50%). In addition, *A. hypochondriacus* registered the highest volume of gas produced. *A. hybridus* and *A. tricolor* reported the lowest gas production. Among the accessions, India exhibited the highest OMD, while Delaware had the lowest. The latter also showed the lowest level of OMCV. In Shaba, the highest gas production was recorded.

In Table 6, in vitro end-products are reported. *A. hypochondriacus* and *A. hybridus* showed the highest and lowest amount of SCFA, respectively. *A. hybridus* and *A. tricolor* reported the greatest percentages of BCFA, while *A. hypochondriacus* had the lowest. Furthermore, *A. hybridus* and *A. tricolor* exhibited the highest levels of acetate, iso-butyrate, iso-valerate, and valerate. *A. cruentus* showed the lowest percentage of propionate, while *A. hypochondriacus* had the highest. These two species produced the greatest percentages of butyrate. *A. hypochondriacus* produced the lowest amounts iso-butyrate, iso-valerate, and valerate. India, Iowa, and Pennsylvania were the accessions with the highest amounts of SCFA. Delaware, Goias, Shaba, and Beijing1 reported the lowest levels of SCFA. *A. hybridus* accessions (Delaware, Ohio, Goias) and *A. tricolor* accessions (Beijing1, Taiwan, and Beijing2) showed the highest levels of BCFA. On the contrary, *A. hypochondriacus* accessions resulted in the lowest level of BCFA. Beijing2 and Ohio showed the highest percentages of acetate. Iowa showed the lowest and highest levels of acetate and propionate, respectively. Shaba had the greatest amount of butyrate.

Table 5. In vitro fermentation characteristics of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species.

Species		OMD (%)	OMCV (mL g^{-1})
		Species Effect	
A. cruentus		73.1 ^{B}	178 ^{B}
A. hybridus		46.8 ^{D}	107 ^{C}
A. hypochondriacus		77.6 ^{A}	182 ^{A}
A. tricolor		48.1 ^{C}	109 ^{C}
	Origin	**Accession Effect**	
A. cruentus	Benin	65.7 ^{D}	152 ^{D}
	Shaba	78.1 ^{AB}	193 ^{A}
	Arizona	75.3 ^{C}	190 ^{AB}
A. hybridus	Delaware	38.9 ^{I}	84.4 ^{H}
	Ohio	55.1 ^{E}	130 ^{E}
	Goias	46.4 ^{G}	106 ^{G}
A. hypochondriacus	India	80.0 ^{A}	185 ^{B}
	Iowa	76.3 ^{BC}	174 ^{C}
	Pennsylvania	76.3 ^{BC}	186 ^{AB}
A. tricolor	Beijing1	42.7 ^{H}	104 ^{G}
	Taiwan	49.9 ^{F}	115 ^{F}
	Beijing2	51.6 ^{F}	109 ^{G}
RMSE		0.87	6.14

OMD = organic matter degradability; OMCV = cumulative volume of gas related to incubated organic matter; RMSE = root mean square error; along the column, uppercase letters indicate $p < 0.01$.

Table 6. In vitro fermentation end-products of the twelve amaranth accessions belonging to *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. tricolor* species.

Species		SCFA	BCFA	Ace	Prop	Iso-But	But	Iso-Val	Val
		mmol g^{-1}		% SCFA					
		Species effect							
A. cruentus		37.2 ^{C}	5.44 ^{B}	49.8 ^{B}	17.3 ^{D}	1.98 ^{B}	22.8 ^{A}	3.50 ^{B}	3.40 ^{B}
A. hybridus		31.6 ^{D}	6.78 ^{A}	52.3 ^{A}	22.4 ^{B}	2.48 ^{A}	15.1 ^{B}	4.24 ^{A}	4.07 ^{A}
A. hypochondriacus		52.6 ^{A}	4.68 ^{C}	49.0 ^{B}	24.4 ^{A}	1.64 ^{C}	20.7 ^{A}	3.17 ^{C}	2.76 ^{C}
A. tricolor		40.3 ^{B}	6.88 ^{A}	52.2 ^{A}	21.0 ^{C}	2.53 ^{A}	16.5 ^{B}	4.35 ^{A}	4.07 ^{A}
	Origin	**Accession effect**							
A. cruentus	Benin	38.5 ^{DE}	5.78 ^{BC}	52.3 ^{ABCD}	17.0 ^{FG}	2.00 ^{BC}	21.7 ^{ABC}	3.77 ^{BCD}	4.06 ^{A}
	Shaba	33.0 ^{EF}	5.46 ^{C}	49.1 ^{DEF}	16.8 ^{G}	1.99 ^{BC}	25.7 ^{A}	3.58 ^{CD}	3.46 ^{AB}
	Arizona	40.0 ^{CD}	5.09 ^{CD}	48.0 ^{EF}	18.0 ^{EFG}	1.94 ^{CD}	21.0 ^{ABCD}	3.15 ^{EF}	2.66 ^{B}
A. hybridus	Delaware	28.7 ^{F}	6.97 ^{A}	51.9 ^{ABCDE}	21.4 ^{CD}	2.60 ^{A}	14.7 ^{E}	4.37 ^{A}	4.13 ^{A}
	Ohio	36.6 ^{DE}	6.62 ^{AB}	53.1 ^{AB}	21.1 ^{CDE}	2.24 ^{B}	16.6 ^{CDE}	4.21 ^{ABC}	4.00 ^{A}
	Goias	29.3 ^{F}	6.74 ^{A}	51.8 ^{ABCDE}	24.7 ^{B}	2.60 ^{A}	14.1 ^{E}	4.14 ^{AB}	4.07 ^{A}
A. hypochondriacus	India	55.0 ^{A}	4.94 ^{D}	50.8 ^{BCDE}	20.6 ^{DEF}	1.84 ^{CD}	22.1 ^{ABC}	3.24 ^{DEF}	2.80 ^{B}
	Iowa	51.7 ^{A}	4.79 ^{D}	47.8 ^{F}	28.0 ^{A}	1.45 ^{E}	16.7 ^{CDE}	3.37 ^{DE}	2.84 ^{B}
	Pennsylvania	51.1 ^{AB}	4.30 ^{E}	48.3 ^{DEF}	24.5 ^{B}	1.64 ^{DE}	23.2 ^{AB}	2.90 ^{F}	2.64 ^{B}
A. tricolor	Beijing1	35.4 ^{DEF}	6.67 ^{A}	52.5 ^{ABC}	19.6 ^{DEFG}	2.50 ^{A}	17.8 ^{BCDE}	4.17 ^{AB}	4.32 ^{A}
	Taiwan	45.0 ^{BC}	7.04 ^{A}	50.1 ^{CDEF}	23.9 ^{BC}	2.57 ^{A}	14.9 ^{E}	4.46 ^{A}	4.03 ^{A}
	Beijing2	40.5 ^{CD}	6.94 ^{A}	53.9 ^{A}	19.6 ^{DEFG}	2.51 ^{A}	16.9 ^{CDE}	4.43 ^{A}	3.97 ^{A}
RMSE		6.40	0.03	1.08	1.11	0.01	5.15	0.03	0.10

Ace = acetate; Prop = propionate; Iso-But = iso-butyrate; But = butyrate; Iso-Val = iso-valerate; Val = valerate; SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids (Iso-But + Iso-Val)/SCFA × 100; RMSE = root mean square error; along the column, uppercase letters indicate $p < 0.001$.

3.5. Correlation

The correlation between in vitro and chemical composition parameters is described in Table 7. The CP content showed a positive correlation ($p < 0.01$) with OMD, OMCV,

and butyrate, but was negatively correlated ($p < 0.05$) with BCFA, iso-butyrate, and iso-valerate. On the contrary, CF and EE were negatively correlated ($p < 0.01$, 0.05, respectively) with OMD, OMCV, and SCFA. However, both parameters showed positive correlations ($p < 0.01$ and 0.05, respectively) with BCFA, acetate, iso-butyrate, iso-valerate, and valerate. Similarly, starch content was positively correlated ($p < 0.01$) with OMD, OMCV, and SCFA ($p < 0.05$). On the contrary, starch was negatively correlated ($p < 0.01$) with BCFA, iso-butyrate, iso-valerate, valerate, and acetate ($p < 0.05$). Total dietary fiber, insoluble dietary fiber, and total dietary fiber and ash contents were negatively ($p < 0.05$) correlated with OMD, OMCV, SCFA, and butyrate and positively ($p < 0.01$) with BCFA, acetate, iso-butyrate, iso-valerate, and valerate. The SDF reported a positive ($p < 0.05$) correlation with SCFA and a negative correlation ($p < 0.05$) with valerate.

Table 7. Correlation between in vitro parameters and chemical composition.

	OMD	OMCV	SCFA	BCFA	Ace	Prop	Iso-But	But	Iso-Val	Val
CP	0.6642 **	0.7342 ***	0.0765 NS	−0.6422 **	−0.4182 NS	−0.3337 NS	−0.5608 *	0.7671 ***	−0.6596 **	−0.3179 NS
CF	−0.7754 ***	−0.7317 ***	−0.6690 ***	0.8604 ***	0.6691 ***	−0.3071 NS	0.8305 ***	−0.4206 NS	0.8321 ***	0.8399 ***
EE	−0.5640 **	−0.4951 *	−0.4083 *	0.4624 *	0.4130 *	−0.1011 NS	0.4913 *	−0.3145 NS	0.4131 *	0.4662 *
Starch	0.6964 ***	0.6613 ***	0.4737 *	−0.6904 ***	−0.4565 *	0.1718 NS	−0.6350 ***	0.3589 NS	−0.6916 ***	−0.7044 ***
IDF	−0.8577 ***	−0.8389 ***	−0.5574 *	0.8937 ***	0.6036 **	−0.1498 NS	0.8407 ***	−0.5747 *	0.8802 ***	0.7927 ***
SDF	0.1493 NS	0.0743 NS	0.5340 *	−0.3098 NS	−0.2836 NS	0.2644 NS	−0.3234 NS	0.0754 NS	−0.2809 NS	−0.4665 *
TDF	−0.8719 ***	−0.8707 ***	−0.4585 *	0.8639 ***	0.5666 *	−0.0609 NS	0.8089 ***	−0.6094 **	0.8536 ***	0.7144 ***
Ash	−0.5372 *	−0.5201 *	−0.6731 **	0.5372 *	0.3638 NS	0.2626 NS	0.6056 **	−0.5910 **	0.4488 *	0.4853 *

CP = crude protein; CF = crude fiber; EE = ether extract; IDF = insoluble dietary fiber; SDF = soluble dietary fiber; TDF = total dietary fiber; OMD = organic matter degradability; OMCV = cumulative volume of gas related to incubated organic matter; Ace = acetate; Prop = propionate; Iso-But = iso-butyrate; Iso-Val = iso-valerate; SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids (Iso-But + Iso-Val)/SCFA × 100. *, **, ***, and NS indicate $p < 0.001$, 0.01, 0.05, and not significant, respectively.

Table 8 provides the correlation between in vitro fermentation parameters, fatty acid classes, and quality index. Myristic acid was positively correlated ($p < 0.05$) with BCFA and iso-valerate. Palmitic acid was positively correlated ($p < 0.05$) with OMD and SCFA, while palmitoleic acid was positively correlated ($p < 0.05$) with SCFA. Differently, oleic acid was highly ($p < 0.001$) and positively correlated with OMD, OMCV, and butyrate. On the contrary, the same fatty acid was negatively correlated with BCFA, acetate, iso-butyrate, and iso-valerate. Linoleic acid was negatively correlated ($p < 0.05$) with OMD. Alpha-linolenic acid was positively correlated with OMD, OMCV, SCFA, and butyrate and negatively with BCFA, acetate, iso-butyrate, iso-valerate, and valerate.

Regarding the classes, MUFA were positively correlated with OMD, OMCV, and butyrate, while they were negatively correlated with iso-valerate. The polyunsaturated fatty acids of the n3 series were positively correlated to OMD, OMCV, SCFA, and butyrate. These fatty acids were negatively correlated with BCFA, acetate, iso-butyrate, iso-valerate, and valerate. The PUFA-n6 series were negatively correlated with OMD.

Table 8. Correlation between in vitro parameters, fatty acid classes, and quality index.

	OMD	OMCV	SCFA	BCFA	Ace	Prop	Iso-But	But	Iso-Val	Val
C14:0	−0.227 NS	−0.294 NS	0.212 NS	0.428 *	0.226 NS	0.053 NS	0.320 NS	−0.282 NS	0.483 *	0.161 NS
C16:0	0.406 *	0.351 NS	0.438 *	−0.273 NS	−0.027 NS	0.058 NS	−0.329 NS	0.071 NS	−0.213 NS	−0.294 NS
C16:1	0.3011 NS	0.2067 NS	0.4265 *	−0.179 NS	−0.172 NS	0.267 NS	−0.368 NS	−0.043 NS	−0.025 NS	−0.249 NS
C17:0	0.4715 *	0.4778 *	0.3964 NS	−0.275 NS	−0.160 NS	−0.301 NS	−0.370 NS	0.413 *	−0.187 NS	−0.179 NS
C18:0	−0.011 NS	−0.068 NS	0.280 NS	0.218 NS	0.202 NS	−0.090 NS	0.099 NS	−0.089 NS	0.295 NS	0.025 NS
C18:1n9	0.680 ***	0.709 ***	0.131 NS	−0.448 *	−0.448 *	−0.367 NS	−0.406 *	0.659 ***	−0.454 *	−0.172 NS
C18:1n7	−0.521 **	−0.518 **	−0.555 **	0.347 NS	0.338 NS	0.274 NS	0.445 *	−0.524 **	0.252 NS	0.314 NS
C18:2n6	−0.409 *	−0.359 NS	−0.365 NS	0.171 NS	0.064 NS	0.074 NS	0.238 NS	−0.160 NS	0.111 NS	0.191 NS
C18:3n3	0.532 **	0.550 **	0.469 *	−0.665 ***	−0.687 ***	0.154 NS	−0.634 ***	0.498 *	−0.648 ***	−0.687 ***
C20:0	−0.432 *	−0.435 *	−0.412 *	0.348 NS	0.372 NS	0.258 NS	0.363 NS	−0.524 **	0.316 NS	0.268 NS
C22:0	−0.461 *	−0.457 *	−0.500 *	0.266 NS	0.333 NS	0.271 NS	0.321 NS	−0.485 *	0.2078 NS	0.201 NS
SFA	0.163 NS	0.099 NS	0.371 NS	0.024 NS	0.119 NS	0.019 NS	−0.072 NS	−0.067 NS	0.095 NS	−0.112 NS
MUFA	0.601 ***	0.627 ***	0.044 NS	−0.395 NS	−0.396 NS	−0.308 NS	−0.342 NS	0.564 **	−0.411 *	−0.124 NS
PUFA	−0.396 NS	−0.345 NS	−0.353 NS	0.157 NS	0.050 NS	0.076 NS	0.223 NS	−0.148 NS	0.097 NS	0.176 NS
SFA/UFA	0.128 NS	0.066 NS	0.336 NS	0.061 NS	0.147 NS	0.010 NS	−0.036 NS	−0.089 NS	0.130 NS	−0.079 NS
n3-PUFA	0.532 **	0.550 **	0.469 *	−0.665 ***	−0.687 ***	0.154 NS	−0.634 ***	0.498 *	−0.648 ***	−0.687 ***
n6-PUFA	−0.409 *	−0.359 NS	−0.365 NS	0.171 NS	0.064 NS	0.074 NS	0.2378 NS	−0.160 NS	0.111 NS	0.191 NS
PI	−0.382 NS	−0.332 NS	−0.341 NS	0.142 NS	0.035 NS	0.079 NS	0.209 NS	−0.13 NS	0.084 NS	0.161 NS

C14:0 = myristic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C17:0 = heptadecanoic acid; C18:0 = stearic acid; C18:1n9 = oleic acid; C18:1n7 = vaccenic acid; C18:2n6 = linoleic acid (LA); C18:3n3 = α-linoleic acid (ALA); C20:0 = arachidic acid; C22:0 = behenic acid; OMD = organic matter degradability; OMCV = cumulative volume of gas related to incubated organic matter; Ace = acetate; Prop = propionate; Iso-But = iso-butyrate; Iso-Val = iso-valerate; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; n3 = n3 polyunsaturated fatty acids; n6 = n6 polyunsaturated fatty acids; PI = peroxidation index. *, **, ***, and NS indicate $p < 0.001$, 0.01, 0.05, and not significant, respectively.

4. Discussion

Across the average of investigated accessions, *A. tricolor* and *A. hypochondriacus* showed the significantly highest thousand seed weight (TSW), with an accession range of 0.79–0.90 g for *A. tricolor* and 0.68–0.85 g for *A. hypochondriacus.* The present TSW range of *A. hypochondriacus* is slightly higher than that found by Pospišil et al. [25], who reported 0.69–0.73 g in *A. hypochondriacus* across the average of nitrogen fertilization levels in a three-year field trial in Zagreb, Croatia. The *A. tricolor* TSW range was also wider as com-

pared with 10 genotypes of *A. tricolor* (0.73–0.85 g) grown in Raipur, India [26]. Given the high polymorphism of accessions within the same amaranth species, differences might be ascribed to the genotype effects. However, growing conditions cannot be ruled out as demonstrated by Pospišil et al. [25], who pointed out a lower TSW of *A. hypochondriacus* under drier than more favorable years. In this study, *A. cruentus* showed lower TSW than the previous two species (0.43–0.75 g), and this somehow contrasts with results found in the literature of similar TSW between *A. hypochondriacus* and *A. cruentus* [25,27]. Nonetheless, our TSW with *A. cruentus* is within the range reported by Rivelli et al. [28] in a comparison of five different accessions of *A. cruentus* tested in South Italy. The *A. hybridus* accessions showed the lowest TWS (0.27–0.34 g) and it is similar to the lowest range found by Rivelli et al. [28]. However, this contrasts with Parveen et al. [29], who reported a TSW of 0.55 g averaging eight *A. hybridus* genotypes.

Seed yield was similar between *A. cruentus* and *A. hypochondriacus*, which agrees with the findings of Pospišil et al. [25], who demonstrated no genotypic differences between *A. cruentus* and *A. hypochondriacus* in two out of three growing seasons. However, we found a larger accession influence on grain yield for *A. cruentus* than for *A. hypochondriacus*, as the coefficient of variation of accessions was 46% and 28%, respectively. *A. hybridus* and *A. tricolor* were both less productive, and the coefficient of variation of accessions was quite narrow for *A. tricolor* (7.1%) as compared to *A. hybridus* (32.8%). Grain yields of the best accessions (Pennsylvania, Benin, Shaba) were well comparable or even higher than most cereals grown in semiarid Mediterranean environments, such as durum wheat [30,31] and oat [32].

In contrast to Pospišil et al. [25], who found an increase in seed yield associated with the increase in TSW in a dry growing season, our findings did not show a significant correlation between seed yield and TSW ($p = 0.667$, data not shown). However, the same author pointed out no correlation between seed yield and TSW with more favorable growing conditions. It is worth mentioning that in the present study, the air temperature was typical of the experimental area and rainfall was well distributed [11]; this suggests that traits other than TSW, such as the seed number per panicle or the number of panicles per plant, might have influenced seed yield of investigated accessions.

The organic matter degradability (OMD) of tested *Amaranthus* spp. was quite low for all samples. In particular, for *A. tricolor* and *A. hybridus* species, the OMD was less than 50%. These results could be ascribed to the presence of insoluble dietary fiber in the tested amaranth, as suggested by the significant negative correlations (Table 7) between the insoluble fiber and OMD and OMCV values (-0.8577 and -0.8389, respectively).

On the other hand, insoluble fiber helps to maintain normal gut function but might decrease feed intake and nutrient digestibility [33], increasing the rate of gut passage [34]. Moreover, Acosta et al. [35] tested the addition of distillers dried grains in pig diet, observing that starch digestibility can be affected by insoluble fiber level. Despite the lack of an enzymatic digestion test in this study, the starch content positively affected the fermentation parameters (0.6964 and 0.6613 for OMD and OMCV, respectively).

Starchy feeds as a source of energy and raw cereal grains, along with legume grains and potato starch, constitute the main dietary starch source in pig rations [36]. The concentration of SCFA was quite low for all tested samples; the presence of insoluble dietary fiber could affect the fermentation pathway, as demonstrated by the limited production of SCFA of *A. hybridus* and some accessions of *A. tricolor* (Beijing1 and Beijing2). *A. hypochondriacus* showed higher production of SCFA and butyrate probably due to the higher proportion of soluble dietary fiber, which is readily fermentable [37]. In this regard, the correlations between chemical composition and fermentation parameters demonstrated a positive correlation between starch, SDF vs. SCFA. As suggested by Weaver et al. [38], SDF produced more SCFA compared to IDF. Furthermore, starch feeds that bypass digestion in the stomach and enzymatic hydrolysis in the small intestine are fermented in the large intestine, producing SCFA [39]. Furthermore, the content of crude protein positively affected the fermentation process, particularly butyrate production (0.7671). The high proportion of

butyrate could be useful for the colonic epithelium as a main energy source for cell growth and differentiation [40], suggesting a potential pre-biotic role of *A. hypochondriacus*.

In swine nutrition, it is well known that the dietary FA composition and the molecular structures (chain length and number of double bonds) influence digestion, absorption, and metabolism, and the bioactivity of the FA [41]. The manipulation of lipids in pig diet, especially in the chain length of dietary FA, may be a strategic tool to improve animal performance [41], as explained by the complexity of digestion and absorption of these molecules. The addition of lipids to diets can, in turn, enhance protein digestibility by slowing the passage rate in the intestine by lipids, which contrasts with the effect of fiber [42]. However, still, little focus has been devoted to the impact of lipids, in terms of fatty acids, particularly on gut health and the development of early nutrition of pigs [43].

In general, pigs digest unsaturated dietary lipids more than saturated lipids [44]. Hence, lipase activity and lipid digestion can be positively influenced by unsaturated long-chain fatty acids (LCFA) and negatively by saturated LCFA [41]. Moreover, among unsaturated LCFA, those of the n3 series are believed to affect the gut microbiota mainly through regulation of the type and number of bacteria in the gut, and regulation of SCFA concentrations [45]. This could explain the results obtained in this study where oleic and alpha-linolenic acids as well as total MUFA and n3-PUFA showed positive correlations with OMD (0.680, 0.532, 0.601, 0.532, respectively) and OMCV (0.709, 0.550, 0.627, 0.550, respectively). Conversely, among unsaturated LCFA, linoleic acid and n6-PUFA showed negative correlations with OMD (-0.409) or were not significantly correlated with OMCV. To further confirm these observations, a positive correlation for alpha-linolenic acid and n3-PUFA with SCFA (0.469) and a non-significant correlation between n6-PUFA and SCFA was found. Among the varieties, *A. hypochondriacus,* with the highest content of alpha-linolenic acid and n3-PUFA (0.54 g $100g^{-1}$), presented the highest OMD (77.6%), OMCV (182 mL g^{-1}), and SCFA production (52.6 mmol g^{-1}) and the highest levels of propionate and butyrate (24.2% and 20.7% SCFA, respectively). From a nutritional point of view, it is noteworthy that the metabolites produced by the gut microbiota significantly influence the host's metabolism and health [46]. The proportion of SCFA, produced by bacterial fermentation in the gut, exerts several effects on the host's metabolism and immune system [47]. SCFA, produced via microbial fermentation of non-digestible carbohydrates and digestible starch in the hindgut, contribute with an energy supply for the host and the colonocytes [48] and have antimicrobial properties limiting the risk of infectious diseases in the gut [41].

5. Conclusions

Among the studied species, *A. hypochondriacus* emerged not only for its desirable nutritive value, agronomic traits, and suitability to Mediterranean growing conditions, but also for potential beneficial effects. Although the thousand seed weight was in the lowest range group in Pennsylvania (*A. hypochondriacus*) and Benin (*A. cruentus*), these accessions outyielded the others, suggesting greater adaptability to the Mediterranean growing conditions. With the highest CP, starch, and SDF content and a good proportion of fatty acids, favorable in vitro fermentation characteristics with the highest SCFA and butyric production, together with the lowest CF and IDF content, *A. hypochondriacus* exhibits not only a suitable nutritive value but also a potentially positive effect on large intestine status.

Nonetheless, further field trials with a larger set of accessions from the available amaranth germplasm in multiple sites will help understand the environmental effect on genotypes and to draw clear conclusions on the effectiveness of this crop. Moreover, additional information on amaranth's nutritive value could be obtained by in vivo digestibility trials and amino acid profile determination.

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Effects of Different Trace Elements and Levels on Nutrients and Energy Utilization, Antioxidant Capacity, and Mineral Deposition of Broiler Chickens

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Abstract: This study investigated the effects of inorganic trace elements (IEs) and sucrose chelated trace elements (SEs) on the growth performance, nutrients and energy utilization, antioxidant capacity, and mineral deposition in broiler chickens, and the efficiency of IEs replaced by SEs at different levels was also evaluated. A total of 448,21-day-old male Arbor Acres broiler chickens with similar body weights were randomly assigned into 6 dietary treatments (8 cages/treatments) in a complete randomized design. Treatments were a basal diet including 2.0 g/kg of IE (IE-2.0) premix, and SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 were basal diets in which IEs were replaced by SE premix at 2.0, 1.5, 1.0, 0.5, and 0 g/kg, respectively. In general, there was a linear and quadratic decrease in growth performance including average daily feed intake (ADFI) and average daily gain (ADG), apparent and true availability of nutrients (DM, OM, and CP), GE, trace elements (Cu, Zn, Mn, Fe, I, and Se), essential AA (Lys, Met, Arg, His, Phe, Thr, and Val), non-essential AA (Asp, Ser, Glu, Gly, and Cys), superoxide dismutase (SOD), and trace elements (Fe, Zn, Cu, and Mn) in the liver, and an increase in feed-to-gain ratio (F/G) and liver malondialdehyde (MDA), with decreasing SE levels ($p < 0.05$). In conclusion, under the conditions of this experiment, using half of the sucrose chelated trace elements (Cu, Fe, Zn, and Mn) instead of inorganic trace elements did not affect the growth performance, nutrients and energy utilization, antioxidant capacity, and liver trace element deposition in broiler chickens.

Keywords: sucrose chelated trace elements; growth performance; mineral deposition; nutrient availability

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1. Introduction

Trace elements are essential nutrients to improve the growth performance, reproduction, and immunity of fast-growing broiler chickens [1]. It has been well known that trace elements are cofactors of enzymes that participate in hormone secretion and the immune defense system [2]. Additionally, trace elements are involved in bone development, feathering, and regulating the appetites of broiler chickens [3]. It has been reported that the deficiencies of trace elements such as iron [4], zinc [5], copper [6], and manganese [7] increased the risks of anemia, parakeratosis, critical dysfunctions, bone abnormalities, or growth retardation in poultry. According to the recommendations of the National Research Council [8], excessive or high levels of inorganic trace elements are supplemented into poultry diets to ensure birds reach their genetic growth potential and prevent diseases.

However, excessive or high levels of inorganic elements to maximize birds' performance may result in excessive mineral excretion due to low availability, which can be harmful to the environment and cause wastes of nutrients [9].

The supplementations of organic trace elements in poultry diets have been proven to minimize the adverse effects of inorganic elements due to their better bioavailability [10,11]. Previous studies reported that organic trace minerals could improve growth performance, enhance immunity, reduce trace element excretions, and possess antioxidant and antimicrobial abilities [12–15]. Sucrose chelated multi-elements (SEs) are new organic trace elements prepared from sugar cane molasses and chelated with copper sulfate, zinc, manganese, and iron. We proposed that the matrix of carbohydrates of SEs could isolate the trace elements from the environment, minimizing mineral excretion without compromising the growth performance and nutrient digestibility. In this study, the effectiveness of sucrose chelated trace elements on growth performance, nutrient availabilities, contents of minerals in livers, and antioxidative enzyme activities were evaluated in broiler chickens.

2. Materials and Methods

2.1. Trace Elements Premix

The inorganic trace element (IE) premix includes 4 g/kg of Cu ($CuSO_4 \cdot 5H_2O$), 35 g/kg of Zn ($ZnSO_4 \cdot H_2O$), 45 g/kg of Mn ($MnSO_4 \cdot H_2O$), 25 g/kg of Fe ($FeSO_4 \cdot H_2O$), 250 mg/kg of I (KIO_3), and 150 mg/kg of Se (Na_2SeO_3).

The sucrose chelated trace element (SE) premix includes 4 g/kg of Cu (sucrose chelated Cu, $C_{12}H_{22}O_{15}SCu$), 35 g/kg of Zn (sucrose chelated Zn, $C_{12}H_{22}O_{15}SZn$), 45 g/kg of Mn (sucrose chelated Mn, $C_{12}H_{22}O_{15}SMn$), 25 g/kg of Fe (sucrose chelated Fe, $C_{12}H_{22}O_{15}SFe$), 250 mg/kg of I (KIO_3), and 150 mg/kg of Se (Na_2SeO_3).

SE and IE premixes were both provided by Nanning Zeweier Feed Co., Ltd. (Nanning, China) and all materials used were of feed grade. The zeolite powder provided by Taihang mineral feed premix Co., LTD (Taian, China) was selected for the diluent.

2.2. Experimental Design

A total of 448,21-day-old male Arbor Acres broiler chicks with similar body weight (BW) (953.21 $\pm$ 26.97 g) were purchased from a commercial chicken farm (Xiling Family Farm, Tai'an, China), individually weighed, and then randomly distributed into 6 dietary treatments with 8 replicates (8 birds per replicate/cage) in a completely randomized design: basal diet including 2.0 g/kg of IEs (IE-2.0), and the SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 treatments were IE-2.0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively. Zeolite powder was used to supplement the insufficient quantity in the formula. The rest of the 64 (8 replicates, 8 birds per replicate/cage) birds were randomly assigned for endogenous measurement.

The basal diet and nutrient levels were formulated according to the standards of Arbor Acres growing broilers (Feeding Standard of Chicken of the People's Republic of China, NY/T 33-2004 [16], Table 1). To ensure the consistency of experimental diets, all diets were formulated and manufactured 1 week prior to the trial and stored in sealed containers at approximately 9 °C. The nutrient components of all feeds were analyzed according to the methods described by the AOAC [17] and the trace elements were measured by flame atomic absorption spectrometry (GB 2009.90-2016).

Table 1. Compositions and nutrient levels of basal diet (DM basis) %.

Items	Ingredients	Nutrient Levels [2]	Value
Corn	64.45	ME, MJ/kg	12.77
Soybean meal	25.10	CP	19.34
Soybean oil	2.50	Ca	0.79
Corn gluten powder	3.75	Available phosphorus	0.32
$CaHPO_4$	1.20	Lys	1.00
Limestone	1.20	Met	0.40
NaCl	0.30	Met+Cys	0.71
L-Lys	0.20		
Met	0.10		
Inorganic trace elements premix	0.20		
Premix [1]	1.00		
Total	100.00		12.77

[1] The premix provided the following per kg of the diet: VA 11 500 IU; VD_3 3 500 IU; VE 30 mg; VK_3 3 mg; VB_1 3.38 mg; VB_2 9.00 mg; VB_6 8.96 mg; VB_{12} 0.025 mg; choline chloride 800 mg; calcium pantothenate 13 mg; niacin 45 mg; biotin 0.08 mg; folic acid 1.20 mg. [2] Metabolizable energy was calculated according to the total excreta collections method, crude protein and calcium were analyzed values, and the other nutrient levels were calculated values.

2.3. Animals and Management

Birds from each replicate (8 birds) were housed in individual wire cages equipped with water troughs and feeders in an environmentally controlled house. The temperature was maintained at 20–22 °C and the relative humidity was kept at about 65% during the experimental period. All birds were vaccinated according to the normal immunization program and had ad libitum access to feed (except the endogenous treatment) and water under a 23-hs-on-1-h-off lighting regime. The experiment spanned 28 days after the 7 days of adaptation (21 d to 27 d of age). All birds were inspected at least twice per day and any mortalities or culls were removed or sacrificed in accordance with the guidelines for the care and use of laboratory animals prescribed by the Animal Nutrition Research Institute of Shandong Agricultural University and the Ministry of Agriculture of China. The BW was measured at the end of the adaptation period (28 d) and test period (55 d), feed intake was recorded daily from each cage, and the average daily gain (ADG, g), average daily feed intake (ADFI, g) and feed-to-gain ratio (F/G) were calculated.

2.4. Sample Collections

The nutrient availability experiment was determined based on the total excreta collecting methods by collecting trays. All excreta from 8 birds per cage were collected continuously on d 30–33. Before the collections for endogenous measurement, all selected birds were fasted for 24 h with only access to water, and feces were then collected for the next 48 h. Feathers and shredded dry skin in excreta were removed carefully, and then excreta were weighed, pooled by replicate, and stored at −20 °C until analysis.

2.5. Nutrient Availability

The excreta samples were dried at 65 °C for 72 h, and the dried samples were finely ground using a mortar and pestle, passed through a 1 mm screen, and then stored in sealed containers for the subsequent analysis of dry matter (DM), organic matter (OM), crude protein (CP), gross energy (GE), and trace element according to AOAC (2012). The CP was analyzed by the Kjeldahl method and calculated based on nitrogen content (CP = nitrogen × 6.25). The DM was analyzed by drying at 103 ± 2 °C for 72 h, the OM was determined by 550 °C ash in a muffle furnace (SX2-4-10; Longkou electric furnace manufacturer, Yantai, China), and the GE was determined using the Parr adiabatic bomb calorimeter (Model 6200, Parr Instruments Co., Moline, IL, USA) [18]. After freeze-drying, samples for amino acid (AA, except tryptophan) analysis were measured using an automatic amino acid analyzer (Hitach-835; Hitachi Limited, Japan) by high-performance liquid chromatography (HPLC) according to the method described by [19]. Trace elements were

analyzed by flame atomic absorption spectrophotometry (SpectrAA 220, Mulgrave, Victoria, Australia) according to [20]. All analyses were performed in triplicate. The measured dietary trace elements are shown in Table 2. And the measured values of trace elements in diets satisfy the feeding standard of Chicken of People's Republic of China, NY/T 33-2004 (Supplementary Table S1).

Table 2. Measured values of trace elements in diets (DM basis) mg/kg.

Treatments [1]	Cu	Zn	Mn	Fe	I	Se
IE-2.0	13.84 ± 0.13	135.2 ± 0.14	127.5 ± 0.13	248.7 ± 0.19	1.35 ± 0.01	0.48 ± 0.01
SE-2.0	13.72 ± 0.15	138.2 ± 0.13	134.3 ± 0.14	259.4 ± 0.21	1.46 ± 0.01	0.51 ± 0.02
SE-1.5	12.61 ± 0.12	125.3 ± 0.12	108.6 ± 0.11	238.9 ± 0.22	1.40 ± 0.01	0.36 ± 0.01
SE-1.0	10.73 ± 0.11	109.3 ± 0.11	88.6 ± 0.09	224.3 ± 0.21	1.39 ± 0.01	0.25 ± 0.01
SE-0.5	8.37 ± 0.09	89.5 ± 0.08	64.8 ± 0.07	212.6 ± 0.20	1.25 ± 0.01	0.22 ± 0.01
SE-0	5.92 ± 0.05	70.7 ± 0.06	39.1 ± 0.04	198.1 ± 0.20	0.95 ± 0.01	0.13 ± 0.01

[1] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder was added to supplement the insufficient quantity in the formula.

The apparent and true availabilities of DM, OM, CP, GE, AA, and trace elements were calculated using the following equations:

$$\text{Apparent availability} = [(\text{TNI} - \text{TNE})/\text{TNI}] \times 100, \text{ and}$$

$$\text{True availability} = [(\text{TNI} - \text{TNE} + \text{TNEE})/\text{TNI}] \times 100,$$

where TNI was the total nutrient intake (g) of DM, OM, CP, GE, AA, and trace elements daily, TNE was the total nutrients in excreta of DM, OM, CP, GE, AA, and trace elements daily, and TNEE was the total nutrients in endogenous excreta of DM, OM, CP, GE, AA, and trace elements daily.

2.6. Liver Trace Minerals and Antioxidant Enzymes

At the end of the experiment (d 55), 8 birds (1 per replicate) were randomly selected from each treatment and killed by cervical dislocation after fasting for 24 h. Livers from each bird were immediately collected and kept at −20 °C for analyzing trace minerals and antioxidant enzymes. About 100 g of liver samples were used for analyzing trace mineral contents including iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) by inductively coupled plasma–atomic emission spectrometry (ICP-AES, Optima 8300, Perkin Elmer, Waltham, MA, USA), which has been well described by [21].

Approximately 10 g of liver samples were used for evaluating the antioxidant enzyme activity of superoxide dismutase (T-SOD) and the content of malondialdehyde (MDA) described by [22]. Briefly, the activity of SOD was measured by a SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), which was determined by the amount of SOD required to obtain 50% inhibition of the rate of nitrite production measured by a spectrophotometer (UV-2000, Unico Instruments Co. Ltd., Shanghai, China) at optical density (OD_{550}) [18]. Additionally, the concentration of MDA was determined by an MDA Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The principle of MDA measurement was the reaction between Thiobarbituric acid (TBA) and MDA to generate a stable pink color that is evaluated by a spectrophotometer (UV-2000, Unico Instruments Co., Ltd., Shanghai, China) at OD_{532} [23].

2.7. Statistical Analysis

The data were analyzed as a completely randomized design and cages were considered as the experimental units. All data were evaluated by one-way ANOVA using PROC GLM followed by Tukey's multiple comparison test (SAS 9.4) with the model $Yi_j = \mu + T_i + e_{ij}$, where μ is the total means, *Ti* is the fixed treatment effects (i = 1, 2, 3, 4, 5, 6), and *eij* is the

residual of the model. Orthogonal polynomial contrasts were used to analyze the linear and quadratic effects of SE levels on growth performance and nutrient availability. A *p*-value less than or equal to 0.05 was used to declare significance.

3. Results

3.1. Growth Performance

In general, there was a linear and quadratic decrease in ADFI and ADG and increase in F/G with the decrease in SE levels ($p < 0.05$) (Table 3). Only SE-0 decreased ADFI and ADG, and increased F/G compared to IE-2.0 ($p < 0.05$). Interestingly, birds fed a lower SE level (SE0.5 and SE-1.0) had similar effects on ADFI, ADG, and F/G compared to birds fed higher IE (IE-2.0) levels ($p > 0.05$).

Table 3. Effects of different trace element levels on the growth performance of broiler chickens [1].

Treatments [2]		ADFI, g	ADG, g	F/G
IE-2.0		150.76 [a]	77.31 [a]	1.95 [b]
SE-2.0		151.68 [a]	79.01 [a]	1.92 [b]
SE-1.5		151.00 [a]	77.44 [a]	1.95 [b]
SE-1.0		150.77 [a]	76.15 [a]	1.98 [b]
SE-0.5		145.37 [ab]	71.97 [ab]	2.02 [ab]
SE-0		144.34 [b]	66.82 [c]	2.16 [a]
SEM [3]		1.328	0.582	0.02
p-value				
Treatment		0.037	0.041	0.071
SE	Linear	<0.001	0.022	0.016
	Quadratic	<0.001	0.031	0.023

ADG = Average daily gain; ADFI = Average daily feed intake; F/G = ADFI, g/ADG, g. [1] Data are means for 8 replicates of 4 broilers per replicate. [2] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [3] Total standard error of the means. [a,b,c] Means within a column with different letters are significantly different ($p < 0.05$).

3.2. Apparent and True Availability of Nutrients and Gross Energy

Overall, with the decrease in SE levels, the apparent and true availability of DM, OM, CP, and GE decreased linearly and quadratically ($p < 0.05$) (Table 4). The SE-0 decreased the apparent and true availability of DM, OM, CP, and GE compared to IE-2.0 ($p < 0.05$), and SE-0.5 and SE-1.0 only decreased the apparent and true availability of OM compared to SE-2.0 ($p < 0.05$). Furthermore, birds fed a lower SE level (SE-1.0) had similar effects on the apparent and true availability of DM, OM, CP, and GE compared to birds fed higher IE (IE-2.0) levels ($p > 0.05$).

Table 4. Effects of different trace element levels on the availability of nutrients and gross energy of broiler chickens [1] %.

Treatments [2]	Apparent Availability				True Availability			
	DM	OM	CP	GE	DM	OM	CP	GE
IE-2.0	80.76 [a]	81.13 [ab]	65.27 [a]	81.1 [a]	84.16 [a]	84.65 [ab]	67.73 [a]	84.47 [a]
SE-2.0	81.68 [a]	82.43 [a]	66.25 [a]	82.23 [a]	85.28 [a]	86.11 [a]	68.83 [a]	85.74 [a]
SE-1.5	81.00 [a]	81.23 [ab]	65.85 [a]	81.12 [a]	84.69 [a]	84.91 [ab]	68.34 [a]	84.48 [a]
SE-1.0	80.77 [a]	80.18 [b]	64.86 [a]	80.58 [a]	84.33 [a]	83.68 [b]	67.31 [a]	83.93 [a]
SE-0.5	79.37 [ab]	80.07 [b]	62.22 [ab]	79.68 [ab]	82.82 [ab]	83.65 [b]	64.65 [ab]	83.09 [ab]
SE-0	77.34 [b]	77.90 [c]	59.86 [b]	78.48 [b]	80.85 [b]	81.43 [c]	62.18 [b]	81.79 [b]
SEM [3]	0.328	0.201	0.544	0.326	0.338	0.209	0.563	0.339

Table 4. *Cont.*

Treatments [2]		Apparent Availability				True Availability			
		DM	OM	CP	GE	DM	OM	CP	GE
p-value									
Treatment		0.011	<0.001	0.017	0.048	0.012	<0.001	0.017	0.047
SE	Linear	<0.001	<0.001	0.006	0.006	<0.001	<0.001	0.006	0.006
	Quadratic	<0.001	<0.001	0.002	0.003	<0.001	<0.001	0.002	0.003

DM = dry matter; OM = organic matter; CP = crude protein; GE = gross energy. [1] Data are means for 8 replicates of 4 broilers per replicate. [2] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [3] Total standard error of the means. [a,b,c] Means within a column with different letters mean significantly different ($p < 0.05$).

3.3. Apparent and True Availability of Trace Minerals

Generally, the apparent and true availabilities of Cu, Zn, Mn, Fe, I, and Se decreased linearly and quadratically with decreasing SE levels ($p < 0.05$) (Table 5). Birds fed SE-1.5, SE1.0, SE-0.5, and SE-0 decreased the apparent and/or true availability of Cu, Zn, Mn, and Fe compared to IE-2.0 ($p < 0.05$); however, SE-2.0 increased the apparent and true availability of Cu, Mn, and Se ($p < 0.05$). Interestingly, birds fed a lower SE level (SE-0.5) had similar effects on the apparent and true availability of I compared to birds fed higher IE (IE-2.0) levels ($p > 0.05$).

Table 5. Effects of different trace element levels on the availability of trace elements of broiler chickens [1] %.

Treatments [2]		Apparent Availability						True Availability					
		Cu	Zn	Mn	Fe	I	Se	Cu	Zn	Mn	Fe	I	Se
IE-2.0		46.10 [b]	57.14 [a]	55.24 [b]	50.0 [a]	78.75 [b]	87.55 [b]	47.18 [b]	58.51 [a]	57.01 [b]	51.18 [a]	80.73 [b]	90.08 [b]
SE-2.0		49.52 [a]	58.68 [a]	60.14 [a]	50.34 [a]	78.32 [b]	89.35 [a]	50.76 [a]	60.18 [a]	62.15 [a]	51.61 [a]	80.39 [b]	92.06 [a]
SE-1.5		42.98 [c]	50.96 [b]	48.36 [c]	38.83 [b]	77.04 [b]	86.22 [b]	44.17 [c]	52.36 [b]	50.08 [c]	39.87 [b]	79.27 [b]	89.02 [b]
SE-1.0		34.89 [d]	43.51 [c]	39.71 [d]	34.58 [c]	78.41 [b]	83.57 [c]	35.80 [d]	44.60 [c]	41.02 [d]	35.43 [c]	80.47 [b]	86.07 [c]
SE-0.5		25.12 [e]	34.37 [d]	24.06 [e]	33.32 [c]	78.23 [b]	82.08 [c]	25.75 [e]	35.27 [d]	24.87 [e]	34.19 [c]	80.31 [b]	84.58 [c]
SE-0		7.38 [f]	25.59 [e]	−1.42 [f]	31.44 [c]	80.64 [a]	74.77 [d]	7.59 [f]	26.38 [e]	−1.47 [f]	32.33 [c]	82.98 [a]	77.09 [d]
SEM [3]		0.364	0.554	0.443	0.531	0.323	0.229	0.381	0.569	0.457	0.544	0.334	0.234
p-value													
Treatment		<0.001	<0.001	<0.001	<0.001	0.049	<0.001	<0.001	<0.001	<0.001	<0.001	0.042	<0.001
SE	Linear	<0.001	<0.001	<0.001	<0.001	0.029	<0.001	<0.001	<0.001	<0.001	<0.001	0.024	<0.001
	Quadratic	<0.001	<0.001	<0.001	<0.001	0.013	<0.001	<0.001	<0.001	<0.001	<0.001	0.009	<0.001

[1] Data are means for 8 replicates of 4 broilers per replicate. [2] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [3] Total standard error of the means. [a,b,c,d,e,f] Means within a column with different letters are significantly different ($p < 0.05$).

3.4. Apparent and True Availability of Amino Acids

For essential AA, there was a linear and quadratic decrease in the apparent and true availability of Lys, Met, Arg, His, Phe, Thr, and Val with the decrease in SEs ($p < 0.05$) (Table 6). Birds fed SE-0 decreased the apparent and/or true availability of Lys, Met, Arg, Thr, and Val compared to IE-2.0 ($p < 0.05$); however, SE-2.0 increased the apparent availability of Phe and the true availability of Lys and Phe ($p < 0.05$). Interestingly, birds fed a lower SE level (SE-0.5) had similar effects on the apparent and true availability of essential AA compared to birds fed higher IE (IE-2.0) levels ($p > 0.05$).

Table 6. Effects of different trace element levels on the availability of essential amino acids of broiler chickens [1] %.

Treatments [2]		Apparent Availability							True Availability						
		Lys	Met	Arg	His	Phe	Thr	Val	Lys	Met	Arg	His	Phe	Thr	Val
IE-2.0		89.48 ^{a}	88.95 ^{a}	89.33 ^{ab}	90.76 ^{ab}	89.36 ^{b}	83.01 ^{ab}	85.70 ^{ab}	90.74 ^{b}	89.74 ^{a}	90.78 ^{ab}	92.06 ^{ab}	90.99 ^{b}	84.47 ^{ab}	87.60 ^{ab}
SE-2.0		90.77 ^{a}	90.58 ^{a}	90.09 ^{a}	91.72 ^{a}	91.37 ^{a}	84.94 ^{a}	87.14 ^{a}	92.25 ^{a}	91.49 ^{a}	91.54 ^{a}	93.09 ^{a}	92.90 ^{a}	86.13 ^{a}	89.05 ^{a}
SE-1.5		89.55 ^{a}	89.54 ^{a}	89.59 ^{ab}	90.25 ^{b}	89.81 ^{b}	83.57 ^{ab}	85.13 ^{ab}	91.00 ^{b}	90.52 ^{a}	91.04 ^{ab}	91.71 ^{b}	91.24 ^{b}	84.87 ^{ab}	86.86 ^{ab}
SE-1.0		89.87 ^{a}	89.59 ^{a}	89.18 ^{ab}	90.50 ^{b}	90.06 ^{ab}	82.48 ^{ab}	85.74 ^{ab}	91.23 ^{ab}	90.10 ^{a}	90.63 ^{b}	91.97 ^{ab}	91.51 ^{b}	83.86 ^{ab}	87.26 ^{ab}
SE-0.5		89.84 ^{a}	89.31 ^{a}	88.92 ^{b}	90.29 ^{b}	89.71 ^{b}	81.32 ^{bc}	84.70 ^{b}	91.33 ^{ab}	90.25 ^{a}	90.71 ^{ab}	91.67 ^{b}	90.96 ^{b}	82.65 ^{bc}	85.91 ^{bc}
SE-0		88.05 ^{b}	84.36 ^{b}	87.88 ^{c}	90.07 ^{b}	89.16 ^{b}	79.44 ^{c}	84.21 ^{b}	89.47 ^{c}	85.34 ^{b}	89.72 ^{c}	91.63 ^{b}	90.27 ^{b}	80.64 ^{c}	85.04 ^{c}
SEM [3]		0.173	0.242	0.128	0.151	0.183	0.335	0.265	0.158	0.232	0.109	0.158	0.179	0.333	0.263
p-value															
Treatment		0.006	<0.001	0.001	0.051	0.029	0.002	0.06	0.002	<0.001	0.003	0.11	0.008	0.002	0.004
SE	Linear	0.001	<0.001	<0.001	0.006	0.002	<0.001	0.002	0.003	<0.001	<0.001	0.012	0.001	<0.001	<0.001
	Quadratic	0.002	<0.001	<0.001	0.007	0.005	<0.001	0.007	0.001	<0.001	0.001	0.012	0.006	<0.001	0.003

[1] Data are means for 8 replicates of 4 broilers per replicate. [2] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [3] Total standard error of the means. ^{a,b,c} Means within a column with different letters are significantly different ($p < 0.05$).

For non-essential AA, linear and quadratic decreases in the apparent and true availability of Asp, Ser, Glu, Gly, and Cys were observed with decreasing SE ($p < 0.05$) (Table 7). Compared to IE-2.0, an increased apparent availability of Glu and true availability of Glu and Gly were observed in SE-2.0 ($p < 0.05$); however, a decreased apparent and/or true availability of Ser and Glu in SE-0.5 and SE-0, and Cys and Tyr in SE-0 were observed ($p > 0.05$). Overall, birds fed a lower SE level (SE-1.0) had similar effects on the apparent and true availability of non-essential AA compared to birds fed higher IE (IE-2.0) levels ($p > 0.05$).

3.5. Antioxidant Enzyme Activities and Liver Mineral Deposition Efficiency

In general, there was a linear and quadratic increase in liver MDA and a decrease in SOD with the decrease in SE levels ($p < 0.05$) (Figure 1). Birds fed SE-0.5 and SE-0 decreased liver SOD compared to IE-2.0 ($p < 0.05$); however, an increased liver SOD in SE-2.0 and SE-1.5 and decreased MDA in ZE-2.0 were observed ($p < 0.05$).

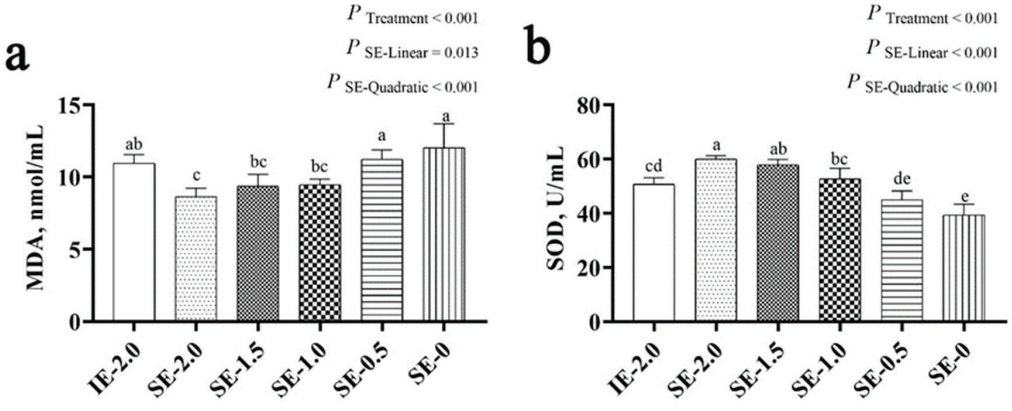

Figure 1. Effect of sucrose chelated organic trace elements on liver MDA and SOD of broilers ($n = 8$). (**a**) Contents of MDA in the liver. (**b**) Activities of SOD in the liver. The activity of MDA and SOD was expressed as units per mL protein of the liver. One unit of SOD was defined as the amount of SOD required to inhibit 50% of the rate of nitrite production at 37 °C. One unit of MDA was defined as the amount of MDA required to generate a stable pink color after reaction with thiobarbituric acid. IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. ^{a,b,c,d,e} Means within a row with different letters are significantly different ($p < 0.05$).

Overall, with decreasing SE levels, the contents of Fe, Zn, Cu, and Mn in the liver decreased linearly and quadratically ($p < 0.05$) (Figure 2). Compared to IE-2.0, decreased liver Fe, Zn, and Mn in SE-0.5 and SE-0 were observed ($p < 0.05$), and only decreased Cu in SE-0 was observed ($p > 0.05$). Interestingly, SEs at lower levels (SE-1.0) had similar effects on the contents of liver Fe, Zn, Cu, and Mn compared to higher IE (IE-2.0) levels ($p > 0.05$).

Table 7. Effects of different trace element levels on the availability of non-essential amino acids of broiler chickens [1] %.

Treatments [2]		Apparent Availability							True Availability						
		Asp	Ser	Glu	Pro	Gly	Cys	Tyr	Asp	Ser	Glu	Pro	Gly	Cys	Tyr
IE-2.0		89.29 ^{ab}	87.83 ^{a}	91.07 ^{b}	89.3	83.06 ^{bc}	90.39 ^{ab}	89.22 ^{a}	90.48 ^{ab}	89.66 ^{ab}	92.78 ^{b}	91.16	85.13 ^{ab}	92.39 ^{ab}	91.60 ^{a}
SE-2.0		90.41 ^{a}	89.03 ^{a}	92.24 ^{a}	89.77	85.31 ^{a}	90.67 ^{a}	89.19 ^{a}	91.72 ^{a}	90.86 ^{a}	94.04 ^{a}	91.71	87.34 ^{a}	92.34 ^{ab}	91.54 ^{a}
SE-1.5		89.63 ^{a}	87.23 ^{ab}	90.96 ^{b}	89.5	85.55 ^{a}	89.03 ^{bc}	87.68 ^{b}	90.94 ^{ab}	89.03 ^{b}	92.84 ^{b}	91.84	86.73 ^{a}	90.81 ^{bc}	89.85 ^{b}
SE-1.0		89.17 ^{ab}	87.55 ^{ab}	90.28 ^{bc}	88.81	84.89 ^{ab}	89.20 ^{bc}	88.20 ^{ab}	90.37 ^{ab}	89.36 ^{ab}	92.15 ^{bc}	90.35	86.07 ^{a}	91.09 ^{bc}	90.16 ^{ab}
SE-0.5		89.17 ^{ab}	85.95 ^{b}	89.31 ^{c}	88.83	84.25 ^{abc}	89.35 ^{bc}	88.64 ^{ab}	90.51 ^{ab}	88.06 ^{b}	91.07 ^{c}	90.49	85.48 ^{ab}	91.22 ^{bc}	90.31 ^{ab}
SE-0		87.97 ^{b}	84.15 ^{c}	87.84 ^{d}	88.97	82.43 ^{c}	87.85 ^{c}	87.94 ^{b}	89.39 ^{b}	86.30 ^{c}	89.75 ^{d}	90.91	83.59 ^{b}	89.58 ^{c}	89.30 ^{b}
SEM [3]		0.197	0.241	0.154	0.177	0.283	0.182	0.189	0.193	0.229	0.165	0.176	0.298	0.174	0.186
p-value															
Treatment		0.045	0.001	<0.001	0.554	0.021	0.002	0.122	0.05	0.002	<0.001	0.11	0.022	0.001	0.008
SE	Linear	0.004	<0.001	<0.001	0.065	0.001	0.008	0.276	0.004	<0.001	<0.001	0.027	0.001	0.001	0.058
	Quadratic	0.002	<0.001	<0.001	0.104	0.001	0.004	0.365	0.002	<0.001	<0.001	0.030	0.004	0.005	0.125

[1] Data are means for 8 replicates of 4 broilers per replicate. [2] IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [3] Total standard error of the means. ^{a,b,c} Means within a column with different letters are significantly different ($p < 0.05$).

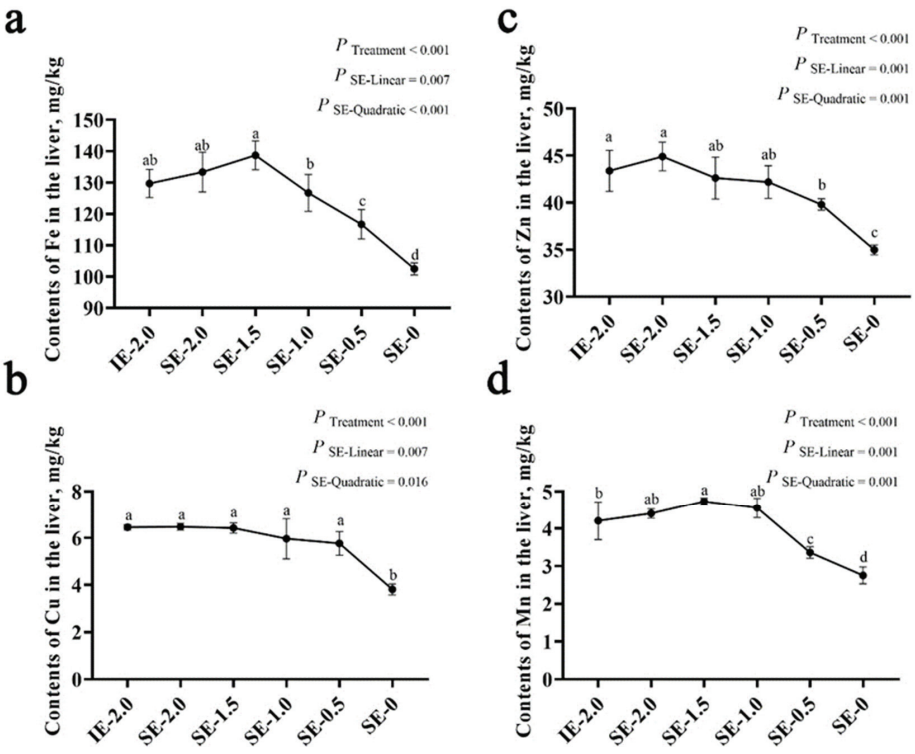

Figure 2. Effects of sucrose chelated organic trace elements on contents of Fe, Cu, Zn, and Mn in the liver of broilers (n = 8). (**a**) Contents of Fe in the liver. (**b**) Contents of Zn in the liver. (**c**) Contents of Cu in the liver. (**d**) Contents of Mn in the liver. IE-2.0: basal diet (2.0 g/kg of inorganic trace element premix); SE-2.0, SE-1.5, SE-1.0, SE-0.5, and SE-0 in which IEs were replaced with 2.0, 1.5, 1.0, 0.5, and 0 g/kg of SEs, respectively, and 0, 0.5, 1.0, 1.5, and 2.0 g/kg of zeolite powder were added to supplement the insufficient quantity in the formula. [a,b,c,d] Means within a row with different letters are significantly different ($p < 0.05$).

4. Discussion

Trace elements are indispensable nutrients in poultry, which are required for promoting growth performance and regulating bone development, appetite, and feathering [24]. However, higher levels of trace elements are supplemented to diets to obtain a better growth performance, while the waste cannot be ignored. Therefore, on the basis of meeting the standard required by the feeding standard of Chicken of People's Republic of China, NY/T 33-2004, we chose several lower levels of SEs (0.5, 1.0, 1.5 or 2.0 g/kg) to find a better solution. It has been reported that broiler chickens supplemented with methionine hydroxy analog chelated manganese (50 mg Mn/kg diet) increased the BW and ADFI compared to those fed the basal diet [25]. However, studies by [26] demonstrated that the feed supplementation of methionine and chelate (1 g methionine chelate/kg diet) including Cu, Fe, Zn, and Mn had no beneficial improvements on the growth performance of broiler chickens. Similarly in our study, there were no significant impacts of SEs (0.5, 1.0, 1.5, 2.0 g/kg feed) and IEs (2.0 g/kg feed) on the ADFI, ADG, and F/G of broiler chickens. Our study at least indicated that the supplementation of SEs at low levels (0.5, 1.0, 1.5 g/kg) could make the birds achieve the same performance as IEs at a high level (2.0 g/kg), which is consistent with those of previous studies [27,28]. This demonstrated that SEs could reduce amounts of in-feed mineral supplementations, which may reduce the amount of excreted minerals and

alleviate environmental pollutions. The promised performance could be due to improved nutrient digestibility and absorptions by oligosaccharides in SE [29].

Impacts of different levels of SEs on the availabilities of DM, OM, CP, and GE were evaluated in this study since nutrient availability is an important parameter correlated with growth performance [30]. In our study, feeding birds at the lower SE level (0.5 g/kg) could achieve similar nutrient availabilities when compared to birds fed higher levels of SEs (1.0, 1.5, 2.0 g/kg) and IEs (2.0 g/kg), indicating that organic trace elements could improve nutrient availabilities, which are consistent with previous studies [31,32]. This may be due to the improved gut health of broiler chickens by the supplementation of oligosaccharides in organic trace minerals. It has been reported that organic trace minerals reduced crypt depth, which is related to greater intestinal maturity [33]. The lower crypt depth in broiler chickens is an indicator of decreased requirements of nutrients for maintenance and production that could promote nutrient availability and growth performance [34]. Additionally, previous reports have indicated that organic trace minerals reduced the number of intestinal goblet cells [35]. Goblet cells are responsible for mucin production for the defense of intestinal microorganisms, but it could also lead to reductions in nutrient absorption [36]. Additionally, the improved nutrient availabilities may be due to the modulation of microbiota in the small intestines by organic trace minerals. Gut microorganisms are an essential layer of the brush border with an ability to absorb nutrients, prevent infectious diseases, and enhance immune systems [37]. Interestingly, improved richness and diversity of the chicken intestinal microbiota by organic trace elements have been reported previously [38]. The products for chelating the trace minerals in our research were oligosaccharides. Oligosaccharides that can be fermented by gut microbiota have been well known for enhancing growth performance, elevating nutrient digestibility and absorption, and increasing the relative abundance of beneficial bacterial species such as *Lactobacillus crispatus* and *Anaerostipes butyraticu* [39,40]. Despite this, analyses on gut microbiota are still necessary in further experiments to understand the true mechanisms of SEs on nutrient availability.

In addition to nutrient and energy availabilities, the effects of sucrose chelated organic trace elements on the availabilities of minerals and amino acids were evaluated in our study. Antagonistic interactions of inorganic trace minerals can cause low availabilities of minerals, which are often reflected in compromised growth performance, increased mineral excretions, and an elevated economic loss and environmental pollution in poultry farms [41]. It has been reported that the organic trace minerals may be better digested and absorbed compared to inorganic elements due to their minimal interacts with antagonists in chicken feeds such as sulfur in dried distillers' grains (DDGs) and iron contamination in corns [42]. This could explain the similar digestibility of minerals when birds were fed low levels of SEs (0.5 g/kg) compared to higher levels of SEs (1.0, 1.5, 2.0 g/kg) and IEs (2.0 g/kg). Another interesting finding of this study was the similar amino acid digestibility when birds were fed low levels of SEs (0.5 g/kg) compared to higher levels of SEs (1.0, 1.5, 2.0 g/kg) and IEs (2.0 g/kg). The improved mineral and amino acid digestibility when fed SEs may also be due to the increased stabilities in the gastrointestinal tract and improved antioxidant activities in broiler chickens [43,44]. Another explanation is that the fermentation of oligosaccharides to short-chain fatty acids in the intestines could be beneficial to digestive enzyme activities, gut morphology, and diversity of microbiota [45].

Commercial broiler chickens in intensive poultry systems at high stock density are sensitive to oxidative stressors, inducing compromised growth performance, gut health, and meat quality [46]. The MDA is a product of lipid peroxidation, and its accumulation is the biomarker for reflecting oxidative stress [47]. The SOD is an enzymatic scavenger that neutralizes the reactive oxygen species (ROS), and its concentration can be used to reflect antioxidant status in poultry [48]. Additionally, the liver is an organ that is particularly susceptible to oxidative stress for inducing liver disorders [49]. The results from this study demonstrated that the full replacement of IEs by SEs could increase concentrations of SOD and reduce MDA in livers of broiler chickens, which is consistent with some recent studies [32,50]. Additionally, birds fed a lower level of SEs (1.5 g/kg) exert a comparable or

even better antioxidant status than IEs at a higher level (2.0 g/kg). This corresponds to a recent study showing that the replacement of IEs by lower levels of SEs increased activities of liver SOD [32]. The elevated antioxidant enzymes could be due to oligosaccharides in SEs that alleviate antioxidant stress [51]. Trace elements can deposit in broiler chicken organs such as the liver, kidney, bone, and pancreas. The contents of trace elements in the liver can reflect the biological efficacy of dietary trace minerals in broiler chickens [52]. A higher Mn concentration was shown in birds fed SEs at a lower level (1.5 g/kg) compared to IEs at a higher level (2.0 g/kg). Mn is an important activator of essential enzymes such as hydrolases and arginase, involved in many crucial nutrient metabolisms and playing significant roles in the antioxidant system [53]. This may also explain the increased concentrations by SEs compared to IEs.

5. Conclusions

The present study indicated that in broiler chicken diets, feed supplementations of SEs at lower levels (1.0 or 1.5 g/kg) could reach the same results of growth performance and nutrient digestibility compared to high levels of SEs (2.0 g/kg) and IEs (2.0 g/kg). Additionally, the full replacement of IEs (2.0 g/kg) by SEs (2.0 g/kg) reduced MDA and increased SOD. This indicated that our advanced chelating technology can reduce trace element requirements of broiler chickens. This may suggest that SEs can reduce the excretion of minerals in broiler chickens, which may reduce environmental pollution. However, further investigations are still necessary to elucidate the mechanisms of the beneficial effects of SEs on growth performance and nutrient digestibility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13071369/s1, Table S1: Trace element Required of Broilers.

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Institutional Review Board Statement: In this experiment, the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University (approval number: SDAUA-2021-0410; date of approval: 10 April 2021).

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The Impact of Different Environments on Productive Performance, Welfare, and the Health of Muscovy Ducks during the Summer Season

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Abstract: The objective of this research was to evaluate the influence of the housing system (deep litter [DL] vs. deep litter with swimming pond [DLSP]) on productive performance, carcass traits, body temperature, blood profile, and the element composition of the femur and tibia in Muscovy ducks. At 5 weeks of age, sexed ducklings (264) were divided into 4 equal groups according to housing system and gender (drakes vs. ducks). The groups were as follows: 66 drakes/DL, 66 drakes/DLSP, 66 ducks/DL, and 66 ducks/DLSP. Each of the four groups was divided into three identical replicated subgroups of 22 animals. Regarding external body temperature, the DL birds had higher temperatures compared with the DLSP birds. In addition, drakes had lower temperature values than ducks. Regarding the blood analysis, the birds did not manifest any deviations in the biochemical traits of the blood. The DLSP birds had greater live weight, weight gain, and feed conversion ratio, but a lower proportion of breast meat than the DL birds. The housing conditions did not affect the fracture toughness of the tibia and femur of the birds; however, Muscovy ducks from the DLSP group had more Ca and Mg in the tibia and more Mg in the femur compared with the DL birds.

Keywords: femur; production; swimming; tibia

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1. Introduction

Waterfowl rearing, including ducks, plays a significant role in global poultry meat production. The production of duck meat has been steadily increasing worldwide since 2000 [1]. In 2020, the total global production of duck meat reached 4,997,577 tons [2]. Among the various duck breeds, Pekin ducks are the most predominant, followed by Muscovy and Mule ducks [1]. Despite the overall growth in production, there are substantial variations in housing systems, particularly in terms of housing conditions. These discrepancies can be attributed in part to the specific requirements of different duck breeds. Housing systems range from indoor conventional setups to free-range systems [3]. The housing conditions significantly impact animal welfare and, consequently, production performance, along with other relevant parameters [4,5].

The scientific community [6–9] frequently emphasizes the evaluation of animal welfare with respect to housing conditions, and in the case of waterfowl, the provision of open water areas has shown potential for improvement. Housing systems that incorporate open water areas allow ducks to engage in species-specific behaviors such as swimming,

bathing, and head-dipping [3]. Access to open water is also crucial for thermoregulation and maintaining feather hygiene. Ducks require the ability to dip their heads and splash water on their feathers to ensure proper cleanliness [6]. The thermoneutral range for ducks is typically between 7 and 23 °C [8]. When the environmental temperature exceeds this range, it can lead to decreased appetite and reduced feed consumption, ultimately impacting growth rate, body weight, and carcass parameters [10].

Moreover, housing ducks in outdoor systems with access to open water has the potential to positively influence stress levels and enhance bird comfort [6]. Blood parameters serve as reliable indicators of overall health status because any physiological, nutritional, or pathological changes within the organism are reflected in these parameters [11,12]. A study by Kraus et al. [13] investigated the impact of housing systems on biochemical blood parameters, some of which may also be related to stress, in laying hens. Their findings suggested that the housing system is a significant factor influencing biochemical blood parameters.

The intensification of duck farming has reduced production costs but has also raised concerns about the welfare of ducks. To optimize animal well-being while maintaining productive performance, it is crucial to focus on housing conditions [4]. One significant aspect of welfare is bone quality, which has been studied in various animal species, including rabbits [14], laying hens [15], and ducks [8]. Poor bone quality can lead to fractures and osteoporosis, posing a significant challenge in poultry. Housing systems can influence not only fracture toughness but also the mineral composition of bones, which is essential in ensuring bone quality [14,15]. Movement plays a key role in maintaining bone health [16]. However, housing systems with open water access also have some disadvantages. Waterfowl can pose higher health risks, raise concerns about water contamination, and lead to increased manure production [3].

It is known from the literature that ducks spend considerable time in the water and thus have weak legs [3]. Therefore, it is appropriate to monitor the quality of the leg bones of ducks that are fattened under intensive conditions and to possibly optimize their housing. This is a pilot study of Muscovy ducks that seeks to determine the element composition of the tibia and femur in detail in different housing conditions. Apart from the element composition, this study also aims to evaluate productive performance, carcass traits, external body temperature, blood parameters, and bone quality properties of the tibia and femur of ducks reared in different housing conditions. The hypothesis is that housing conditions and the gender of Muscovy ducks will influence their productive performance, carcass traits, body temperature, bone quality, and blood profile. The study follows on from a previous study by Krunt et al. [8], aiming to evaluate the issue in greater detail with regard to meat production in the same housing conditions.

2. Materials and Methods

2.1. Animals and Housing

This study included 264 Muscovy ducks in total. Ducklings were sexed after hatching and were then housed in deep litter in controlled conditions and fed *ad libitum* (20.5% CP and 12.2 MJ·kg^{-1} ME). At the age of 5 weeks, they were divided into 4 equal groups according to housing system (deep litter [DL] vs. deep litter with swimming pond [DLSP]) and gender (drakes vs. ducks). The groups were as follows: 66 drakes/DL, 66 drakes/DLSP, 66 ducks/DL, and 66 ducks/DLSP. Each of the four groups was divided into three identical subgroups of 22 animals because of the replication of observations. DL housing systems in closed-sided houses (all animals were protected by the roof of the house against bad weather to keep the housing clean and dry) and DLSP in open-sided houses were used as housing systems. Animals housed in DLSP groups had free access to a swimming pond (10 m × 6 m × 3 m) for the duration of the study. Moreover, the DLSP housing system included vegetation (trees and bushes) close to the swimming pond, which provided much-needed shade. The area around the pond was covered with gravel to prevent vegetation from being eaten, which would affect feed intake and productive parameters. The environmental conditions of the housing were natural (temperature, lighting) and

identical in both housing systems. Regarding the temperature, the average measured value (mean day and night) during the study was 19.03 °C (max. 37.7 °C; min. 7 °C; rh. 65%), while lighting consisted of 16 h of light and 8 h of darkness on average. As bedding, wheat straw was used in both housing systems. A density of 4 animals per m^2 was maintained in all groups. The study took place in summer, specifically from June 2022 to August 2022.

2.2. Feeding and Water Access

All groups were fed *ad libitum* with commercial feed mixture for ducks (pelleted) that contained 20% CP and 11.2 MJ·kg^{-1} of metabolizable energy (ME), 9 g·kg^{-1} Ca, 4 g·kg^{-1} P, 0.6 g·kg^{-1} Mg, 6.5 g·kg^{-1} K, 1.6 g·kg^{-1} Na, 1.2 g·kg^{-1} Cl, 80 mg·kg^{-1} Mn, 80 mg·kg^{-1} Zn, 70 mg·kg^{-1} Fe, 6 mg·kg^{-1} Cu, 1.1 mg·kg^{-1} I, and 0.15 mg·kg^{-1} Se. Access to fresh water was also unlimited in all groups and replications. In the housing systems that included a swimming pond, fresh water was supplied via channels.

2.3. Body Temperature Analysis

The body temperature analysis consisted of the measurement of external body temperature. Body temperature measurements were taken from 12 randomly selected animals in each replication (36 animals per gender and housing system, 144 animals in total). An assessment of body temperature was made every week, on the same day (Monday) at the same time (between 12:00 and 12:30) from the age of 5 weeks until slaughter (13 weeks of age).

External body temperature was measured using a thermographic camera (Bosch, GTC 400 C Professional, Stuttgart, Germany) at the core of the body, where the temperature was the highest (Figure 1). Three images of each animal were obtained, and the average value was used for further evaluation. Each animal was handled for less than 1.5 min. All birds were treated gently to avoid disrupting their well-being.

2.4. Biochemical Blood Analysis

Biochemical blood analysis included assessment of albumin (ALB), globulin (GLOB), alanine transaminase (ALT), amylase (AMY), aspartate aminotransferase (AST), total cholesterol (CHOL), gamma-glutamyl transferase (GGT), glucose (GLU), glutamate pyruvate transaminase (GPT), triglycerides (TAG), total proteins (TP), creatinine (CR), and urea (U). Moreover, the following elements were examined in the blood serum: calcium (Ca), chlorine (Cl), magnesium (Mg), phosphorus (P), sodium (Na), and potassium (K).

At the age of 13 weeks, all the animals were slaughtered by jugular venesection. Blood samples were taken from 12 animals (randomly chosen) from each replication and each group. The blood for biochemical analysis was collected in empty sterile tubes. In total, 144 tubes were collected for biochemical analysis (36 tubes were collected per housing system and gender). After the collection, blood samples were centrifuged and the obtained serum was stored at −20 °C until the analysis. The evaluation of biochemical traits was made using commercial kits (Erba Lachema, s.r.o., Brno, Czech Republic) on the automatic analyzer XL-200 (Erba Lachema s.r.o., Brno, Czech Republic) at the Department of Veterinary Sciences of the Faculty of Agrobiology, Food, and Natural Resources at the Czech University of Life Sciences Prague.

2.5. Productive Performance and Carcass Traits

The examined productive traits consisted of live weight, average daily weight gain, average daily feed consumption, and feed conversion ratio. The birds' weight was recorded weekly when every bird was weighed individually, and their feed consumption was noted every day per group and per replication. The fifth week of age was counted as the start of the experiment while the end was 13 weeks of age. Health status was monitored. No animals died during the experiment.

After slaughter, 8 birds per experimental group and per replication (96 birds in total) were randomly selected for carcass analysis. Carcass dissection was conducted according

to [17], where SW was slaughter weight, SEW (semi-eviscerated weight) was the manually eviscerated carcass, calculated as carcass weight after removal of the trachea, esophagus, gastrointestinal tract, crop, spleen, pancreas, gallbladder, and gonads. EW (eviscerated weight) was calculated as the SEW after removing the head, feet, heart, liver, gizzard, glandular stomach, and abdominal fat. The DoP (dressing out percentage) was calculated as the SEW divided by SW $\times$ 100. The AF (abdominal fat) was measured as a proportion of the SEW. The breast, thigh, and wing yields were calculated as a percentage of EW.

2.6. Bone Quality and Element Composition Analysis

The bone quality analysis involved determining bone weight, length, width, and breaking strength. From a chemical point of view, dry matter, ash, and selected elements, including boron (B), calcium (Ca), cadmium (Cd), cobalt (Co), chrome (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), vanadium (V), and zinc (Zn), were assessed. The tibia and femur bones were used for the bone quality and element composition analysis. At the age of 13 weeks, all the animals were slaughtered by jugular venesection. Before slaughter, a 12 h fasting period was applied. Bones were taken from 6 animals from each replication (randomly chosen) and each group, from the right leg. In total, 72 tibia bones and 72 femur bones were used for the analysis.

After slaughter, the bones were de-fleshed without boiling, afterward individually packed into plastic bags, sealed, and frozen at a temperature of −20 °C in the freezer until the beginning of the analysis. Before the analysis, the bones were taken out of the freezer, thawed for 24 h, and cleaned again of all excess tissue. Length and width were measured using an electronic sliding caliper (DIN 862; IP54; Shut Geometrical Metrology; Gröningen, The Netherlands) with 0.01 mm precision. The measurements were made in the middle of the bones, and each bone was measured three times. Fracture toughness was determined using the Instron device (Instron Universal Testing Machine; model 3342; Instron Ltd., Norwood, MA, USA), which calculates the required force (in *N*) to break the bone. The analysis involved a 50-kg-load cell at a 50-kg-load range with a crosshead speed of 50 mm·min^{-1} with bone supported on a 3.35-cm span, according to [18].

Chemical analysis (dry matter, ash, and element composition) was performed as follows: the dry matter content of bones was determined by drying the samples in the oven at a temperature of 105 °C for 24 h. After drying, the bones were weighed on the Ohaus (Model: Traveler TA502, Parsippany, NJ, USA) digital laboratory scale with 0.01 g precision to calculate dry matter content. The ash content was determined by burning the samples in the oven at a temperature of 550 °C. The ashed samples were subsequently treated with concentrated HCl and HNO_3 acids and the determination of element composition was analyzed using the ICP-OES iCAP 7000 (Thermo Fisher Scientific, Waltham, MA, USA). The limit of detection (LD) was calculated using the equation: LD = 3.29 σ0 (where σ0 is a blank sample standard deviation). The samples and standards were matrix matched. Several procedural blanks were included throughout the analysis.

The bone quality analysis was conducted in the laboratory of the Department of Animal Science of the Faculty of Agrobiology, Food, and Natural Resources at the Czech University of Life Sciences Prague. The chemical analysis (dry matter, ash, and element composition) was conducted in the laboratory of the Department of Soil Science and Soil Protection of the Faculty of Agrobiology, Food, and Natural Resources at the Czech University of Life Sciences Prague.

2.7. Statistical Analysis

The effect of gender and housing system was assessed using a mixed model and the MIXED procedure recommended by SAS (SAS Institute Inc., Cary, NC, USA, 2011):

$$Y_{ijk} = \mu + HS_i + G_j + (HS \times G)_{ij} + e_{ijk},$$

where Y_{ijk} is the value of the trait, μ is the overall mean, HS_i is the effect of the housing system (deep litter, deep litter with swimming pond), G_j is the effect of gender (drakes, ducks), $(HS \times G)_{ij}$ is the effect of the interaction between housing system and gender, e_{ijk} is the random residual error. The significance of the differences among groups was tested using Duncan's multiple-range test. The value of $p \leq 0.05$ was considered significant for all measurements.

3. Results

3.1. Body Temperature

The statistically evaluated results of external body temperature are listed in Table 1. Figure 1 is an image taken by the thermographic camera to show how external body temperature was measured. Furthermore, since the interactions are superior to the individual effects, the interactions between the housing system and gender are displayed in Figure 2 for external body temperature. The effect of gender and housing system was calculated as significant ($p < 0.001$; $p < 0.05$) for external body temperature. Regarding the housing system, the animals with the highest values were those kept on litter compared with animals that had access to the water area. When considering the effect of gender, ducks had a higher temperature than drakes. The interaction between the housing system and gender was statistically significant for external body temperature.

Table 1. External body temperature in relation to housing system and gender of 13-week-old Muscovy ducks.

Housing System	Gender	External Body Temperature (°C)
DL [1]		36.6 [a]
DLSP [2]		36.1 [b]
	Drakes	35.9 [b]
	Ducks	36.8 [a]
p-Value		
Housing system		0.0231
Gender		0.0001
SEM [3]		0.019

[1] DL, deep litter; [2] DLSP, deep litter with swimming pond; and [3] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

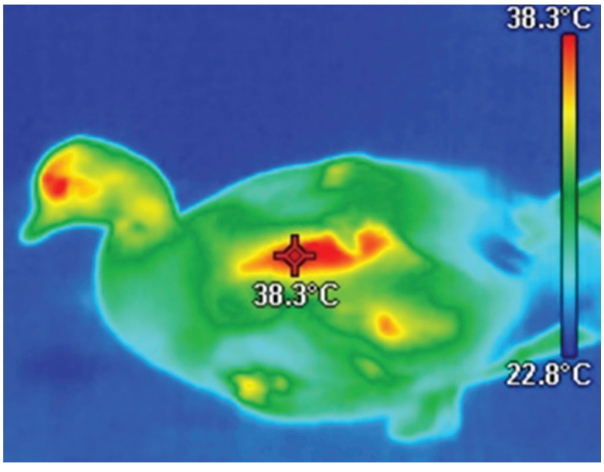

Figure 1. Measurement of external body temperature using a thermographic camera.

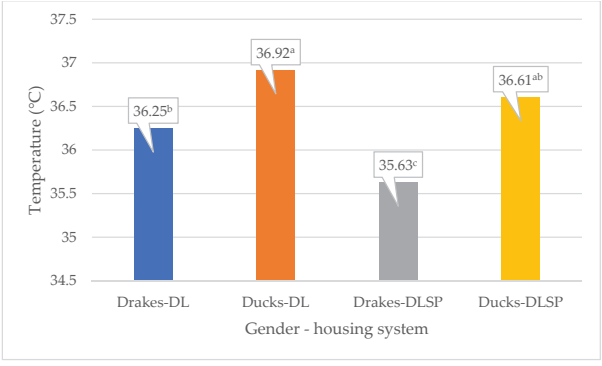

Figure 2. The significant interaction ($p < 0.001$) between gender and housing system for external body temperature in 13-week-old Muscovy ducks. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

3.2. Biochemical Blood Composition

In terms of biochemical blood traits (Tables 2 and 3), the housing system was confirmed as significant in concentrations of Ca ($p < 0.05$) and GLU ($p < 0.05$), where higher values were found for the DL group compared with the DLSP group. Gender influenced concentrations of Ca ($p < 0.05$), GLU ($p < 0.05$), CR ($p < 0.05$), AMY ($p < 0.05$), GPT ($p < 0.05$), and GGT ($p < 0.05$). Except for CR, higher values were detected in ducks compared with drakes.

Table 2. Effect of housing system and gender on biochemical properties and element composition of 13-week-old Muscovy duck blood.

Housing System	Gender	ALB [1] (g·L^{-1})	GLOB [2] (g·L^{-1})	ALB/GLOB [3]	TP [4] (g·L^{-1})	Ca [5] (mmol·L^{-1})	P [6] (mmol·L^{-1})	Na [7] (mmol·L^{-1})	K [8] (mmol·L^{-1})	Mg [9] (mmol·L^{-1})	Cl [10] (mmol·L^{-1})
DL [11]		15.8	15.9	0.997	32.3	2.95 ^{a}	2.37	156.9	3.55	0.884	105.2
DLSP [12]		15.8	15.7	1.005	32.2	2.94 ^{b}	2.36	156.8	3.54	0.881	105.3
	Drakes	15.8	15.8	0.999	32.2	2.94 ^{b}	2.36	156.9	3.53	0.871	105.2
	Ducks	15.8	15.8	1.003	32.3	2.96 ^{a}	2.37	156.8	3.56	0.894	105.4
p-Value											
Housing system		0.5089	0.1038	0.4980	0.5089	0.0446	0.8310	0.6793	0.6729	0.7836	0.4975
Gender		0.4120	0.8968	0.4079	0.2659	0.0095	0.3563	0.6675	0.3870	0.1040	0.3417
SEM [13]		0.040	0.053	0.002	0.081	0.003	0.009	0.115	0.014	0.005	0.107

[1] ALB, albumin; [2] GLOB, globulin; [3] ALB/GLOB, albumin/globulin ratio; [4] TP, total protein; [5] Ca, calcium; [6] P, phosphorus; [7] Na, sodium; [8] K, potassium; [9] Mg, magnesium; [10] Cl, chlorine; [11] DL, deep litter; [12] DLSP, deep litter with swimming pond; and [13] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

Table 3. Effect of housing system and gender on biochemical properties of 13-week-old Muscovy duck blood.

Housing System	Gender	CHOL [1] (mmol·L^{-1})	GLU [2] (mmol·L^{-1})	CR [3] (µmol·L^{-1})	U [4] (mmol·L^{-1})	TAG [5] (mmol·L^{-1})	AMY [6] (IU·L^{-1})	AST [7] (IU·L^{-1})	ALT [8] (IU·L^{-1})	GPT [9] (IU·L^{-1})	GGT [10] (IU·L^{-1})
DL [11]		4.51	12.85 ^{a}	18.3	1.54	0.921	51.1	52.0	40.8	20.2	2.50
DLSP [12]		4.43	12.16 ^{b}	18.6	1.49	0.884	52.5	52.2	40.0	19.8	2.47
	Drakes	4.50	12.14 ^{b}	18.7 ^{a}	1.53	0.883	50.3 ^{b}	51.1	40.0	19.7 ^{b}	2.46 ^{b}
	Ducks	4.44	12.87 ^{a}	18.2 ^{b}	1.50	0.922	53.3 ^{a}	53.1	40.8	20.3 ^{a}	2.51 ^{a}
p-Value											
Housing system		0.0626	0.0466	0.2372	0.4661	0.0780	0.3257	0.8473	0.1265	0.0846	0.2734
Gender		0.1630	0.0388	0.0491	0.6460	0.0688	0.0357	0.0745	0.2659	0.0141	0.0442
SEM [13]		0.023	0.180	0.147	0.035	0.011	0.750	0.589	0.269	0.116	0.012

[1] CHOL, cholesterol; [2] GLU, glucose; [3] CR, creatinine; [4] U, urea; [5] TAG, triglycerides; [6] AMY, amylase; [7] AST, aspartate aminotransferase; [8] ALT, alanin aminotransferase; [9] GPT, glutamate pyruvate transaminase; [10] GGT, gama glutamyl transferase; [11] DL, deep litter; [12] DLSP, deep litter with swimming pond; and [13] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

3.3. Growth Performance and Carcass Value

The results of productive performance and growth performance are displayed in Table 4 and Figure 3. Table 5 shows the results of selected carcass traits. According to the live weight of birds at 5 weeks of age (start of the experiment), the significant differences between the DLSP and DL groups are caused by sexual dimorphism between drakes and ducks because the live weight of the groups was counted as an average. Figure 3 shows that groups were created based on similar weight. Birds from the DLSP group had significantly higher live weight ($p < 0.001$) at the end of the experimental period, higher average daily weight gain ($p < 0.001$), lower feed consumption ($p < 0.001$), and lower feed conversion ratio ($p < 0.001$). All selected parameters were significantly higher for drakes than for ducks. However, the productive traits differed significantly between the groups, and were not reflected in most of the monitored carcass traits. A higher value ($p < 0.05$) of the AF proportion from SEW was found in DL birds compared with DLSP birds. In addition, the breast meat was significantly affected by housing conditions ($p < 0.05$) in the DL birds compared with the DLSP birds. As expected, drakes had a significantly higher SW ($p < 0.001$), SEW ($p < 0.001$), and EW ($p < 0.001$) than ducks. Furthermore, drakes were characterized by higher DoP ($p < 0.001$) and higher ($p < 0.05$) thigh proportions compared with ducks. However, ducks had a higher wing ($p < 0.001$) and breast ($p < 0.001$) proportion compared with drakes.

Table 4. Effect of housing system and gender on productive performance of 13-week-old Muscovy ducks.

Housing System	Gender	LW5 [1] (g)	LW13 [2] (g)	ADWG [3] (g)	ADFC [4] (g)	FCR [5]
DL [6]		1167 [a]	3874 [b]	48.34 [b]	177.59 [a]	3.79 [a]
DLSP [7]		1114 [b]	3932 [a]	50.30 [a]	172.30 [b]	3.52 [b]
	Drakes	1258 [a]	4851 [a]	64.16 [a]	212.07 [a]	3.31 [b]
	Ducks	1024 [b]	2955 [b]	34.48 [b]	137.82 [b]	4.00 [a]
p-Value						
Housing system		0.0004	0.0002	0.0003	0.0024	0.0001
Gender		0.0001	0.0001	0.0001	0.0001	0.0001
SEM [8]		36.374	285.910	4.486	11.237	0.113

[1] LW5, live weight at 5 weeks of age; [2] LW13, live weight at 13 weeks of age; [3] ADWG, average daily weight gain; [4] ADFC, average daily feed consumption; [5] FCR, feed conversion ratio; [6] DL, deep litter; [7] DLSP, deep litter with swimming pond; and [8] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

Table 5. Effect of housing system and gender on carcass value characteristics of Muscovy ducks at 13 weeks of age.

Housing System	Gender	SW [1] (g)	SEW [2] (g)	EW [3] (g)	DoP [4] (%SEW)	AF [5] (%SEW)	Wings (%EW)	Thighs (%EW)	Breasts (%EW)
DL [6]		3742	2810	2407	74.73	0.97 [a]	17.53	21.55	33.45 [a]
DLSP [7]		3723	2780	2392	74.50	0.75 [b]	17.33	21.80	32.34 [b]
	Drakes	4825 [a]	3644 [a]	3123 [a]	75.54 [a]	0.93	16.95 [b]	22.02 [a]	31.85 [b]
	Ducks	2640 [b]	1945 [b]	1675 [b]	73.68 [b]	0.78	17.91 [a]	21.33 [b]	33.94 [a]
p-Value									
Housing system		0.7330	0.4915	0.7062	0.3915	0.0181	0.4951	0.3653	0.0021
Gender		0.0001	0.0001	0.0001	0.0001	0.1126	0.0017	0.0153	0.0001
SEM [8]		123.110	95.647	81.722	0.167	0.047	0.155	0.143	0.216

[1] SW, slaughter weight; [2] SEW, semi-eviscerated weight; [3] EW, eviscerated weight; [4] DoP, dressing out percentage; [5] AF, abdominal fat; [6] DL, deep litter; [7] DLSP, deep litter with swimming pond; and [8] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

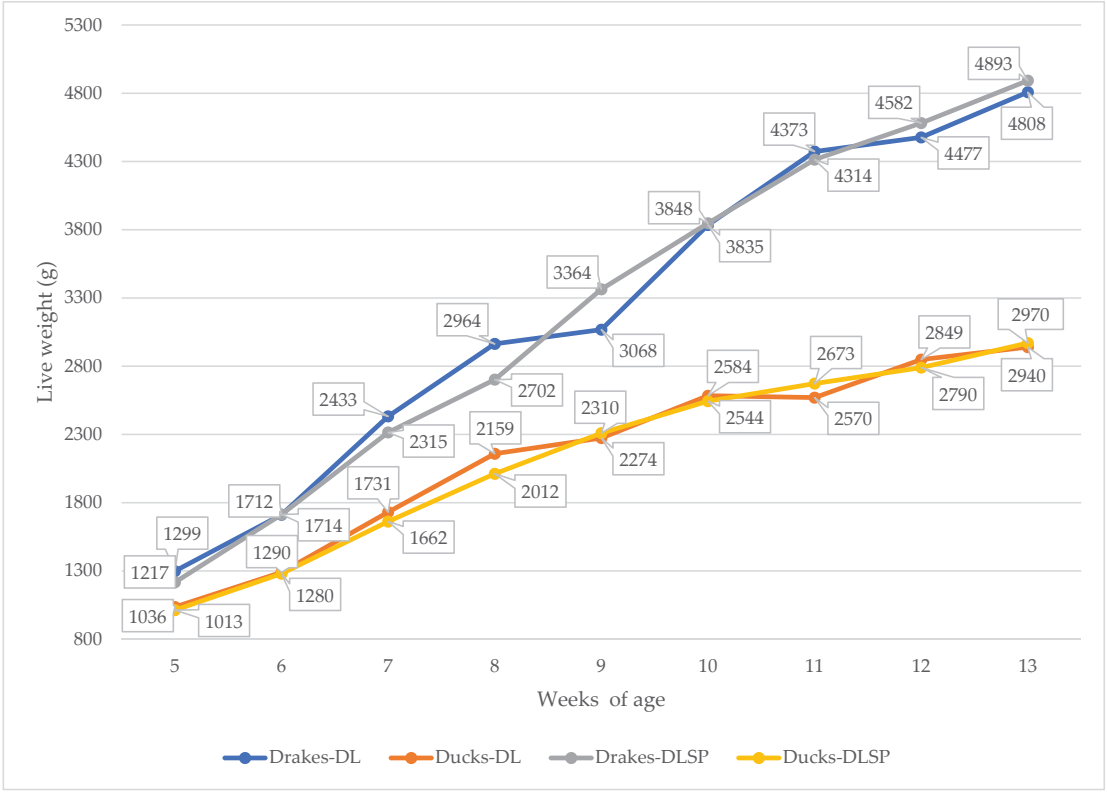

Figure 3. Growth performance of Muscovy ducks (5–13 weeks of age) with regard to housing system and gender.

3.4. Bone Quality and Element Composition

The results for basic bone quality parameters, including fracture toughness, bone length, width, and weight, are displayed in Table 6 for the tibia and in Table 7 for the femur. Only the effect of gender was statistically significant. Gender significantly affected all evaluated parameters in both the tibia and the femur, with drakes having significantly higher values ($p < 0.001$) in all evaluated parameters than ducks in both of the observed bones.

Table 6. Effect of housing system and gender on basic tibia properties of 13-week-old Muscovy ducks.

Housing System	Gender	Fracture Toughness (N)	Length (mm)	Width (mm)	Weight (g)
DL [1]		383.4	112.1	8.12	11.1
DLSP [2]		378.8	113.6	8.10	11.3
	Drakes	482.3 [a]	124.0 [a]	9.5 [a]	15.3 [a]
	Ducks	278.9 [b]	102.4 [b]	7.3 [b]	7.5 [b]
p-Value					
Housing system		0.3674	0.4672	0.8834	0.6228
Gender		0.0001	0.0001	0.0001	0.0001
SEM [3]		17.157	3.235	0.301	0.563

[1] DL, deep litter; [2] DLSP, deep litter with swimming pond; and [3] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

Table 7. Effect of housing system and gender on basic femur properties of 13-week-old Muscovy ducks.

Housing System	Gender	Fracture Toughness (N)	Length (mm)	Width (mm)	Weight (g)
DL [1]		367.3	68.9	9.5	8.5
DLSP [2]		359.5	70.4	9.6	8.0
	Drakes	455.2 [a]	76.0 [a]	11.1 [a]	10.2 [a]
	Ducks	278.0 [b]	63.5 [b]	8.7 [b]	4.9 [b]
p-Value					
Housing system		0.5614	0.2876	0.9554	0.4654
Gender		0.0001	0.0001	0.0001	0.0001
SEM [3]		15.878	1.618	0.311	0.583

[1] DL, deep litter; [2] DLSP, deep litter with swimming pond; and [3] SEM, standard error of the mean. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

The element composition, dry matter, and ash results are shown in detail in Table 8 for the tibia and in Table 9 for the femur. For the tibia, the statistically significant effect of the housing system was calculated for the following elements: B ($p < 0.05$), Ca ($p < 0.05$), Cr ($p < 0.001$), Cu ($p < 0.05$), K ($p < 0.05$), Mg ($p < 0.001$), Mn ($p < 0.05$), P ($p < 0.001$), S ($p < 0.001$), and V ($p < 0.05$). Furthermore, the significant effect of gender was found to be significant in B ($p < 0.001$), Cr ($p < 0.05$), Fe ($p < 0.05$), Mg ($p < 0.001$), Mn ($p < 0.05$), Na ($p < 0.001$), Ni ($p < 0.05$), P ($p < 0.001$), Pb ($p < 0.05$), S ($p < 0.05$), and Zn ($p < 0.05$) and also for dry matter ($p < 0.001$) and ash contents ($p < 0.001$). For the femur, the effect of the housing system was significant in B ($p < 0.05$), Cd ($p < 0.05$), Co ($p < 0.05$), Fe ($p < 0.05$), K ($p < 0.05$), Mg ($p < 0.05$), Mn ($p < 0.001$), Na ($p < 0.05$), P ($p < 0.001$), Pb ($p < 0.05$), and S ($p < 0.05$) and for dry matter contents ($p < 0.001$). The effect of gender was calculated as statistically significant in B ($p < 0.05$), Cd ($p < 0.05$), Co ($p < 0.05$), Fe ($p < 0.001$), K ($p < 0.05$), Pb ($p < 0.001$), S ($p < 0.05$), V ($p < 0.001$), and Zn ($p < 0.05$) and also for dry matter ($p < 0.001$) and ash content ($p < 0.05$).

Table 8. Effect of housing system and gender on tibia element composition and chemical attributes of 13-week-old Muscovy ducks.

Housing System	Gender	B [1] (mg·kg^{-1})	Ca [2] (g·kg^{-1})	Cd [3] (mg·kg^{-1})	Co [4] (mg·kg^{-1})	Cr [5] (mg·kg^{-1})	Cu [6] (mg·kg^{-1})	Fe [7] (mg·kg^{-1})	K [8] (g·kg^{-1})	Mg [9] (g·kg^{-1})	Mn [10] (mg·kg^{-1})
DL [11]		91.5	271.4 [b]	0.13	1.19	2.66 [b]	20.2 [a]	62.1	3.2 [b]	4.3 [b]	9.98 [b]
DLSP [12]		101.9	277.0 [a]	0.13	1.21	3.41 [a]	17.5 [b]	60.0	3.5 [a]	4.6 [a]	11.45 [a]
	Drakes	90.3 [b]	274.9	0.13	1.21	3.36 [a]	18.1	65.7 [a]	3.4	4.7 [a]	10.22 [b]
	Ducks	103.1 [a]	273.8	0.13	1.18	2.75 [b]	19.6	56.7 [b]	3.4	4.2 [b]	11.25 [a]
p-Value											
Housing system		0.0208	0.0142	0.6182	0.7895	0.0002	0.0171	0.5859	0.0418	0.0001	0.0063
Gender		0.0006	0.5899	0.6182	0.7895	0.0016	0.1907	0.0415	0.9837	0.0001	0.0495
SEM [13]		9.853	1.149	0.003	0.044	0.105	0.565	2.204	0.081	0.040	0.282

Housing System	Gender	Na [14] (g·kg^{-1})	Ni [15] (mg·kg^{-1})	P [16] (g·kg^{-1})	Pb [17] (mg·kg^{-1})	S [18] (g·kg^{-1})	V [19] (mg·kg^{-1})	Zn [20] (mg·kg^{-1})	DM [21] (%)	Ash (%)
DL		8.6	0.49	114.4 [a]	1.55	7.3 [b]	0.67 [b]	411.6	88.7	56.7
DLSP		8.8	0.57	107.8 [b]	1.92	7.8 [a]	1.02 [a]	428.9	88.8	56.1
	Drakes	8.9 [a]	0.63 [a]	108.9 [b]	1.40 [b]	7.7 [a]	0.78	404.7 [b]	86.1 [b]	58.5 [a]
	Ducks	8.4 [b]	0.43 [b]	112.8 [a]	2.07 [a]	7.4 [b]	0.92	435.4 [a]	91.3 [a]	54.5 [b]
p-Value										
Housing system		0.7854	0.4021	0.0001	0.0983	0.0009	0.0016	0.3129	0.2708	0.2015
Gender		0.0001	0.0237	0.0004	0.0044	0.0014	0.1865	0.0017	0.0001	0.0001
SEM		0.216	0.044	0.621	0.121	0.106	0.060	19.037	0.261	0.412

[1] B, boron; [2] Ca, calcium; [3] Cd, cadmium; [4] Co, cobalt; [5] Cr, chrome; [6] Cu, copper; [7] Fe, iron; [8] K, potassium; [9] Mg, magnesium; [10] Mn, manganese; [11] DL, deep litter; [12] DLSP, deep litter with swimming pond; [13] SEM, standard error of the mean; [14] Na, sodium; [15] Ni, nickel; [16] P, phosphorus; [17] Pb, lead; [18] S, sulfur; [19] V, vanadium; [20] Zn, zinc; and [21] DM, dry matter. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

Table 9. Effect of housing system and gender on femur element composition and chemical attributes of 13-week-old Muscovy ducks.

Housing System	Gender	B [1] (mg·kg^{-1})	Ca [2] (g·kg^{-1})	Cd [3] (mg·kg^{-1})	Co [4] (mg·kg^{-1})	Cr [5] (mg·kg^{-1})	Cu [6] (mg·kg^{-1})	Fe [7] (mg·kg^{-1})	K [8] (g·kg^{-1})	Mg [9] (g·kg^{-1})	Mn [10] (mg·kg^{-1})
DL [11]		124.5 ^{a}	276.6	0.13 ^{b}	1.14 ^{b}	3.40	87.7	106.9 ^{b}	4.8 ^{b}	4.51 ^{b}	10.5 ^{b}
DLSP [12]		94.9 ^{b}	277.9	0.17 ^{a}	1.44 ^{a}	3.44	18.9	122.3 ^{a}	5.3 ^{a}	4.62 ^{a}	13.3 ^{a}
	Drakes	93.4 ^{b}	280.0	0.16 ^{a}	1.44 ^{a}	3.49	19.3	129.7 ^{a}	5.3 ^{a}	4.61	12.3
	Ducks	126.1 ^{a}	274.5	0.13 ^{b}	1.37 ^{b}	3.34	87.3	99.5 ^{b}	4.9 ^{b}	4.52	11.5
p-Value											
Housing system		0.0048	0.6499	0.0055	0.0055	0.8175	0.1227	0.0459	0.0019	0.0134	0.0001
Gender		0.0020	0.8679	0.0224	0.0055	0.4225	0.1269	0.0001	0.0079	0.0733	0.0879
SEM [13]		9.391	1.496	0.007	0.058	0.092	22.563	4.108	75.560	0.024	0.264

Housing System	Gender	Na [14] (g·kg^{-1})	Ni [15] (mg·kg^{-1})	P [16] (g·kg^{-1})	Pb [17] (mg·kg^{-1})	S [18] (g·kg^{-1})	V [19] (mg·kg^{-1})	Zn [20] (mg·kg^{-1})	DM [21] (%)	Ash (%)
DL		9.40 ^{a}	0.67	116.2 ^{a}	1.34 ^{b}	8.3 ^{a}	0.83	489.1	88.5 ^{b}	62.2
DLSP		8.96 ^{b}	0.64	109.2 ^{b}	2.07 ^{a}	8.0 ^{b}	0.75	452.5	89.9 ^{a}	62.2
	Drakes	9.14	0.67	112.0	1.25 ^{b}	8.0 ^{b}	0.59 ^{b}	447.7 ^{b}	87.5 ^{b}	62.9 ^{a}
	Ducks	9.21	0.64	113.4	2.16 ^{a}	8.3 ^{a}	0.99 ^{a}	493.9 ^{a}	91.1 ^{a}	61.5 ^{b}
p-Value										
Housing system		0.0399	0.8437	0.0001	0.0024	0.0030	0.4258	0.0877	0.0001	0.9923
Gender		0.7499	0.8544	0.2419	0.0002	0.0010	0.0001	0.0317	0.0001	0.0389
SEM		0.180	0.086	0.699	0.139	0.075	0.058	18.73	0.053	17.515

[1] B, boron; [2] Ca, calcium; [3] Cd, cadmium; [4] Co, cobalt; [5] Cr, chrome; [6] Cu, copper; [7] Fe, iron; [8] K, potassium; [9] Mg, magnesium; [10] Mn, manganese; [11] DL, deep litter; [12] DLSP, deep litter with swimming pond; [13] SEM, standard error of the mean; [14] Na, sodium; [15] Ni, nickel; [16] P, phosphorus; [17] Pb, lead; [18] S, sulfur; [19] V, vanadium; [20] Zn, zinc; and [21] DM, dry matter. Values marked with different superscript letters in each column are significantly different ($p \leq 0.05$).

4. Discussion

4.1. Body Temperature

The duration of time that animals spend in the water plays a crucial role in regulating their body temperature [8]. This behavior enables animals to cool their bodies, enhance evaporation, and mitigate heat stress [7]. Furthermore, outdoor-reared ducks with free access to water have shown improved stress levels and enhanced bird comfort [6]. However, the use of open water sources can lead to increased waste production and negatively impact the condition of the litter used in the housing system [19].

In the present study, the housing system had a significant influence on external body temperature, although the temperature decline between the DL and the DLSP groups was relatively modest. It was particularly intriguing to note the differences in external temperature between genders. Krunt et al. [8] found significant variations in internal body temperature between drakes and ducks, with lower values observed in drakes. They proposed that these gender differences could be attributed to hormonal activity, considering that progesterone, for example, inhibits vasodilation in women [20] and domestic mammals [21].

Moreover, the higher body temperature in females compared with males has been confirmed in various species, including mice [22], Japanese quails [23], and farm-reared emus [24]. The authors of these studies attributed the temperature oscillations to differences in circadian modulation of the hypothermic response [22], variations in calorie intake and body weight [23], and metabolic changes [24], which can increase heat production capacity. The normal range of body temperature for Muscovy ducks typically falls between 38 and 42 °C [25]. Naturally, when measuring temperature using external methods that do not interfere with the body, the recorded values were lower.

4.2. Biochemical Blood Composition

Serum analysis is commonly used to determine and predict disease and infection; it can also be used to track the health status of animals [26]. The results of the blood composition in the present paper are shown in Tables 3 and 4. According to our data, the type of housing system only significantly influenced the concentration of GLU and Ca

in the blood. However, studies such as [27] or [28] proposed that greater movement or swimming in ducks reduces serum triacylglycerol and cholesterol. The birds in the present study did not differ in terms of these traits. Glucose is the main energy source of mammals and birds [29]. In our study, higher values of GLU were found in the DL group, which could mean a reduced need for energy due to lower physical activity [8] compared with the DLSP group, where birds could swim, thereby expending greater energy. Moreover, gender appeared as significant in the present paper. The differences could be linked to body size and different energy needs, as reported by [30]. Differences in Ca between housing systems were very low, varying only by 0.01 mmol·L^{-1}; they were also low between genders, varying only by 0.02 mmol·L^{-1}. Calcium is typically presented in three fractions—the ionized fraction, the protein-bound fraction, and the fraction complexed to anions [31]. Similar concentrations of Ca serum in birds could indicate similar levels of parathormone and vitamin D or the hormone, calcitonin, which influence the level of Ca in the blood. Other investigated elements were sodium, chlorine, and potassium. ATP pump based on sodium and potassium is important for balancing water in cells and maintaining biomass energy metabolism [32]. It is known that the concentration of these elements can be reduced by heat stress [33]. According to our results, it is clear that summer temperatures did not reduce these elements in both groups of birds. In addition, the concentration of creatinine differs significantly according to gender, with higher concentrations of serum in males than in females. Conversely, serum CR is found in the muscles and is described as a waste product, which is attributed to higher muscle mass [34]. Therefore, the higher body weight and muscle mass of males led to increased CR. Moreover, serum amylase is typically used to assess pancreas activity. It reflects stability in the rate of entry and removal from the blood. A small increase does not usually indicate pancreatitis, but some other condition [35]. Liver enzymes (GPT, GGT) serve as indicators of the pathological state that appears in hepatocytes and symbolizes abnormal (declining or rising) liver function [36].

4.3. Growth Performance and Carcass Value

Based on the findings, birds from the housing system with access to the swimming pond (DLSP) exhibited higher final live weight, average daily weight gain, and lower feed conversion rates compared with birds in standard deep litter housing (DL). These results align with the findings of Abo Ghanima et al. [28], who also reported improved productive performance in swimming birds, attributing it to the beneficial effects of natural swimming behavior for ducks. Similarly, Rehman et al. [26] concluded that the presence of swimming ponds enhanced feed efficiency and resulted in higher final weights compared with birds without access to swimming facilities.

However, contrasting results were reported by Damaziak et al. [37], who found superior weight gain and feed conversion ratios in intensively fattened birds compared with those provided with space for running. These discrepancies suggest that the type of movement may play a crucial role in the observed variations among birds. Based on the collective findings of the aforementioned studies, it can be inferred that swimming appears to be a more natural activity for waterfowl, generally leading to improved performance, while running may require additional energy expenditure and potentially hinder the growth of birds.

The differences observed between drakes and ducks can be attributed to significant sexual dimorphism, characterized by larger body dimensions in drakes [8]. However, while the final weight was higher in DLSP birds compared with DL birds, there was no statistical difference in eviscerated weight. This could be explained by the presence of a greater number of feathers on the bodies of DLSP birds, as it is known that birds from outdoor runs with access to swimming ponds tend to exhibit improved hygiene, cleaner feathers, and overall better feather condition when compared with birds in intensive housing conditions, where cleanliness and hygiene issues may arise. Additionally, feather growth is significantly enhanced in outdoor systems [3].

The absence of exercise opportunities, such as swimming in this case, in intensive housing conditions had an impact on the proportion of abdominal fat in birds. Birds from the DL group had a higher proportion of abdominal fat compared with the DLSP birds. Similar results were reported by Farghly et al. [38], who observed higher levels of abdominal fat in groups of birds without access to swimming compared with groups where swimming was allowed for varying durations as per the experimental design. Conversely, DLSP birds exhibited reduced breast muscle compared with DL birds due to increased water-based movement and related activities.

In other studies [28,39], the researchers did not observe a decline in breast muscle, possibly because they focused on comparing intensive, semi-intensive, and extensive systems without considering the factor of swimming in their investigations. The variations observed between genders in terms of DoP, the percentage of wings, thighs, and breasts could be attributed to the higher carcass weight of drakes compared with ducks. This is a factor that is considered when calculating the share of each body part, influencing its proportion. Thus, there is a relationship with overall body size. As a result of different growth patterns, drakes exhibited particularly well-developed thighs, surpassing the corresponding ratio in ducks. Additionally, when expressed in grams, drakes displayed greater length and weight across all body parts [40].

4.4. Bone Quality and Element Composition

In the case of poultry, particularly laying hens, the focus often lies on examining the impact of various nutritional factors on bone composition [15]. Nevertheless, specific attention has been paid to the influence of duck nutrition on bone quality in certain studies [41], while other research endeavors [8] have explored the potential effect of physical activity, as observed in fattened ducks, on bone quality—a relationship that has been demonstrated in other animal species [14].

Within the scope of this study, the authors conducted a comparative analysis between deep litter housing and an alternative housing system that offered swimming opportunities in ponds. Although only numerical differences were observed across the treatments, no significant variations were detected in terms of tibia and femur length, width, weight, or fracture toughness. Rodenburg et al. [3] noted that ducks inherently possess weaker leg and thigh joints, but swimming enables them to alleviate the weight-bearing burden on their joints. Interestingly, our findings indicate that the inclusion of swimming did not exert any adverse effects on tibia or femur strength. Moreover, housing treatment did not produce any discernible impacts on length, width, or weight parameters, aligning with the findings of Farghly and Mahmoud [8]. The findings suggest that the use of swimming ponds by ducks may not have notably reduced terrestrial locomotion, thereby having a limited impact on bone development or fragility. Furthermore, the study supports the assertion made by previous researchers regarding the influence of gender, as observed in Muscovy ducks exhibiting sexual dimorphism, with males displaying significantly larger physical dimensions than females.

Bone quality is influenced by various factors, including its architectural structure, organic composition, and mineral constituents. The mineral content, predominantly composed of calcium and phosphorus, is commonly assessed through bone ash analysis [42]. In the context of housing conditions for laying hens, studies have shown that bones from hens housed in cages exhibit lower ash content compared with those from floor pens [43] or free-range systems [44], which provide greater opportunities for animal mobility. The ash content and percentage of dry matter in bones also play a significant role in the incidence of osteoporosis in adult animals [44]. Additionally, when considering the effect of gender, factors such as live weight (which can vary substantially between genders) and growth rate, are likely to be the primary influencing factors [45]. In a study conducted by Corr et al. [46], it was observed that lighter broiler chickens exhibited higher mineral content in their bones compared with their heavier counterparts. However, in the current investigation, it was found that heavier males displayed a higher ash content in both bones studies compared

with females. As highlighted by González-Cerón et al. [45], ash content can serve as an indicator of bone mineralization.

Interestingly, Rath et al. [42] reported contrasting findings, noting that fast-growing birds tend to exhibit lower levels of bone mineralization compared with slower-growing birds. Additional studies [46,47] have also suggested that slower-growing birds demonstrate superior bone mineralization and density compared with their faster-growing counterparts. These differences between males and females in our study could potentially be attributed to the divergent growth rates, with females experiencing faster growth and reaching physical maturity earlier than males [3].

The structure of the bone is determined by the minerals that are involved in the whole process. Calcium (Ca) and phosphorus (P) play a leading role and are the key elements markedly influencing bone strength. However, regarding the quality of the bone matrix, other elements are also involved, namely, sodium (Na), magnesium (Mg), potassium (K), copper (Cu), zinc (Zn), manganese (Mn), chrome (Cr), iron (Fe), and lead (Pb), all of which influence bone strength [48]. A mutual relationship between Ca and P is widely recognized, and Mg is connected to these as an antagonist of Ca [49]. The importance of Mg was mentioned in a study by Krunt et al. [14], with the authors believing that Mg plays a key role in bone strength. A study by Shastak and Rodehutscord [50] confirmed that rats with a deficiency of Mg in their diet showed a worse bone growth rate and lower bone strength than their counterparts with a normal diet. In addition, a deficiency of Na results in increased activity of osteoclasts and bone resorption [51]. In contrast, a surplus of Na in the feed mixture negatively affects Ca excretion from the organism. Moreover, osteoporosis is exposed to Na [52]. K is considered an element that favorably affects bone homeostasis by influencing the acid–base balance [53]. However, the benefits of Cu for bone health are not consistent in the scientific literature [48]. There could be a link with the inhibition of osteoclastic resorption through lysine crosslinks in collagen and elastin, which are affected by Cu as an enzymatic cofactor [54]. Another cofactor is Zn, which influences the proliferation and bone mineralization via protein gene expression (e.g., alkaline phosphatase or osteocalcin) [55]. Cr, Al, Pb, Cd, and Co could have a potential negative effect on bone health, while B, Si, Fe, and Sr could have a positive effect. Manganese (Mn) may influence the bone positively or negatively, depending on its content [48]. Furthermore, vanadium (V) plays an important role in the organism, such as the regulation of the glucose metabolism [56]. It also has an osteogenic effect; hence it influences the differentiation and mineralization of components in the cell bone extracellular matrix or in the formation of collagen [19]. In addition, it has been found that cobalt (Co) affects both angiogenesis and osteogenesis through alkaline phosphatase or osteocalcin alone; alternatively, the effect could be influenced by other trace elements [57].

5. Conclusions

During the fattening period, the birds exhibited a decline in their external body temperature when provided with unrestricted access to swimming ponds. Remarkably, the birds residing in the housing systems offering swimming opportunities demonstrated superior productive performance and a lower proportion of abdominal fat compared with birds in standard deep litter housing. These findings provide a compelling argument in discussions on the potential disparity in production outcomes between alternative and intensive systems.

Despite discernible variations in the elemental composition of the tibia and femur between birds housed under different systems, the present study suggests that the housing system itself did not exert a substantial influence on bone quality, and specifically fracture toughness, at the investigated age. Nevertheless, it is imperative to exercise caution when interpreting these results, particularly if birds were reared under identical conditions until reaching the reproductive phase. This investigation has provided novel insights into the bone quality of waterfowl raised under intensive conditions.

In future research endeavors, we recommend focusing attention on evaluating the meat quality of ducks fattened in accordance with the housing conditions examined in this study. By focusing on these aspects, we will further enhance our understanding of the broader implications associated with the studied housing systems.

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Institutional Review Board Statement: The welfare of the ducks was carefully considered during the experiment. The animals were not subjected to pain, suffering, distress, or lasting harm. Feed and water were provided *ad libitum*. The study was carried out in line with the guidelines of Act No. 246/1992 on protection against animal cruelty. The study was approved by the ethics committee of the Czech University of Life Sciences Prague, which allowed the use of live animals (approval no. 08/2022).

Informed Consent Statement: Not applicable.

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Article

Effects of Dairy Cows Management Systems on the Physicochemical and Nutritional Quality of Milk and Yogurt, in a North-Eastern Romanian Farm

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Abstract: The study's objective was to investigate changes in the fatty acid composition of cow milk in general and in 80 Romanian Spotted cows' husbandry and feeding systems in particular (grazing–GC group vs. stabulation–SC group). The ultimate objective was to determine if the changes that happened in the milk also transferred to the finished product. Also, the influence of the quality of raw milk produced by both systems was evaluated when yogurt was made from it. The milk was gathered in May, July and September and used for both the yogurt-making process and the study, which lasted from May to October. In comparison to milk from SC, milk from grazed caws had larger percentages of fat and dry matter throughout the summer (GC) season. Moreover, pasture-based rations (MGC) contained more PUFA than MCS did. Data research revealed that not only do factors such as milk origin and initial quality have a substantial impact on yogurt quality parameters, but also technologies such as milk fermentation have a considerable impact on the fatty acid profile of yogurt. In comparison to cows kept permanently in stables, grazed cows (MGC) had fat with a lower concentration of saturated fatty acids and a higher proportion of rumenic, vaccenic and oleic acids (MSC). When fresh milk is processed into yogurt and other dairy products, the fatty acid profiles alter, with saturated fatty acids predominating over unsaturated ones. The findings show that pasture-fed cows have a positive impact on milk quality, particularly in terms of fatty acid profile, as well as on yogurt's ultimate nutritional and dietary quality.

Keywords: cows feeding; milk; yogurt; fatty acids; quality

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1. Introduction

Cow milk is a significant source of energy, high-quality protein, lipids, lactose, micro- and macroelements, vitamins and enzymes that support healthy human growth, development as well as essential organism processes [1].

The majority of milk lipids are in the form of triacylglycerols, which consist of a molecule of glycerol bonded to three ways of fatty acids. Over the last few decades,

milk fatty acid composition has gained the interest of manufacturers and consumers as it influences various nutritional, physical and flavour properties of dairy products [2].

Dietary lipids from dairy products play a significant role in human nutrition. The complexity of milk fat arises from the fact that it is composed of more than 400 different fatty acids, which makes milk fat the most complex of all all-natural fats. Nevertheless, the majority of these acids are consumed in trace amounts, and only about 15 acids are consumed in quantities exceeding 1% [3]. C16:0, C18:1, C14:0 and C18:0 are the most abundant, ordered from largest to smallest.

Milk composition is influenced by a number of variables, such as the state of the environment or the animal feeding, which impact on the quality of the raw material and of the subsequent manufactured dairy products [4]. Indirectly or directly, several variables can influence milk composition, such as animal health, farm management, different feeding methods, seasonal fluctuations and environmental factors.

Researchers have become interested in the prospect of enhancing the diet of humans due to the change in the milk's fatty acid (FA) composition by changing the ruminants' diets [5]. Unsaturated fats are beneficial for human health, whilst the saturated ones, primarily those rich in C12:0, C14:0 and C16:0 FAs, are linked to cardiovascular disorders [6]. The effect of milk fat on human health is currently seen much more favourable than it was in the past [7,8]. Despite this, this field of study continues to be a very compelling topic for knowledge advancement.

Multiple factors affect the fatty acid composition of cow milk, such as breed, season, lactation stage, number of lactations, cows' age, geographic location and, which is most relevant, the diet. Cows' nutrition accounts for 95% of the variation in cow milk fat yield and, mostly, quality (fatty acids profile), via the dietary fatty acids and the ruminal biohydrogenation processes [9,10].

However, milk from exclusively pasture-raised cattle contains much more polyunsaturated FA (PUFA), conjugated linonic acid (CLA), n-3 FA and branched FA [11]. In addition to being superior to concentrate-based milk production methods, pasture-based dairying systems that include fresh and preserved forages as well as occasional concentrate supplementation result in acceptable and sustainable productivity [12]. Slots et al. [13] claim that to produce milk with a high content of PUFA and a high level of potential antioxidants, an extensive production form with a high level of pasture is advised. Consumers may find the fatty acid (FA) profile of pasture-raised milk fat to be more palatable because of the increased levels of CLA and linolenic acid and lower levels of saturated hypercholesterolemic fatty acids [14].

The quality of fermented dairy products is subject to change due to variations in raw milk compounds, including fatty acids, that respond differently or interfere throughout several technological processes, including heat treatment, homogenization or pasteurization [15,16], standardization, fermentation, inoculation microorganisms culture type, duration of fermentation and storage conditions [17]. Apart from these, the high nutritional content of yogurts and other fermented milk beverages is greatly determined by the manufacturing technique, as well as by any potential improvement in bioavailability brought on by the fermentation process. The fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* produces yogurt, a coagulated milk product, that usually has improved nutritional and dietary traits, in comparison with raw milk [18].

However, high milk protein and fat yields can be supported in a number of ways, including increasing feed intake (if necessary, assisted by feed additives), offering a well-balanced diet, and supplying enough levels of minerals [7,19,20]. Monitoring diet composition, gathering and/or purchasing high-quality forage, and using silage inoculants are all crucial [21]. Despite all of these, the increased cost of improving cow nutrition may have contributed to Romania's sharp decline in bovine herds in recent years (2,092,414 heads in 2016 to 1,910,900 heads in 2020) [22].

Cows maintained on pasture from May through October not only lowers food costs but also alters the lipid content of milk, which may have a good effect on consumer health.

The milk yields at grazing period can be higher compared to those obtained in the rest of the year or compared to those obtained from animals raised exclusively in stables. In Romania, in the area of Suceava county, seasonal grazing and supplements with freshly mowed grass for cows can be easily organised [23,24]. Consumers prefer to buy dairy products labelled as ecological or natural, because they currently believe that milk from cows that graze is healthier than that issued from cows fed almost exclusively from feedstock, indoors [25].

We hypothesise that the experimental factor (cows feeding) affects mostly the lipids profile of raw milk and of the subsequent produced yogurts, while the proximate composition of the dairy products is pretty similar, regardless of the cows' dietary specificity. For these reasons, the primary goal of this investigation was to examine the variations in the chemical composition and fatty acids profile of milk from cows maintained on pasture (MGC–milk from grazed cows) and of that produced by cows exclusively maintained in stable (MSC–milk from stable cows), in light of the growing attention that consumers pay to the quality attributes of milk and milk products. The milk from the two cows' groups was processed to prepare a yogurt (using whole, not skimmed milk), analysed to assess the differences given by the cows' maintenance system and of raw milk quality on its physical, chemical, textural and nutritional quality.

2. Materials and Methods

2.1. Animals and Feeding

The research was carried out at the Best Cows Sadova farm, located in the village of Sadova, Suceava County, Romania. The territory of the village has a total area of 6786 ha, out of which were: forest vegetation 3974 ha (58.56%), agricultural land, predominantly hay and pastures, 2575 ha (38.00%) and land with other uses. All investigations were carried on the basis of the Statement on Research Bioethics no. 32/03 May 2022, issued by the Committee of Ethics and Bioethics, Faculty of Food and Animal Sciences, Iasi University of Life Sciences.

Within the farm, 80 heads of Bălțată Românească breed (Romanian Spotted with Brown, part of the Siemmental breed family) are raised. They were randomly allotted in two groups: GC (n = 40), cows mostly fed on pasture with corn silage supplementation and less concentrated feedstuffs and SC, (n = 40) cows fed indoors benefiting from diet based on hay, corn silage and increased quantities of energy and protein concentrated feedstuffs (Table 1). Average body weight of cows was 700 kg, and average individual daily milk yield was 25 kg, with 4% fat. These values were used to calculate the nutritional requirements and to elaborate diets, in accordance with INRA France methodology [26]. Chemical composition values of the feedstuffs available at the farm was assessed within an accredited laboratory and were used to formulate the diets. The values are presented in Table 1, along with the diet nutritional specifications.

The period in which the study was carried out was from May to mid-October. The milk for analysis as well as for the production of yogurt was collected throughout 3 consecutive days in the last week of May, July and September, from the afternoon milking, daily. Animals have had passed the midterm of a normal lactation period (305 days), reaching lactation days 186–192 at the moment of the first samples collection, knowing they calved grouped within January 10–16 period. Grazed cows were kept in stable throughout the night, without feed access but with ad libitum water. They were released on pasture at 5 AM and brought back in stable at 9 PM. After morning and afternoon milking, they were provided, under a covered shelter, corn silage and concentrate mix (corn crumbles, rapeseed meal, minerals, salt and premix), twice a day (at 6 AM and 5 PM). Cows kept exclusively in stable received two main meals per day after morning and afternoon milking, silage and concentrate mix (corn crumbles, minerals, salt and premix). After their intake, they were provided hay, throughout the day, between milking moments.

Table 1. Nutritional requirements and diets provided daily to dairy cows (live weight 700 kg, daily milk yield 25 kg, milk fat 4%).

Daily Diet Type, Structure and Proximate Composition	*Nutritional Requirements:* IDM (kg) Up to 19.5	MLDU Up to 17.10	NEMU 16.6	PDI-N (g) 1645.00	PDI-E (g) 1645.00	Ca (g) 136.00	P (g) 76.00
Cows maintained on pasture (GC)	*Covered:*						
Pasture–graminaea, 66.0 kg (9.5 kg IDM); Corn silage, 20.5 kg (5 kg IDM); Corn crumbles, 2.9 kg (2.5 kg IDM); Rapeseed meal, 1.8 kg (1.6 kg IDM); Limestone, 0.1 kg (0.1 kg IDM); Sodium bicarbonate, 0.1 kg (0.1 kg IDM); Salt, 0.1 kg (0.1 kg IDM); Premix Vitafort Bio, 0.1 kg (0.1 kg IDM) ***Diet proximate composition per 1000 g IDM*** 87.3 g CAsh, 912.7 g OM, 230.8 g CP, 45.1 g EE, 183.2 g CF, 230.3 g ADF, 355.5 g NDF, 453.5 g NFC	19.0	15.9	17.2	1705	1645	138	78
Cows maintained in stable (GS)	*Covered:*						
Meadow hay–graminaea, 11.6 kg (9 kg IDM); Corn silage, 14.4 kg (3.5 kg IDM); Corn grains crumbles, 4.3 kg (3.8 kg IDM); Rapeseed meal, 3.0 kg (2.7 kg IDM); Limestone, 0.2 kg (0.2 kg IDM); Sodium bicarbonate, 0.1 kg (0.1 kg IDM); Salt, 0.1 kg (0.1 kg IDM); Premix Vitafort Bio, 0.1 kg (0.1 kg IDM) ***Diet proximate composition per 1000 g IDM*** 82.0 g CAsh, 918.0 g OM, 220.9 g CP, 45.2 g EE, 171.5 g CF, 215.6 g ADF, 332.8 g NDF, 480.5 g NFC	19.5	13.8	17.3	1661	1654	139	76

IDM—ingested dry matter (maximal physiological threshold); Cash—crude ash; OM—organic matter; CP—crude protein; EE—ether extract; CF—crude fibre; ADF—acid detergent fibre; NDF—neutral detergent fibre; NFC—non-fibrous carbohydrates; MLDU—milk load digestive units (maximal physiological threshold); NEM U—net energy for milk units; PDI—N (protein digestible at intestinal level—synthesised on dietary nitrogen basis). PDIE (protein digestible at intestinal level—synthesised on dietary energy basis).

2.2. Raw Milk Collecting, Sampling and Analysis

Grazed cows were milked using individual cans via a mobile mechanical collecting device, and the milk was deposited in a separate tank, while cows in stabulation were milked in the parlour of the stable, and the milk was stored in the main tank of the farm. Usually, at the farm level, the milk is stored up to 14 h, at 4 °C, between collections run every morning by a regional processing company to collect the milk from evening and morning milkings. The milk hygienic status was within the limits of maximum 100,000 germs/mL (regular checks of the collecting company indicated levels below 50,000 germs/mL) and the udder health status was within the maximal threshold of 400,000 somatic cells/mL (regular checks of the collecting company indicated levels below 160,000 somatic cells/mL). The milk used in our study (150 L) was extracted from the two storage tanks of the farm. Due to the fact that cows were milked separately, in accordance with their allotting, the yielded milk was coded accordingly: MGC (milk from GC group, grazed cows) and MSC (milk from stabulation cows). Milk was transported to the dairy processing centre by a truck equipped with a thermo-regulated refrigeration tank with separate cells. The temperature of the milk in the transportation tank was kept at 5 °C. Five samples of 500 mL each were collected in sterile containers from each group cell and taken to the laboratory in special boxes equipped with ice packs and then stored under refrigeration conditions at 4 °C for 24 h. Prior to analysing, an average sample was formed per group from each of the five original samples, and milk was thoroughly homogenised and introduced to subsequent analytical laboratory investigations (10 replications per analysed trait/method).

After calibrating the pH meter (WTW InoLab, Xylem Analytics GmbH, Weilheim, Germany), the pH was measured using a glass electrode with a temperature probe (buffer solutions pH 4 and 7). Total solids (TS) in milk were assessed by the AOAC method no. 925.23 [27], and the samples were dehydrated in a Memmert UFE 700 forced air oven (Memmert GmbH, Schwabach, Germany). Water (W) content resulted from the difference, according to the relation (1).

$$W\ (\%) = 100\% - TS\ (\%) \quad (1)$$

Fat of milk (Fat %) was assessed through the acid-butyrometric Gerber method [28], using a Nova Safety Funke Gerber thermo-regulated centrifuge (Funke Gerber GmbH, Berlin, Germany) and Funke Gerber Milch 65 °C calibrated butyrometers (Funke Gerber GmbH, Germany). Regarding the non-fat solid (SNF) content, this acetate was calculated by difference, in accordance with relation (2).

$$SNF\ (\%) = TS\ (\%) - Fat\ (\%) \quad (2)$$

Crude ash (total minerals) content was assessed via incineration at 550 °C, in a Super Therm C311 furnace (SuperTherm SRL, Romania) after prior combustion on a Bunsen funnel, until samples ceased to smoke, in accordance with AOAC 945.46 specifications [29,30].

The crude protein (CP), true protein (TP), casein, non-casein nitrogen (NCN), whey proteins and non-protein nitrogen (NPN) contents were determined by using Kjeldahl method applied on a Velp Scientifica DK 6 digestion unit and UDK 7 distillation system (VelpScientifica, Usmate, Italy) according to standard protocol of IDF [31]. The total nitrogen content was multiplied by 6.38, which generated the crude protein content. The TP in the milk sample were determined by treating with 12% trichloroacetic acid. The nitrogen (%) was converted to NPN and NCN contents by using the conversion factor 3.60 and 6.25, respectively. Protein (nitrogen) fractions were calculated using Equations (3)–(5):

$$TP = CP - NPN \quad (3)$$

$$Casein\ (N\%) = Total\ protein\ (N\%) - NCN\ (N\%) \quad (4)$$

$$Whey\ protein = NCN - NPN \quad (5)$$

2.3. Yogurt Analysis

2.3.1. Yogurt Preparation

From each type of milk (MGC and MSC), two quantities of 25 L were used as raw matter for yogurt processing, and two corresponding groups were formed. Yogurt samples were codified based on their originating raw matter: YGC samples (yogurt produced from milk cows maintained on pasture) and YSC samples (yogurt produced from milk cows maintained in stable). According to Attia et al. [32], milk was pasteurized using a thermal treatment installation (milk pasteuriser type IPL-1M, produced by ICPIAS S.A., Cluj-Napoca, Romania) at 85–90 °C for 20–30 min, then chilled to 43 °C, and the probiotic starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus*, YF-L812 commercial product, Chr. HANSEN, Hørsholm, Denmark), were inoculated (starting from the standard 50 U culture per 250 L of milk, according to the manufacturer's guidelines). The mixture was afterwards incubated in plastic cups (the capacity of the plastic cups used for yogurt was 250 mL (diameter 65 mm and height 108 mm)) at 43 °C, using a laboratory incubator (IT 40 thermostatic chamber, produced by Electronic April S.R.L., Cluj-Napoca, Romania) until a hard coagulum and a pH range of 4.3 to 4.5 were obtained (5–6 H). The samples of yogurt were then kept at 4 °C for 24 h, prior to further analyses.

2.3.2. Texture Analysis

Textural analysis of yogurt assortments was carried out with the Mark 10 ESM 300 texturometer (Mark-10 Inc., Copiague, NY, USA), equipped with a digital dynamometer of 25 N (resolution 0.005 N). Analysis was carried out on 3 yogurt samples from each yogurt type, following a non-stationary manner, for two complete cycles of displacement of the probe in the yogurt mass. The determinations were performed directly in the glass in order not to destroy the clot. The cylindrical probe used for texture was matched to the diameter of the glass so that the distance from the probe to the wall of the glass was 13 mm. For all analysed samples, the temperature was 4 ± 1 °C. This test procedure results in the texture curve profile [33]. Regarding the principle of the method, it consists of determining the texture by exerting compressive stress on the coagulum using a Brookfield TA4/1000 cylindrical probe (h = 20 mm, D = 38.1 mm, Brookfield AMETEK Inc., Middleborough, MA, USA). The force was recorded continuously during the experiment. As a working method, throughout the course of the experiment, the cylindrical probe exerts different compression forces depending on the firmness of the clot. The data were recorded by obtaining the texture profile from which a series of textural parameters are determined such as: cohesiveness, elasticity, hardness, gumminess, consistency, resilience, adhesiveness, adhesive and coagulum breaking force. After returning the probe from the clot mass to its initial position, a new compression cycle is performed by reintroducing the probe into the clot mass to determine the resilience of the clot. Experimental results were obtained through 10 repetitions.

2.3.3. Physical and Chemical Analyses

Both pH and acidity determinations were performed according to the methods described by Say et al. [34]. Briefly, 10 g of yogurt sample was dissolved in 10 mL of distilled water. The mixture was allowed to equilibrate at room temperature. The pH of the samples was then determined by a pH meter (WTW InoLab GmbH, Weilheim, Germany).

The titratable acidity was measured and calculated. Samples of 10 g each were quickly dissolved in 30 mL of distilled water and carefully blended. In the obtained solution, a few drops of phenolphthalein indicator were added. To ensure full neutralization, it was titrated against a standard 0.1 N NaOH until a light pink colour appeared and persisted for at least 10–15 s. Lactic acid, the primary organic acid in yogurt samples, was used to compute the titratable acidity and is shown in Equation (6).

$$\text{Titrable acidity } (\%) = \frac{\text{vol. 0.1 NaOH (mL)} \times 0.1 \times 0.009 \times 100}{\text{mass of sample (g)}} \quad (6)$$

The proportion of free whey is used to represent the degree of syneresis (i.e., the spontaneous release of the watery part of yogurts due to gel contraction). The Wijesinghe et al. [35] approach was used to measure it. In a nutshell, 10 g of each yogurt sample was placed separately on a sheet of filter paper and allowed to rest on top of a funnel. The quantity of residual yogurt was weighed after draining under vacuum for 10 min, and the syneresis was computed using Equation (7).

$$\text{Free whey } (\%) = \frac{\text{mass of initial sample(g)} - \text{mass of sample after filtration(g)} \times 100}{\text{weight of initial sample (g)}} \quad (7)$$

The total solids content of yogurt according to IDF [36]. The proximate analysis (moisture, crude protein, fat and ash) was assessed using the AOAC method [37] as follows: moisture (Method No. 990.20), crude protein (Method No. 945.46), fat content (Method No. 905.02) and ash (Method No. 991.20). Experimental results were obtained through 10 repetitions.

2.4. Fatty Acids Analysis in Milk and Yogurt

2.4.1. Fat Extraction

From raw milk, fat was extracted using the Roese-Gottlieb method [38], and the yogurt fat extraction was performed using the Folch method [39]. Both for the determinations performed on milk and those performed on yogurt, 10 repetitions were performed.

2.4.2. Preparation of Fatty Acid Methyl Esters

The IDF standard method (ISO 15884:2002) was used in the process of converting fatty acids into their corresponding fatty acid methyl esters (FAME) [40].

2.4.3. Analysis of Fatty Acid Composition by GC Method

Gas chromatography was utilized to analyse the fatty acid (FA) composition using an HP 6890 GC System (HP Agilent co., Münster, Germany) with a flame-ionization detector (FID). The lipid phase was utilized, and the film thickness was 0.2 μm in a capillary column CP Sil 88 (Chrom8 International BV, Lelystad, The Netherlands) with a length of 100 m and an internal diameter of 0.25 mm. The following settings were used for the analysis: helium was used as the carrier gas, and the gas flow rate was 1.5 mL/min. The column temperature ranged from 60 °C (for 1 min) to 180 °C (Δt = 5 °C/min), the detector temperature was 250 °C, and the injector temperature was 225 °C. The volume of the sample injection was 0.4 μL (split mode 50:1). To identify the fatty acids included in the examined products, the retention times of fatty acids were compared to the retention times of methyl esters of fatty acids of reference milk fat (BCR Reference Materials) of the CRM 164 symbol and the literature data [41,42]. The cis-9, trans-11 CLA isomer was identified using a combination of CLA methyl esters from Sigma-Aldrich, St. Louis, Missouri, in the United States. Positional trans isomers of C18:1 were identified using standards of methyl esters (Sigma Aldrich, St. Louis, MO, USA), whereas trans isomers of C18:2 acid (cis, trans, and trans, cis) were identified using a combination of standards of C18:2 isomers (Supelco, Bellefonte, PA, USA). The individual fatty acids were expressed as mean relative percentages of the total FAME identified.

The hypocholesterolemic fatty acids were calculated using Equation (8) (DFA) [43].

$$DFA = UFA + C18:0 \quad (8)$$

The index of hypercholesterolemic fatty acids (OFA) was calculated using Equation (9) [43].

$$OFA = C12:0 + C14:0 + C16:0 \quad (9)$$

2.5. Data Analysis

For physicochemical indices of raw milk and yogurts, the data issued from 10 analytical repetitions were conducted in triplicate for each sample and subsequently subjected to statistical computation, using the GraphPad Prism 9.4.1 software (Graph Pad Ltd., Palo Alto, CA, USA). Table data are presented as mean and standard deviation. Significant differences among results were identified using analysis of variance (ANOVA). Tukey's test was applied to determine which pairwise comparisons were significant. For all tests, p-values of $p < 0.05$ were considered [44].

3. Results

3.1. Raw Milk Quality

The results of this study highlight the fact that the feeding system, as well as the period in which the milk was harvested, has a significant effect ($p < 0.05$) on the chemical composition of the milk. Total lactation average milk solids content from cows on the MGC system was significantly higher than that of MCS ($p < 0.05$) systems. Maximum solids contents were recorded in September (12.90 ± 0.08% for MGC and 12.79 ± 0.14% for MCS), and minimum contents were observed during early lactation (May) for each diet (Table 2).

The cows from the MCS feeding system produced milk with significantly higher ($p < 0.05$) total lactation average milk fat content (4.21 ± 0.05%) than that of MGC (4.32 ± 0.09%). Regarding the fat content, the lowest level was recorded in May at MGC (4.18 ± 0.04%), but the highest value was also noted in the case of milk from MGC collected in September, namely, 4.41 ± 0.06%, compared to 4.23 ± 0.04% as obtained at MCS ($p < 0.05$). Mean analysis of fat content was significantly higher in MGS compared to MCS ($p < 0.05$).

Table 2. The chemical composition and the fraction of protein of raw milk.

Physical–Chemical Trait	System of Exploitation	The Experimental Period			Overall
		May	July	September	
pH	MGC	6.51 ± 0.02^{xA}	6.44 ± 0.03^{yB}	6.45 ± 0.02^{yB}	6.47 ± 0.04^{y}
	MCS	6.52 ± 0.04^{xA}	6.50 ± 0.04^{xA}	6.51 ± 0.04^{xA}	6.51 ± 0.04^{x}
Water (W) (%)	MGC	87.32 ± 0.10^{xA}	87.13 ± 0.13^{xB}	87.10 ± 0.08^{xB}	87.18 ± 0.14^{x}
	MCS	87.23 ± 0.14^{xA}	87.24 ± 0.14^{xA}	87.22 ± 0.14^{xA}	87.23 ± 0.14^{x}
Total solids (TS) (%)	MGC	12.68 ± 0.10^{xC}	12.87 ± 0.13^{xB}	12.90 ± 0.08^{xAB}	12.82 ± 0.14^{x}
	MCS	12.77 ± 0.14^{yA}	12.78 ± 0.14^{yA}	12.80 ± 0.14^{yA}	12.78 ± 0.14^{y}
Fat (%)	MGC	4.18 ± 0.04^{xC}	4.37 ± 0.05^{xB}	4.41 ± 0.06^{xAB}	4.32 ± 0.09^{x}
	MCS	4.20 ± 0.06^{yA}	4.20 ± 0.06^{yA}	4.23 ± 0.04^{yA}	4.21 ± 0.05^{y}
Solid non-fat (SNF) (%)	MGC	8.63 ± 0.10^{xB}	8.50 ± 0.15^{xA}	8.49 ± 0.06^{xBA}	8.54 ± 0.12^{x}
	MCS	8.57 ± 0.17^{yA}	8.58 ± 0.17^{yA}	8.57 ± 0.18^{yA}	8.57 ± 0.17^{x}
Ash (%)	MGC	0.75 ± 0.03^{xA}	0.79 ± 0.02^{yA}	0.80 ± 0.05^{xA}	0.78 ± 0.04^{x}
	MCS	0.77 ± 0.05^{xA}	0.76 ± 0.05^{xA}	0.78 ± 0.05^{yA}	0.77 ± 0.04^{x}
The protein fractions of milk					
Crude protein (CP) (%)	MGC	3.45 ± 0.07^{xA}	3.46 ± 0.07^{xA}	3.48 ± 0.07^{xA}	3.46 ± 0.07^{x}
	MCS	3.34 ± 0.04^{yB}	3.32 ± 0.04^{yB}	3.36 ± 0.04^{yA}	3.34 ± 0.09^{y}
True protein (TP) (%)	MGC	3.16 ± 0.04^{xB}	3.15 ± 0.05^{xB}	3.19 ± 0.05^{xA}	3.17 ± 0.08^{x}
	MCS	3.04 ± 0.06^{yA}	3.02 ± 0.06^{yA}	3.06 ± 0.06^{yA}	3.04 ± 0.06^{y}
Casein (%)	MGC	2.44 ± 0.06^{xA}	2.42 ± 0.06^{xA}	2.48 ± 0.06^{xA}	2.45 ± 0.06^{x}
	MCS	2.30 ± 0.05^{yA}	2.29 ± 0.06^{yA}	2.33 ± 0.04^{xA}	2.33 ± 0.07^{y}
Whey protein (WP) (%)	MGC	0.44 ± 0.02^{xC}	0.42 ± 0.02^{xB}	0.47 ± 0.02^{xAC}	0.44 ± 0.02^{x}
	MCS	0.41 ± 0.01^{xA}	0.40 ± 0.02^{xA}	0.42 ± 0.02^{yA}	0.41 ± 0.02^{y}

MGC = milk from grazed cows; MCS = milk from cows maintained permanently in stable; SEM = standard error of mean. Averages with different letters "A", "B", "C" in the rows and "x", "y" in the columns indicate statistically significant differences ($p < 0.05$).

The analysis of the data regarding the ash content highlights a constant level throughout the period, the average being 0.78 ± 0.04% at MGC and 0.77 ± 0.04% at MCS ($p > 0.05$).

The analysis of the data regarding the protein fraction of milk highlights differences between MGC and MCS ($p < 0.05$) for crude protein, true protein and casein, but evaluating the data according to the period, we notice that differences appear in the case of TP to MGC between the milk collected in the months May and July compared to September ($p < 0.05$) (Table 2). In the case of WP, no differences were noted ($p > 0.05$) generated by the rearing system or the period in which the milk was collected.

Data on the profile of fatty acids in milk are reported in Table 3. Myristic (C14:0), Palmitic (C16:0) and Stearic (C18:0) acid contributed most to total FAs. Their concentration (overall) was 10.36, 28.05 and 9.81 g/100 g total FAs for MGC and 12.13, 35.31 and 8.91 g/100 g for MCS, respectively. The saturated FAs contributed 62.37 g in the case of MGC and 71.34 g in the case of MCS ($p < 0.05$), and monounsaturated FAs contributed

5.62 g/100 g in the case of MGC and 2.41 g/100 g in the case of MCS ($p < 0.05$). For the polyunsaturated FAs, the overall was 4.66 g for MGC and 2.66 g for MCS ($p < 0.05$). The content of CLA was 0.93 g/100 g total FAs. Table 3 demonstrates how the time of milk collection had a substantial impact on the amount of FAs present in MGC lacking C12:0, C17:0, C18:0 trans-11, and C18:2. In the case of PUFA, the highly significant differences caused by the milk harvesting time were also found in May, where an average value of 5.05 g/100 g was obtained in contrast to 4.69 g/100 g (July) and 4.26 g/100 g (September) ($p < 0.05$). Moreover, differences for SFA, DFA and OFA were identified within the MGC ($p < 0.05$). When it came to UFA, the May result (10.75 g/100 g) was significantly higher than the values from July and September (10.07 g/100 g and 10.04 g/100 g, respectively). For MGC, the highest DFA/OFA ratio values were in May (0.49 g/100 g) and July (0.50 g/100 g), as opposed to September, when the average value was 0.46 g/100 g ($p < 0.05$).

Table 3. The main fatty acids in cow's milk (g/100 g fatty acids methyl esters).

Physical–Chemical Trait	Type of Nutrition	The Experimental Period			Overall
		May	July	September	
Butyric acid-C4:0	MGC	3.08 ± 0.31^{xB}	3.29 ± 0.14^{Xa}	2.95 ± 0.20^{xB}	3.11 ± 0.26^{x}
	MCS	2.82 ± 0.20^{yA}	2.83 ± 0.20^{yA}	2.81 ± 0.20^{xA}	2.82 ± 0.19^{y}
Caprionic acid-C6:0	MGC	1.99 ± 0.19^{xB}	2.11 ± 0.14^{xA}	1.82 ± 0.15^{yC}	1.97 ± 0.20^{y}
	MCS	2.07 ± 0.04^{xA}	2.08 ± 0.04^{xA}	2.06 ± 0.04^{xA}	2.07 ± 0.04^{x}
Caprylic acid-C8:0	MGC	1.19 ± 0.07^{yB}	1.45 ± 0.07^{xA}	1.24 ± 0.06^{yB}	1.29 ± 0.13^{x}
	MCS	1.30 ± 0.02^{xA}	1.31 ± 0.02^{yA}	1.29 ± 0.02^{xA}	1.30 ± 0.02^{x}
Capric acid-C10:0	MGC	3.03 ± 0.16^{yA}	2.91 ± 0.12^{yB}	2.44 ± 0.12^{yC}	2.79 ± 0.29^{y}
	MCS	3.13 ± 0.08^{xA}	3.14 ± 0.08^{xA}	3.12 ± 0.08^{xA}	3.13 ± 0.07^{x}
Lauric acid-C12:0	MGC	3.18 ± 0.10^{yB}	3.17 ± 0.09^{yB}	2.64 ± 0.33^{yA}	2.99 ± 0.33^{y}
	MCS	3.50 ± 0.08^{xA}	3.51 ± 0.08^{xA}	3.49 ± 0.08^{xA}	3.50 ± 0.08^{x}
Myristic acid-C14:0	MGC	9.61 ± 0.48^{yC}	11.22 ± 0.31^{yA}	10.24 ± 0.32^{yB}	10.36 ± 0.77^{y}
	MCS	12.13 ± 0.09^{xA}	12.14 ± 0.09^{xA}	12.12 ± 0.09^{xA}	12.13 ± 0.08^{x}
Myristoleic acid-C14:1	MGC	1.20 ± 0.04^{xB}	1.15 ± 0.04^{xC}	1.26 ± 0.04^{xA}	1.20 ± 0.06^{x}
	MCS	0.65 ± 0.04^{yA}	0.66 ± 0.04^{yA}	0.64 ± 0.04^{yA}	0.65 ± 0.04^{y}
Pentadecylic acid-C15:0	MGC	1.26 ± 0.05^{yA}	1.15 ± 0.03^{yB}	1.12 ± 0.04^{yC}	1.18 ± 0.07^{y}
	MCS	1.34 ± 0.03^{xA}	1.35 ± 0.03^{xA}	1.33 ± 0.03^{xA}	1.34 ± 0.03^{x}
Palmitic acid-C16:0	MGC	28.64 ± 1.07^{yA}	27.85 ± 0.83^{yB}	27.65 ± 0.67^{yB}	28.05 ± 0.95^{y}
	MCS	35.31 ± 0.31^{xA}	35.32 ± 0.31^{xA}	35.30 ± 0.31^{xA}	35.31 ± 0.30^{x}
Palmitoleic acid-C16:1	MGC	2.12 ± 0.09^{xA}	1.94 ± 0.09^{xC}	2.02 ± 0.07^{xB}	2.02 ± 0.11^{x}
	MCS	1.14 ± 0.04^{yA}	1.15 ± 0.04^{yA}	1.13 ± 0.04^{yA}	1.14 ± 0.04^{y}
Margaric acid-C17:0	MGC	0.72 ± 0.11^{xA}	0.67 ± 0.06^{xA}	0.60 ± 0.07^{xB}	0.66 ± 0.10^{x}
	MCS	0.62 ± 0.02^{yA}	0.63 ± 0.02^{xA}	0.61 ± 0.02^{xA}	0.62 ± 0.02^{y}
Stearic acid-C18:0	MGC	9.56 ± 0.43^{xB}	11.08 ± 0.82^{xA}	8.80 ± 0.37^{xC}	9.81 ± 1.11^{x}
	MCS	8.91 ± 0.48^{yA}	8.92 ± 0.48^{yA}	8.90 ± 0.48^{xA}	8.91 ± 0.46^{y}
Vaccenic acid-C18:1 trans-11	MGC	2.39 ± 0.14^{xB}	2.30 ± 0.14^{xB}	2.50 ± 0.14^{xA}	2.40 ± 0.16^{x}
	MCS	0.62 ± 0.06^{yA}	0.63 ± 0.06^{yA}	0.61 ± 0.06^{yA}	0.62 ± 0.06^{y}
C18:2	MGC	2.52 ± 0.20^{xA}	2.46 ± 0.18^{xA}	1.96 ± 0.18^{xB}	2.31 ± 0.31^{x}
	MCS	1.51 ± 0.03^{yA}	1.52 ± 0.03^{yA}	1.50 ± 0.03^{yA}	1.51 ± 0.03^{y}

Table 3. *Cont.*

Physical–Chemical Trait	Type of Nutrition	The Experimental Period			Overall
		May	July	September	
C18:3	MGC	1.13 ± 0.10^{xA}	0.91 ± 0.05^{xC}	1.02 ± 0.05^{xB}	1.02 ± 0.12^{x}
	MCS	0.24 ± 0.02^{yA}	0.25 ± 0.02^{yA}	0.23 ± 0.02^{yA}	0.24 ± 0.02^{y}
Arachidic acid C20:0	MGC	0.19 ± 0.02^{xA}	0.17 ± 0.02^{yB}	0.13 ± 0.02^{yC}	0.16 ± 0.03^{y}
	MCS	0.19 ± 0.02^{xA}	0.20 ± 0.02^{xA}	0.18 ± 0.02^{xAB}	0.19 ± 0.02^{x}
Rumeric acid-CLA	MGC	1.40 ± 0.08^{xA}	1.32 ± 0.07^{xB}	1.28 ± 0.05^{xB}	1.33 ± 0.08^{x}
	MCS	0.91 ± 0.08^{yA}	0.92 ± 0.08^{yA}	0.90 ± 0.08^{yA}	0.91 ± 0.08^{y}
Monounsaturated fatty acid-MUFA	MGC	5.71 ± 0.22^{xA}	5.39 ± 0.22^{xB}	5.78 ± 0.19^{xA}	5.62 ± 0.27^{x}
	MCS	2.41 ± 0.08^{yA}	2.44 ± 0.08^{yA}	2.38 ± 0.08^{yA}	2.41 ± 0.08^{y}
Polyunsaturated fatty acid-PUFA	MGC	5.04 ± 0.23^{yA}	4.69 ± 0.18^{xB}	4.26 ± 0.22^{xC}	4.66 ± 0.38^{x}
	MCS	2.66 ± 0.09^{xA}	2.69 ± 0.09^{yA}	2.63 ± 0.09^{yA}	2.66 ± 0.09^{y}
Saturated fatty acid-SFA	MGC	62.45 ± 1.26^{yB}	65.06 ± 0.67^{yA}	59.61 ± 0.62^{yC}	62.37 ± 2.42^{y}
	MCS	71.34 ± 0.61^{xA}	71.45 ± 0.61^{xA}	71.23 ± 0.61^{xA}	71.34 ± 0.60^{x}
Unsaturated fatty acids-UFA	MGC	10.75 ± 0.33^{xA}	10.07 ± 0.32^{xB}	10.04 ± 0.32^{xB}	10.29 ± 0.46^{x}
	MCS	5.07 ± 0.07^{yA}	5.13 ± 0.07^{yA}	5.01 ± 0.07^{yA}	5.07 ± 0.08^{y}
DFA	MGC	20.31 ± 0.49^{xB}	21.15 ± 0.83^{xA}	18.84 ± 0.51^{xC}	20.10 ± 1.15^{x}
	MCS	13.99 ± 0.48^{yA}	14.06 ± 0.48^{yA}	13.92 ± 0.48^{yA}	13.99 ± 0.46^{y}
OFA	MGC	41.42 ± 1.18^{yB}	42.24 ± 0.78^{yA}	40.53 ± 0.58^{yC}	41.40 ± 1.11^{y}
	MCS	50.95 ± 0.27^{xA}	50.98 ± 0.27^{xA}	50.92 ± 0.27^{xA}	50.95 ± 0.26^{x}
DFA/OFA	MGC	0.49 ± 0.02^{xA}	0.50 ± 0.03^{xA}	0.46 ± 0.01^{xB}	0.49 ± 0.02^{x}
	MCS	0.27 ± 0.01^{yA}	0.28 ± 0.01^{yA}	0.27 ± 0.01^{yA}	0.27 ± 0.01^{y}

DFA = desirable hypocholesterolemic fatty acids, OFA = hypercholesterolemic fatty acid, DFA/OFA = desirable hypocholesterolemic fatty acids/hypercholesterolemic fatty acid. Averages with different letters "A", "B", "C" in the rows and "x", "y" in the columns indicate statistically significant differences ($p < 0.05$).

In the case of milk collected from MCS, differences given by the period of milk collection were reported only in the case of C20:0, where the average value obtained in May was 0.19 g/100 g, 0.20 g/100 g in July and 0.18 g/100 g in September.

In comparison to the indoor period, the milk fat produced during the grazing period (MGC) contained more long-chain FAs and fewer medium-chain FAs (MCS). The milk fat in the grazing period had higher monounsaturated fat (5.62 g/100 g) and polyunsaturated fat (4.66 g/100 g total FAs) ($p < 0.05$) and lower saturated fat (62.37 g/100 g; p 0.05) than the milk fat in the indoor period (MCS) ($p < 0.05$), which showed that milk fat from the indoor period (MCS) (35.31 g/100 g) had higher palmitic acid than milk fat from the grazing period (MGC) (28.05 g/100 g).

The concentration of CLA was higher in the grazing period (MGC) (1.33 g/100 g total FAs) than in the indoor period (0.91 g/100 g total FAs) ($p < 0.05$). Also, higher concentrations in the grazing period (MGC) were also observed in the case of MUFA, PUFA and UFA ($p < 0.05$) compared to those obtained for milk fat from the indoor period (MCS).

For the DFA, the average value obtained was 20.10 g/100 g for milk fat produced in the grazing period (MGC) and 13.99 g/100 g for milk fat from the indoor period (MCS) ($p < 0.05$). For OFA, milk fat produced in the grazing period (MGC) was 41.40 g/100 g lower compared to that from MCS which was 50.95 g/100 g ($p < 0.05$). Regarding the DFA/OFA ratio, the mean value for MGC was 0.49 g/100 g and 0.27 g/100 g for MCS ($p < 0.05$).

3.2. Yogurts Quality

3.2.1. Texture Testing

TPA parameters (cohesiveness, springiness, hardness, gumminess, firmness, resilience adhesiveness, adhesiveness force and breaking force) of the yogurt samples are given in Table 4.

Table 4. Texture parameters of yogurt.

Physical–Chemical Trait	System of Exploitation	The Experimental Period			Overall
		May	July	September	
Cohesiveness	YGC	0.21 ± 0.03^{xC}	0.26 ± 0.03^{xB}	0.28 ± 0.03^{xAB}	0.25 ± 0.04^{x}
	YCS	0.19 ± 0.04^{xC}	0.24 ± 0.04^{xB}	0.24 ± 0.04^{xAB}	0.23 ± 0.05^{x}
Springiness	YGC	0.49 ± 0.05^{yC}	0.54 ± 0.05^{yB}	0.56 ± 0.05^{yAB}	0.53 ± 0.06^{y}
	YCS	0.57 ± 0.04^{xB}	0.62 ± 0.04^{xA}	0.62 ± 0.04^{xA}	0.60 ± 0.05^{x}
Hardness (*N*)	YGC	1.62 ± 0.05^{xC}	1.67 ± 0.05^{xB}	1.69 ± 0.05^{xAB}	1.66 ± 0.05^{x}
	YCS	1.62 ± 0.05^{xB}	1.67 ± 0.05^{xA}	1.67 ± 0.05^{xA}	1.65 ± 0.05^{x}
Gumminess (*N*)	YGC	0.35 ± 0.04^{xC}	0.40 ± 0.04^{xB}	0.42 ± 0.04^{xAB}	0.39 ± 0.05^{x}
	YCS	0.35 ± 0.04^{xB}	0.40 ± 0.04^{xA}	0.40 ± 0.04^{xA}	0.38 ± 0.05^{x}
Firmness	YGC	5.16 ± 0.04^{xA}	5.21 ± 0.04^{xA}	5.23 ± 0.04^{xA}	5.20 ± 0.05^{x}
	YCS	4.67 ± 0.44^{yA}	4.72 ± 0.44^{yA}	4.72 ± 0.44^{yA}	4.71 ± 0.43^{y}
Resilience	YGC	0.23 ± 0.04^{yC}	0.28 ± 0.04^{Bx}	0.30 ± 0.04^{xAB}	0.27 ± 0.05^{y}
	YCS	0.29 ± 0.03^{xB}	0.29 ± 0.04^{xB}	0.34 ± 0.03^{xA}	0.30 ± 0.04^{x}
Adhesiveness (*mJ*)	YGC	-0.23 ± 0.04^{xA}	-0.18 ± 0.04^{xB}	-0.16 ± 0.04^{xB}	-0.19 ± 0.05^{y}
	YCS	-0.19 ± 0.04^{yA}	-0.14 ± 0.04^{yB}	-0.14 ± 0.04^{xB}	-0.16 ± 0.05^{x}
Adhesiveness force (*N*)	YGC	-0.33 ± 0.04^{yA}	-0.28 ± 0.04^{yB}	-0.26 ± 0.04^{xB}	-0.29 ± 0.05^{y}
	YCS	-0.27 ± 0.05^{xA}	-0.22 ± 0.05^{xB}	-0.22 ± 0.05^{xB}	-0.24 ± 0.05^{x}
Breaking force *(N)*	YGC	1.48 ± 0.04^{xC}	1.53 ± 0.04^{xB}	1.55 ± 0.04^{xAB}	1.52 ± 0.05^{x}
	YCS	1.45 ± 0.04^{xB}	1.50 ± 0.04^{xA}	1.50 ± 0.04^{yA}	1.48 ± 0.05^{y}

N—newtons; mJ—millijoule. Averages with different letters "A", "B", "C" in the rows and "x", "y" in the columns indicate statistically significant differences ($p < 0.05$).

The study of the data pertaining to the texture of the yogurt reveals that the time (season) during which the milk was gathered and processed had a bearing on the qualitative indexes ($p > 0.05$).

The cohesiveness is a parameter that is strongly influenced by the protein content and the specificity of the internal links of the protein structure that forms the clot. Analysing the data obtained for the texture, for the two yogurt samples, it is observed that the cohesiveness of YGC has an overall value of 0.25, and for the YSC, the value is lower, being 0.23 ($p < 0.05$). Since both values are close to 0 in the analysed samples, it highlights a low resistance to deformation as a result of weak internal bonds.

For the springiness, the overall was 0.53 for YGC and 0.60 for YCS ($p < 0.05$). The hardness of the clot was measured in order to represent the resistance of the clot to the compression load exerted by the probe. Since the parameter largely depends on the product's elasticity, YGC has a higher hardness (1.66 N) than YSC (1.65 N) but with no significant difference ($p > 0.05$).

For the gumminess, average values of 0.39 N were obtained in the case of YGC and 0.38 N in the case of YCS, the differences being also insignificant ($p > 0.05$). No changes were reported even during the determinations between the two types of milk used in processing (Table 4).

Clot resilience was another characteristic that was being watched, and for YGC, the values were 0.27 and 0.30 for YSC ($p < 0.05$). The ability of the clot to return to its original height after compression or the resilience of the clot was shown to be generally better for YGC compared to YSC, with both varieties having values close to 0, values that thus show that the analysed samples does not recover its original height.

The adhesiveness of the clot for YGC was −0.19 mJ and −0.16 mJ for the YSC ($p < 0.05$). For the adhesive force, the mean value in the case of YGC was −0.29 N and −0.24 N for YSC ($p < 0.05$). Regarding the clot breaking force, the average values were 1.52 N for the YGC and 1.48 N for the YSC ($p < 0.05$).

3.2.2. Physiochemical Results

The physiochemical properties, pH, the percentage of titratable acidity and the percentages of syneresis of yogurt samples are summarised in Table 5. The average values obtained for the two types of yogurt did not differ enough to exceed any statistically significant threshold ($p > 0.05$).

Table 5. Results regarding the physico-chemical assessments of yogurt.

Physical–Chemical Trait	System of Exploitation	The Experimental Period			Overall
		May	July	September	
pH	YGC	4.42 ± 0.05^{xA}	4.45 ± 0.05^{xA}	4.40 ± 0.05^{xAB}	4.43 ± 0.05^{x}
	YCS	4.41 ± 0.02^{xB}	4.45 ± 0.02^{xA}	4.43 ± 0.02^{xBA}	4.43 ± 0.03^{x}
Acidity (%)	YGC	0.92 ± 0.02^{xB}	0.93 ± 0.02^{xA}	0.90 ± 0.02^{yC}	0.91 ± 0.02^{x}
	YCS	0.89 ± 0.02^{yC}	0.93 ± 0.02^{xA}	0.91 ± 0.02^{xB}	0.91 ± 0.03^{x}
Syneresis (%)	YGC	3.23 ± 0.01^{yB}	3.28 ± 0.01^{xA}	3.21 ± 0.01^{yB}	3.24 ± 0.03^{y}
	YCS	3.26 ± 0.04^{xB}	3.30 ± 0.04^{xA}	3.28 ± 0.04^{xBA}	3.28 ± 0.04^{x}
Total solids (TS) (%)	YGC	14.74 ± 0.21^{xA}	14.79 ± 0.21^{xA}	14.83 ± 0.21^{xA}	14.79 ± 0.20^{x}
	YCS	14.48 ± 0.09^{yA}	14.52 ± 0.09^{yA}	14.50 ± 0.09^{yA}	14.50 ± 0.09^{y}
Fat (%)	YGC	3.60 ± 0.07^{xB}	3.65 ± 0.07^{xB}	3.69 ± 0.07^{xAB}	3.64 ± 0.08^{x}
	YCS	3.58 ± 0.04^{xA}	3.62 ± 0.04^{xA}	3.60 ± 0.04^{yA}	3.60 ± 0.05^{y}
Protein (%)	YGC	3.36 ± 0.05^{xA}	3.40 ± 0.05^{xA}	3.38 ± 0.05^{xA}	3.38 ± 0.06^{x}
	YCS	3.38 ± 0.06^{xA}	3.38 ± 0.06^{xA}	3.38 ± 0.06^{xA}	3.36 ± 0.06^{x}
Ash (%)	YGC	0.80 ± 0.04^{xB}	0.85 ± 0.04^{xA}	0.83 ± 0.04^{xA}	0.82 ± 0.04^{y}
	YCS	0.82 ± 0.03^{xB}	0.86 ± 0.03^{xA}	0.84 ± 0.03^{xBA}	0.84 ± 0.03^{x}

Averages with different letters "A", "B", "C" in the rows and "x", "y" in the columns indicate statistically significant differences ($p < 0.05$).

Regarding the syneresis process in the case of YGC, differences are also observed in the control months, the highest value being obtained in July (3.28%) compared to 3.23% obtained in May or 3.21% obtained in September ($p < 0.05$). The same effect was noticed in the case of YCS. The final comparison of means revealed an average value for YGC of 3.24% and of 3.28% for YCS, values indicating significant differences ($p < 0.05$).

Significant differences were also reported in the case of TS, where for YGC the average was 14.79%, and for YCS, an average value of 14.50% was obtained ($p < 0.05$). Regarding the fat content, the data analysis revealed fluctuations in the case of YGC, the averages being 3.60% in May, 3.65% in July and 3.69% in September. The overall fat content in cases of YGC was 3.64%, and for YCS it was 3.60% ($p < 0.05$). The protein level, both for YGC and YCS, remained constant during the entire period of determinations, the final average values being 3.36% and 3.38%, respectively ($p > 0.05$). In the case of ash content, the average value recorded at YGC was 0.82% and at YCS 0.84% ($p < 0.05$). Data on the characterization

of the fatty acid profile of yogurts obtained from the two types of milk revealed several differences (Table 6).

Table 6. The main fatty acids in yogurt (g/100 g fatty acids methyl esters).

Physical–Chemical Trait	Type of Nutrition	The Experimental Period			Overall
		May	July	September	
Butyric acid-C4:0	YGC	2.90 ± 0.22^{xA}	2.95 ± 0.22^{xA}	2.99 ± 0.22^{xA}	2.95 ± 0.22^{x}
	YCS	2.57 ± 0.28^{yA}	2.61 ± 0.28^{yA}	2.59 ± 0.28^{yA}	2.59 ± 0.27^{y}
Caprionic acid-C6:0	YGC	2.09 ± 0.26^{xA}	2.14 ± 0.26^{xA}	2.18 ± 0.26^{xA}	2.14 ± 0.25^{x}
	YCS	2.05 ± 0.09^{xA}	2.09 ± 0.09^{xA}	2.07 ± 0.09^{xA}	2.07 ± 0.09^{x}
Caprylic acid-C8:0	YGC	1.32 ± 0.20^{xA}	1.37 ± 0.20^{xA}	1.41 ± 0.20^{xA}	1.36 ± 0.20^{x}
	YCS	1.34 ± 0.08^{xA}	1.38 ± 0.08^{xA}	1.36 ± 0.08^{xA}	1.36 ± 0.07^{x}
Capric acid-C10:0	YGC	3.12 ± 0.22^{yA}	3.17 ± 0.22^{yA}	3.21 ± 0.22^{yA}	3.16 ± 0.22^{y}
	YCS	3.86 ± 0.11^{xA}	3.90 ± 0.11^{xA}	3.88 ± 0.11^{xA}	3.88 ± 0.11^{x}
Lauric acid-C12:0	YGC	3.45 ± 0.21^{yA}	3.50 ± 0.21^{yA}	3.54 ± 0.21^{yA}	3.50 ± 0.20^{y}
	YCS	3.86 ± 0.11^{xA}	3.90 ± 0.11^{xA}	3.88 ± 0.11^{xA}	3.88 ± 0.11^{x}
Myristic acid-C14:0	YGC	11.50 ± 0.21^{yA}	11.55 ± 0.21^{yA}	11.59 ± 0.21^{yA}	11.55 ± 0.20^{y}
	YCS	12.59 ± 0.10^{xA}	12.63 ± 0.10^{xA}	12.61 ± 0.10^{xA}	12.61 ± 0.10^{x}
Myristoleic acid-C14:1	YGC	0.92 ± 0.21^{xA}	0.97 ± 0.21^{xA}	1.01 ± 0.21^{xA}	0.97 ± 0.20^{x}
	YCS	0.93 ± 0.08^{xA}	0.97 ± 0.08^{xA}	0.95 ± 0.08^{xA}	0.95 ± 0.08^{x}
Pentadecylic acid-C15:0	YGC	1.22 ± 0.21^{yA}	1.27 ± 0.21^{yA}	1.31 ± 0.21^{yA}	1.27 ± 0.20^{y}
	YCS	1.43 ± 0.07^{xA}	1.47 ± 0.07^{xA}	1.45 ± 0.07^{xA}	1.45 ± 0.07^{x}
Palmitic acid-C16:0	YGC	27.85 ± 0.75^{yA}	27.90 ± 0.75^{yA}	27.94 ± 0.75^{yA}	27.89 ± 0.72^{y}
	YCS	35.96 ± 0.44^{xA}	36.00 ± 0.44^{xA}	35.98 ± 0.44^{xA}	35.98 ± 0.42^{x}
Palmitoleic acid-C16:1	YGC	0.71 ± 0.21^{yA}	0.76 ± 0.21^{yA}	0.80 ± 0.21^{yA}	0.76 ± 0.20^{y}
	YCS	1.13 ± 0.08^{xA}	1.17 ± 0.08^{xA}	1.15 ± 0.08^{xA}	1.15 ± 0.08^{x}
Margaric acid-C17:0	YGC	0.50 ± 0.20^{xA}	0.55 ± 0.20^{xA}	0.59 ± 0.20^{xA}	0.54 ± 0.20^{y}
	YCS	0.61 ± 0.07^{xA}	0.65 ± 0.07^{xA}	0.63 ± 0.07^{xA}	0.63 ± 0.07^{x}
Stearic acid-C18:0	YGC	11.73 ± 0.31^{xA}	11.78 ± 0.31^{xA}	11.82 ± 0.31^{xA}	11.77 ± 0.30^{x}
	YCS	8.91 ± 0.68^{yA}	8.95 ± 0.68^{yA}	8.93 ± 0.68^{yA}	8.93 ± 0.66^{y}
Vaccenic acid-C18:1 trans-11	YGC	2.90 ± 0.28^{xA}	2.95 ± 0.28^{xA}	2.99 ± 0.28^{xA}	2.95 ± 0.27^{x}
	YCS	1.07 ± 0.08^{yA}	1.11 ± 0.08^{yA}	1.09 ± 0.08^{yA}	1.09 ± 0.08^{y}
C18:2	YGC	1.73 ± 0.22^{xA}	1.78 ± 0.22^{xA}	1.82 ± 0.22^{xA}	1.78 ± 0.22^{x}
	YCS	1.60 ± 0.08^{xA}	1.64 ± 0.08^{xA}	1.62 ± 0.08^{yA}	1.62 ± 0.08^{y}
C18:3	YGC	1.00 ± 0.21^{xA}	1.05 ± 0.21^{xA}	1.09 ± 0.21^{xA}	1.04 ± 0.21^{x}
	YCS	0.57 ± 0.09^{yA}	0.61 ± 0.09^{yA}	0.59 ± 0.09^{yA}	0.59 ± 0.09^{y}
Arachidic acid C20:0	YGC	0.43 ± 0.20^{xA}	0.48 ± 0.20^{xA}	0.52 ± 0.20^{xA}	0.48 ± 0.20^{x}
	YCS	0.44 ± 0.06^{xA}	0.48 ± 0.06^{xA}	0.46 ± 0.06^{xA}	0.46 ± 0.06^{x}
Rumeric acid-CLA	YGC	1.51 ± 0.22^{xA}	1.56 ± 0.22^{xA}	1.60 ± 0.22^{xA}	1.56 ± 0.22^{x}
	YCS	1.27 ± 0.08^{yA}	1.31 ± 0.08^{yA}	1.29 ± 0.08^{yA}	1.29 ± 0.08^{y}
Monounsaturated fatty acid-MUFA	YGC	4.53 ± 0.62^{xA}	4.68 ± 0.62^{xA}	4.80 ± 0.62^{xA}	4.67 ± 0.61^{x}
	YCS	3.14 ± 0.17^{yA}	3.26 ± 0.17^{yA}	3.20 ± 0.17^{yA}	3.20 ± 0.17^{y}

Table 6. *Cont.*

Physical–Chemical Trait	Type of Nutrition	The Experimental Period			Overall
		May	July	September	
Polyunsaturated fatty acid-PUFA	YGC	4.24 ± 0.61^{xA}	4.39 ± 0.61^{xA}	4.51 ± 0.61^{xA}	4.38 ± 0.60^{x}
	YCS	3.44 ± 0.23^{yA}	3.56 ± 0.23^{yA}	3.50 ± 0.23^{yA}	3.50 ± 0.23^{y}
Saturated fatty acid-SFA	YGC	66.10 ± 2.30^{yA}	66.65 ± 2.30^{yA}	67.09 ± 2.30^{yA}	66.61 ± 2.26^{y}
	YCS	73.64 ± 1.15^{xA}	74.08 ± 1.15^{xA}	73.86 ± 1.15^{xA}	73.86 ± 1.13^{x}
Unsaturated fatty acids-UFA	YGC	8.78 ± 1.21^{xA}	9.08 ± 1.21^{xA}	9.32 ± 1.21^{xA}	9.06 ± 1.19^{x}
	YCS	6.58 ± 0.38^{yA}	6.82 ± 0.38^{yA}	6.70 ± 0.38^{yA}	6.70 ± 0.38^{y}
DFA	YGC	20.50 ± 1.43^{xA}	20.85 ± 1.43^{xA}	21.13 ± 1.43^{xA}	20.83 ± 1.40^{x}
	YCS	15.49 ± 0.96^{yA}	15.77 ± 0.96^{yA}	15.63 ± 0.96^{yA}	15.63 ± 0.93^{y}
OFA	YGC	42.80 ± 0.88^{yA}	42.95 ± 0.88^{yA}	43.07 ± 0.88^{yA}	42.94 ± 0.85^{y}
	YCS	52.41 ± 0.45^{xA}	52.53 ± 0.45^{xA}	52.47 ± 0.45^{xA}	52.47 ± 0.43^{x}
DFA/OFA	YGC	0.48 ± 0.03^{xA}	0.49 ± 0.03^{xA}	0.49 ± 0.03^{xA}	0.48 ± 0.03^{x}
	YCS	0.30 ± 0.02^{yA}	0.30 ± 0.02^{yA}	0.30 ± 0.02^{yA}	0.30 ± 0.02^{y}

DFA = desirable hypocholesterolemic fatty acids, OFA = hypercholesterolemic fatty acid, DFA/OFA = desirable hypocholesterolemic fatty acids/hypercholesterolemic fatty acid. Averages with the letter "A" in the rows and different letters "x", "y" in the columns indicate statistically significant differences ($p < 0.05$).

4. Discussion

4.1. Raw Material Milk Quality

The results of this study were intended to inform an investigation into variations in milk content in relation to feeding procedures and seasonality (May through October). Special attention was paid to chemical makeup and fatty acid profile. We also wanted to understand if changes in raw milk quality and cow care procedures affected the physical, chemical, textural and nutritional aspects of the processed product (yogurt).

The feeding system and its relationship with seasonal fluctuation had an impact on the fat concentrations. All seasons have a similar chemical make-up for the MCS system. On the other hand, between spring and autumn, MGC showed significant seasonal fluctuations in chemical composition. In contrast to the spring and summer, autumn showed higher fat percentages; no discernible differences in protein percentages were found between the seasons.

It is possible that the consumption of unsaturated fatty acids, which are normally found in forage, is what caused the MGC spring milk to have the lowest level of lipids measured. Some studies found that a high concentration of these fatty acids in the rumen can inhibit some microbial species in the rumen, with the production of CLA isomers produced in the rumen inhibiting fatty acid synthesis, thereby inducing a low concentration of fat in milk. These findings were found in some of the studies [45,46].

However, the majority of these studies only make straightforward comparisons between pasture-based systems and zero-grazing systems. This is due to the fact that diet plays a significant role in determining the FA composition of bovine milk, which has resulted in a greater number of studies being reported in comparison to other factors [47]. According to the results of our research, pasture-based rations (MGC) had higher concentrations of PUFA than MCS. Ellis et al. [10] discovered that the MCS group had a greater SFA than the MGC.

It was to be expected that milk concentrations of C14:0 and C16:0 would be higher in MCS, behaving differently to C18:0. Similar findings were made in earlier research, which found that when compared to alfalfa silage, grass silage and fresh pasture, corn silage increased the proportions of C14:0 and C16:0 while decreasing C18:0 (and also C18:1) [48–50].

Furthermore, a different study found that replacing corn silage with more fresh pasture (mostly ryegrass) boosted the concentration of unsaturated fatty acids (UFA) at the expense of saturated fatty acids (SFA) [51]. Several authors have discovered bigger differences between cows consuming grazed grass compared to diets high in preserved forages, revealing that milk from grazing cattle had higher proportions of UFA, C18:0 and C18:1 acids and lower amounts of SFA, C16:0 and C14:0 acids [52–55].

Considering the season, our findings revealed that C14:0, C16:0 and SFA were higher in the summer, while C18:0, C18:1, MUFA and PUFA were primarily higher in the spring. It is significant to note that seasonal fluctuation in milk composition is notably connected to dietary elements related to variations in pasture availability and quality throughout the year. The results from earlier studies on this subject were inconsistent. According to Auldist et al. [56], winter and spring were the seasons with the highest concentrations of MUFA, SCFA and PUFA, respectively.

When compared to winter milk, Collomb et al. [57] and Frelich et al. [58] showed that summer milk had larger contents of MUFA and PUFA, notably C18:1, and lower concentrations of SFA. The proportion of roughage to concentrates has an impact on these results.

4.2. Yogurts Quality

4.2.1. Texture Analysis

The rheological and textural properties of fermented dairy products are determined by their structure and protein network configuration [59]. The most crucial factor in defining yogurt texture is hardness or firmness. It is regarded as the amount of force necessary to cause a specific deformation and is used to determine how hard the yogurt is.

Proteins, mainly casein, interact with cultures of lactic acid bacteria to form a gel-like structure, giving yogurt its characteristic texture. When the milk has a higher protein content, the value recorded for hardness is higher. The samples' YGC hardness levels were therefore greater than YCS. Also, the coagulum typically gets harder as the acidity rises, perhaps as a result of the protein interactions getting stronger. Our findings agreed with those found by Wen et al. [60] and Sah et al. [61].

Jasińska et al. [62] affirm that the yogurt manufactured from the total mixed ration-fed cow milk showed higher hardness, compared to the yogurt made from milk produced by the cows kept on the traditional feeding regime. The coagulum typically gets harder as the acidity rises, perhaps as a result of the protein–protein connections getting stronger.

Cohesiveness measures how effectively a product resists a second deformation in comparison to its resistance under the first deformation [63].

In comparison to values found in YCS, the YGC cohesiveness was higher. This might be associated with the higher levels of protein content, which result in denser sample structures. Our findings support previous studies that have been reported by Lucatto et al. [64] and Al-Bedrani et al. [65]. The ratio of the positive force area during the second penetration to that during the first penetration is the definition of cohesiveness. It can be calculated as the rate of material disintegration caused by mechanical force. Cohesiveness is expressed in tensile strength. Cohesiveness is a measure of how well a thing holds together.

The YGC and YCS samples' gumminess values revealed a generally higher value for MGC-produced products. Gumminess is the result of cohesion and hardness. High gumminess yogurt also has a high hardness value. Foods that are semisolid and have a high degree of cohesion but little hardness are said to be "gumily".

Resilience is another texture characteristic of yogurt sample data identified by TPA. It has to do with the product's capacity to return to its initial position following application of deformation. For this parameter, the average value obtained at YGC was significantly lower than it was at YCS.

As a result, the technological processes used to produce the yogurt are regarded as being a deciding factor by the TPA results. However, given that the technological processes used to produce the yogurt we produced were the same, we can conclude that the

differences between YGC and YCS were provided by the raw milk composition. Ganesan et al. [66] and also Giri and Osman [67] provide evidence to support the idea that the chemical makeup of milk may have an impact on the textural profiles of yogurt samples.

4.2.2. Physicochemical Analysis

The titratable acidity is expressed as the percentage of lactic acid present in the yogurt samples. The data analysis did not show any differences between the two types of milk used to make yogurt in terms of pH or acidity, respectively. The values found in the YGC and YCS results are consistent with findings from earlier studies [68].

Protein serves as a catalyst for bacterial growth during fermentation, while lactose serves as the carbon source that will be converted into lactic acid and lower pH.

Due to their essential role in the coagulation, ripening, and shelf life of curd, pH regulation and acidity are unquestionably crucial factors in the manufacturing of yogurt. LAB's fermentation of lactose to lactic acid lowers the pH of yogurt, which diminishes the electrostatic attraction between casein micelles and changes the distribution of calcium between the micelle and serum phases [60]. As a result, several milk combinations are required in industry to provide effective acidity and pH. Additionally, these mixes will help avoid yogurt syneresis [69].

Low solid content, high incubation temperatures, insufficient storage temperatures, high acidity, etc., are the main causes of syneresis [70]. According to Rani et al. [71], the stabiliser in the yogurt samples binds free water molecules and traps them in the casein network, according. This activity will make the sample more viscous, which will lead to a reduction in syneresis. Intense syneresis in yogurt is a bad quality trait, which might cause consumers to reject it. However, the physical and sensory properties of yogurt gels are greatly influenced by the total solids content of the yogurt milk, especially the protein content.

According to the TS content data, a higher value was obtained in the case of YGC compared to YSC. Aly et al. [72] and Haj et al. [73] reported values that were comparable to those we found.

The animals' feeding regimen also had an impact on the fat content of the milk and, in turn, that of the yogurt. As a result, YGC's fat content was higher than YCS's in both cases. These values were in accordance with the literature data [18].

One of the most significant parts of milk is the fat. From a practical standpoint, fat influences customer preference for dairy products, especially when it constitutes a significant portion of the dairy product.

Because milk fat contains a wide range of fatty acids (FA) with various chain lengths, the majority of which are saturated fatty acids (SFA), and only a small amount of mono unsaturated fatty acids (MUFA) and poly unsaturated fatty acids (PUFA) (2–5%) [3,74], the composition of milk fat is incredibly complex. In the current study, it was shown that diet affects milk's FA content, which has been highlighted in cases where milk-based products are involved.

Regarding OFA, a higher content was noted in the fat from YSC (for all analysed periods) compared to YGC. The DFA/OFA ratio, a very important parameter for consumers, revealed significant differences between the two types of yogurt. Lactic cultures had a beneficial effect in increasing, through fermentative processes, the proportion of DFA in comparison with that measured in raw milk and, subsequently, in improving the DFA/OFA ratio, due to the increase in hypocholesterolemiant fatty acids proportion. It is interesting to notice that the beneficial effect reflected on the DFA/OFA ratio had more amplitude in the milk with lower fatty acid profile quality (milk yielded by cows in stabulation). This aspect is encouraging, and the subsequent metabolic phenomena of fermentation and of microorganisms for certain lipidic profiles of raw milk is worth investigating, to find out ways in rendering higher nutritional quality and more sanogenic products even from milk yielded by animals that could not benefit from rearing on pasture.

Also, it is known that sensory and textural attributes of dairy products derived from milk produced by cows fed on pasture are better than those issued from milk produced by cows under totally optimised feeding conditions [75]. Moreover, a direction of research to follow can be the investigation of antioxidant properties of certain molecules on milk and dairy products stability and sanogenic effects on consumers, supposing that carotenes and tocopherols should be found in higher proportions in milk produced by cows fed mostly on pasture. Also, the effect of a cow feeding system on the volatile and aroma-generating compounds in milk and yogurt should be investigated, knowing that other studies reported higher concentrations of such compounds [76]. Apart from the milk origin, the type of probiotic starter culture has its own effect on yogurt sensory properties, especially on developing flavour compounds (aldehydes, ketones, carboxylic acids), and a mixed research model (dairy cows feeding × yogurt starter culture) must be investigated to discover an optimal solution to bring to market some products more appealing and yet healthier to consumers.

5. Conclusions

The findings of this study showed that the time between milk collection and feeding had an impact on the components of cow milk, particularly fat and implicit FA. The study's findings also enable us to draw the generalization that the MGC beat the MSC in terms of both traditional and holistic criteria. There were statistically significant variations in DFA, OFA and the DFA/OFA. If these discoveries have an impact on human health, more investigation is needed. Because of the fermentation processes caused by the injected lactic cultures, the fatty acid profile in yogurt was improved over the initial profile in raw milk.

Textural, physical and chemical analyses of the YGC samples were superior to those of the YSC samples, demonstrating how grazing by cows affects the quality of raw milk and ultimately processed yogurt.

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Essential Oils as a Dietary Additive for Laying Hens: Performance, Egg Quality, Antioxidant Status, and Intestinal Morphology: A Meta-Analysis

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Abstract: This meta-analysis aimed to evaluate the effects of dietary supplementation with essential oils (EOs) on egg production and quality, antioxidant status in blood serum, and the intestinal morphology of laying hens. The data used were obtained from 38 peer-reviewed publications. The effect size was evaluated by weighted mean differences (WMD) between the experimental treatments (diets added with EOs) and the control treatments (diets without EOs). EO supplementation increased ($p < 0.001$) egg production (WMD = 2.171%), egg weight (WMD = 0.636 g), egg mass (WMD = 1.679 g/d), and decreased the feed conversion ratio (WMD = −0.074 g/g; $p < 0.001$). In addition, greater ($p < 0.05$) eggshell thickness (WMD = 14.262 mm), eggshell strength (0.080 kg/cm^2), albumen height (WMD = 0.201 mm), Haugh unit (WMD = 1.102), and yolk color (WMD = 0.071) were observed in response to EO supplementation. In blood serum, the dietary inclusion of EOs increased ($p < 0.05$) the levels of superoxide dismutase (WMD = 1.147 U/mL), glutathione peroxidase (WMD = 879.553 U/mL), and total antioxidant capacity (WMD = 1.163 U/mL). In the duodenum, jejunum, and ileum, a higher ($p < 0.05$) villus height (VH), crypt depth (CD), villus width, and VH/CD ratio was observed in response to EO supplementation. In conclusion, the dietary inclusion of essential oils can be used as a nutritional strategy to improve egg production and quality, the antioxidant status of blood serum, and intestinal morphology in laying hens.

Keywords: phytogenic additives; essential oils; laying hens; egg production; meta-regression

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1. Introduction

Eggs from laying hens are a high-quality food because they contain nutrients such as proteins, vitamins, minerals, and lipids, which are important in human nutrition [1]. Currently, the demand for eggs from laying hens has increased due to the increase in the human population worldwide [2]. However, laying hens in commercial environments are frequently exposed to a wide variety of stressors such as nutritional (presence of oxidized fats or mycotoxins in the feed), environmental (i.e., heat or cold stress), and physiological (e.g., high rate of egg production and aging) [3,4]. According to Puppel et al. [5], each of these factors contributes to the increase in the production of reactive oxygen species (ROS) in the animal, which can result in alterations in the redox balance and lead to oxidative stress (OS) in birds [4,6]. In laying hens, OS can negatively affect productive performance, egg quality, and bird health [3,7]. Some authors [4,8] have mentioned that including natural antioxidants in the diets of laying hens and broilers can help reduce ROS overproduction and result in lower OS.

According to Nehme et al. [9], EOs are currently among the most economically relevant natural products with antioxidant properties. EOs are volatile and aromatic oily liquids derived from plants, which are obtained by distillation and contain complex mixtures of low molecular weight molecules, mainly terpenes, terpenoids, and phenylpropanoids [10,11]. Although EOs have been used mainly in human pharmacology and cosmetology, their

use in domestic animal feed has increased in recent years [12,13]. Beef cattle, dairy cows, and small ruminants are some animals in which EOs are being evaluated more frequently, mainly as nutritional strategies to manipulate rumen fermentation and reduce environmental impact [11,13,14]. However, some recently published review articles [8,15–17] suggest that the inclusion of EOs in diets for poultry can be used successfully to improve the oxidative status in blood, the health of the intestinal epithelium, and the quality of derived products (meat and eggs).

Particularly in laying hens, some studies have evaluated the effects of EOs as a dietary additive on productive performance [18,19], egg quality [20,21], antioxidant status in blood serum [10,22], and intestinal morphology [23,24]. However, the observed results have been inconsistent and contradictory in several of these studies. For example, some studies have reported up to 7% higher egg production, 15% better feed conversion, 81% higher total blood antioxidant capacity, and 24% longer intestinal villi in laying hens supplemented with EOs [10,18,19]. However, in other studies [22–24] using laying hens, less than 2% of benefits in egg production, feed conversion, and intestinal villi have been detected in response to EO supplementation. These results could be attributed to the high variability between studies regarding age and breed/strain of laying hens, supplementation periods, doses, and primary bioactive metabolites of the EOs used [8,15]. It has been reported that this type of variability can be overcome by using meta-analytic methods [25]. Meta-analysis is a statistical method with high analytical power [26] that uses rigorous procedures that allow one to statistically combine and analyze datasets from various experiments to obtain objective evidence on the effect of a treatment [27,28].

The hypothesis of the present meta-analysis establishes that the inclusion of EOs in the diets of laying hens will benefit egg production and quality, antioxidant status in blood serum, and intestinal morphology. Therefore, this study aimed to evaluate, through a meta-analytic approach, the effects of dietary supplementation with essential oils on egg production and quality, antioxidant status in blood serum, and the intestinal morphology of laying hens.

2. Materials and Methods

2.1. Literature Search

A systematic and exhaustive search of the literature published in English was carried out to identify experiments that evaluated the effects of including EOs in diets for laying hens. The bibliographic search was carried out in the Scopus, ScienceDirect, PubMed, and Web of Science databases and was restricted to documents published between January 2012 and April 2023. The keywords used in the searches were the following: essential oils, laying hens, egg production, egg quality, antioxidant status, and intestinal morphology. The PRISMA guidelines [29] were used in the identification, selection, choice, and inclusion of all documents, as shown in Figure 1.

2.2. Inclusion Criteria

Initial searches returned 315 potential documents, which were reduced to 254 after removing duplicate documents. Subsequently, the rest of the documents were reviewed in detail, and only those that met the following inclusion criteria were included in the final database [27,28,30]: (1) scientific articles published in peer-reviewed journals and written in English; (2) studies conducted with laying hens under confined conditions (i.e., studies under grazing conditions were not considered); (3) studies reporting data on egg production, egg quality, blood serum antioxidant status, or intestinal morphology; (4) studies that used similar diets for the control and experimental treatments, except for the inclusion of EOs in the diets; (5) studies that indicated the doses of EOs (mg/kg DM) added to the experimental diets or provided sufficient information to estimate them; (6) studies that reported the means of the experimental treatments (diets added with EOs) and control (diets without EOs), the number of experimental units (n), and the standard error (SEM) or standard deviation of treatment means.

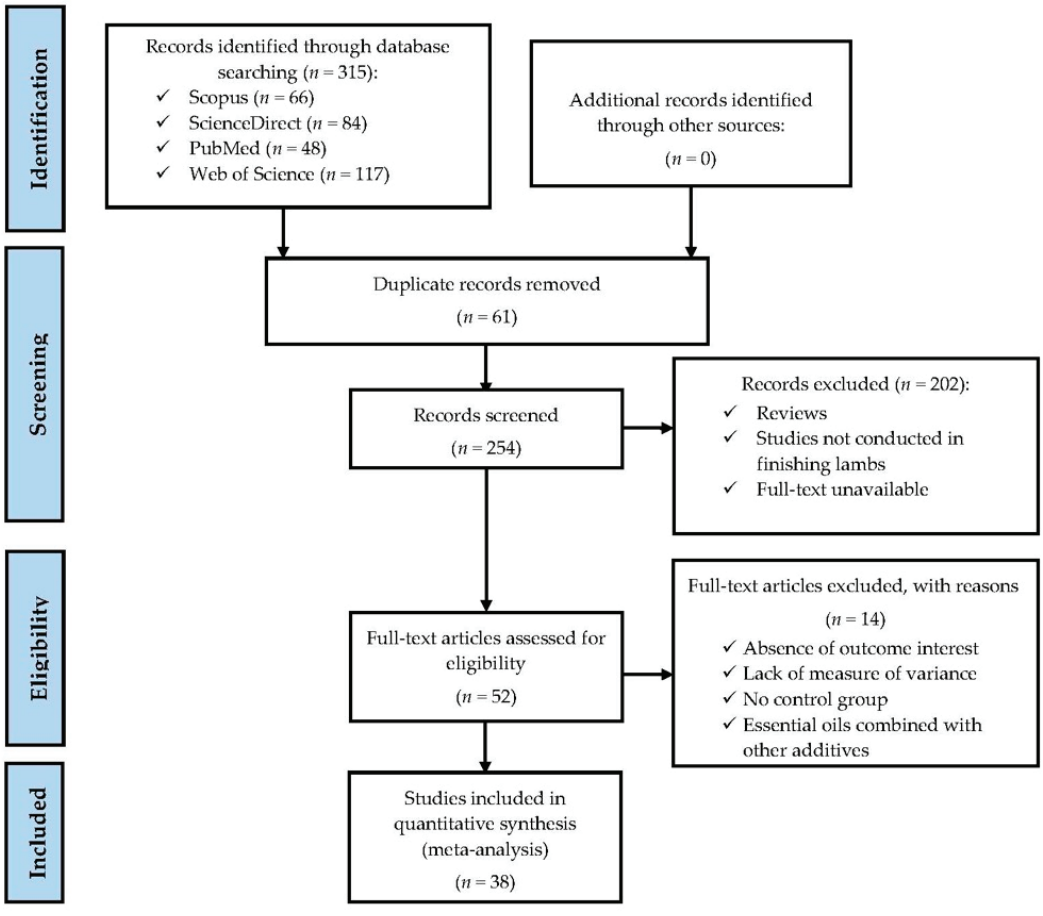

Figure 1. A PRISMA flow diagram detailing the literature search strategy and study selection for the meta-analysis.

2.3. Data Extraction

After applying the inclusion criteria, only 38 peer-reviewed scientific articles were selected (Table 1). First, from each of the selected articles, the following information was extracted: (1) author and year of publication; (2) age of laying hens (in weeks); (3) breed/strain of laying hens; (4) supplementation period (days); (5) doses of EOs added to the experimental diets (mg/kg DM); (6) the primary bioactive metabolite of the EOs. Subsequently, the following response variables were collected from the selected scientific articles: average daily feed intake (ADFI), egg production (EP), egg weight (EW), egg mass (EM), feed conversion ratio (FCR), eggshell thickness (ET), eggshell strength (ES), albumen height (AH), Haugh unit (HU), yolk color (YC), yolk index (YI), malondialdehyde (MDA) in the yolk, enzyme antioxidants in blood serum (superoxide dismutase (SOD), glutathione peroxidase (GPx), total antioxidant capacity (TAC) and MDA), and intestinal morphology (villus height (VH), crypt depth (CD), villus width (VW), and VH/CD ratio) in the duodenum, jejunum, and ileum. The following data were obtained from each of these response variables: (1) means of control treatments (diets without EOs) and experimental treatments (diets added with EOs); (2) n; (3) SEM or SD. When the SD was reported, the values were used directly; however, when the publications did not report a SD, its value was estimated using the SEM value and n through the equation proposed by Higgins and Thomas [31]:

$SD = SEM \times \sqrt{n}$. Finally, only the response variables reported in at least three different publications were analyzed since other authors [27,28,30] have mentioned that this allows one to obtain statistically robust results.

Table 1. Description of the studies included in the meta-analysis database.

Reference	Hen's Age, Weeks	Breed/Strain	Supplementation Period, Days	Dose (mg/kg Feed)	Primary Bioactive Compound
Abdel-Wareth and Lohakare [18]	32–38	Bovans Brown	42–84	74–295	Menthol
Abo Ghanima et al. [19]	28–76	ISA Brown	56–336	300	Blend
Abo Ghanima et al. [20]	28–76	ISA Brown	56–336	300	Thymol, carvacrol, eugenol
Akbari et al. [32]	42	Lohmann White	56	100–200	Menthol, thymol, blend
Arslan et al. [23]	48	Brown Nick H&N	84	50–200	Blend
Bozkurt et al. [21]	36–43	Lohmann White	56–112	24	Blend
Bozkurt et al. [33]	52	Lohmann White	70	24	Blend
Bozkurt et al. [34]	22	Lohmann Brown	252	24	Blend
Bozkurt et al. [35]	82	White Leghorn	175	24	Carvacrol
Beyzi et al. [36]	120	White Leghorn	70	300	Carvacrol
Cheng et al. [10]	65	Lohmann White	56	75–150	Blend
Cufadar et al. [37]	40	Super Nick H&N	84	50–250	Blend
Ding et al. [38]	54–62	Lohmann White	28–84	50–150	Blend
Feng et al. [24]	60–69	Hy-Line Brown	28–84	5–20	Blend
Gao et al. [39]	58–62	Not reported	28–56	8–24	Blend
Ghanem et al. [40]	24	Lohmann Brown	90	50–150	Cinnamaldehyde
Gul et al. [41]	22	Lohmann White	56	200–600	Blend
He et al. [42]	30	Hy-Line White	49	50–150	Carvacrol
Kavan et al. [43]	60	Hy-Line White	56	100–200	Blend
Kaya et al. [44]	36	Lohmann White	56	150–300	Carvacrol
Laptev et al. [45]	52	Lohmann White	27	90	Blend
Marume et al. [46]	18	White Leghorn	56	1000, 2000	Blend
Migliorini et al. [47]	59–67	Not reported	28–84	50–200	Blend
Mousavi et al. [48]	40–45	Hy-Line White	35–70	100–200	Menthol
Mousavi et al. [49]	40–45	Hy-Line White	35–70	100–200	Menthol
Olgun [50]	21	Super Nick H&N	84	25–600	Blend
Puvaca et al. [51]	55–59	Lohmann Brown	28–56	40–80	Terpinen-4-ol
Ramírez et al. [52]	70	ISA Brown	56	80–150	Thymol
Reshadi et al. [53]	66	Lohmann White	84	250	Pulegone
Rodjan et al. [54]	36	Hisex Brown	28	9.6–77.3	Diallyl trisulfide
Rodjan et al. [2]	36	Hisex Brown	28	9.6–77.3	Diallyl trisulfide
Saleh et al. [55]	24	Bovans Brown	42	250–500	Blend
Torki et al. [56]	42	Lohmann White	56	40	Blend
Torki et al. [57]	30–41	Lohmann White	42–84	150–200	Alpha-pinene, carvonene
Torki et al. [58]	42–52	Lohmann White	28–84	250–500	Linalool, limonene, blend
Wang et al. [59]	52–55	Hy-Line Brown	21–42	100	Blend
Xiao et al. [1]	55	Golden Phoenix	56	300	Blend
Yu et al. [22]	26	Hy-Line Brown	28–56	200–600	Trans-anethole

2.4. Calculations and Statistical Analysis

In this study, the metafor package [26] of the statistical software R version 4.1.2 was used to perform all of the statistical analyses. The effects of EO supplementation on egg production and quality, antioxidant status in blood serum, and the intestinal morphology of laying hens were evaluated by examining the weighted mean differences (WMD) between the experimental treatments (diets supplemented with EOs) and control treatments (diets

without EOs). Treatment means were weighted by the inverse of the variance following the method proposed by Der-Simonian and Laird [60] for a random effects model.

2.5. Heterogeneity and Publication Bias

The consistency of the results between studies was assessed with the chi-square (Q) test, in which, due to its relatively low power, a significance level of $p \leq 0.10$ was used [61]. As a complement, the I^2 statistic was used to quantify the proportion of variation due to heterogeneity [62]. Values of $I^2 < 25\%$ indicate low heterogeneity, I^2 between 25 and 50% indicates moderate heterogeneity, and $I^2 > 50\%$ indicates high heterogeneity [62]. Finally, Egger's regression asymmetry test [63] and Begg's adjusted rank correlation [64] were used to assess the presence of publication bias. Both tests were considered significant when $p \leq 0.05$.

2.6. Meta-Regression and Subgroup Analysis

Sources of heterogeneity in the response variables were assessed with meta-regression analysis using the method of moments of Der-Simonian and Lair [60] as this method is well-established for estimating between-study variance. The criteria considered to be able to apply the meta-regression analysis to a response variable were the following: (1) response variable reported in ten or more different scientific articles [65]; (2) *p*-value ≤ 0.10 for the Q test or $I^2 > 50\%$ [61]; (3) *p*-value > 0.05 for the Egger [63] and Begg [64] tests. The age of the laying hens (in weeks), the period of supplementation (in days), and the dose of EOs (mg/kg DM) added to the experimental diets were used as continuous covariates. Likewise, the breed/strain of the laying hens and the primary bioactive metabolite of the EOs were used as categorical covariates. When meta-regression was statistically significant ($p \leq 0.05$) for continuous covariates, WMD was assessed with subgroup analyses as follows: age of laying hens ($\leq$45 and >45 weeks), period of supplementation ($\leq$56 and >56 days), and EOS dose ($\leq$150 and >150 mg/kg DM). On the other hand, the WMD of the categorical covariates that were significant ($p \leq 0.05$) in the meta-regression were analyzed with the following subgroups: breed/strain of laying hens (Hisex Brown, Hy-Line White, Hy-Line Line Brown, Super Nick H&N, White Leghorn, Lohmann Brown, Lohmann White, ISA Brown, and Bovans Brown) and primary bioactive metabolite of EOs (trans-anethole, limonene, linalool, carvonene, alpha-pinene, diallyl trisulfide, terpinen-4-ol, cinnamaldehyde, carvacrol, thymol, menthol, and blend).

3. Results

3.1. Study Attributes

In the database of the present meta-analysis, the age of the laying hens used in the different studies ranged from 22 to 120 weeks. The main breeds/strains of laying hens used in the studies were Lohman White (31.6%), Hy-Line White (10.5%), ISA Brown (7.9%), Lohman Brown (7.9%), and Hy-Line Brown (7.9%), while the remaining studies used another five different breeds/strains of laying hens. Likewise, the supplementation periods with EOs ranged between 27 and 336 days. At the same time, the doses of EOs used in the present meta-analysis ranged between 24 and 2000 mg/kg DM. The EOs with mixtures of primary bioactive compounds were the most used (52.6%) among the different studies. In addition, a significant proportion of the studies used EOs with menthol (10.5%), thymol (7.9%), and carvacrol (13.1%) as the primary bioactive compound while in the remaining studies (15.9%), the EOs used contained another ten different primary bioactive compounds.

3.2. Performance

EOs supplementation did not affect the ADFI ($p > 0.05$; Table 2). However, the EP, EW, and EM increased in response to EO supplementation ($p < 0.001$). In contrast, the dietary inclusion of EOs decreased FCR ($p < 0.001$).

Table 2. Egg performance of laying hens supplemented with essential oils.

Item	N (NC)				Heterogeneity		Egger Test [1]	Begg Test [2]
		Control Means (SD)	WMD (95% CI)	*p*-Value	*p*-Value	I^2 (%)	*p*-Value	*p*-Value
ADFI, g/d	31 (132)	2.235 (0.87)	−0.047 (−0.410; 0.316)	0.798	<0.001	89.69	0.110	0.062
Egg production (EP), %	31 (132)	83.82 (8.08)	2.171 (1.570; 2.772)	<0.001	<0.001	86.20	0.281	0.478
Egg weight (EW), g/d	30 (115)	59.48 (4.35)	0.636 (0.470; 0.802)	<0.001	<0.001	77.69	0.086	0.169
Egg mass (EM), g/d	23 (95)	50.28 (7.38)	1.679 (1.118; 2.240)	<0.001	0.113	45.70	0.120	0.264
FCR, g/g	32 (135)	2.23 (0.27)	−0.074 (−0.094; −0.054)	<0.001	<0.001	80.43	0.301	0.537

N: the number of studies; NC: the number of comparisons between the essential oil treatment and control treatment; SD: standard deviation; WMD: weighted mean differences between control and treatments with microalgae; CI: confidence interval of WMD; *p*-Value to χ^2 (Q) test of heterogeneity; I^2: proportion of total variation of size effect estimates that is due to heterogeneity; [1]: Egger's regression asymmetry test; [2]: Begg's adjusted rank correlation; ADFI: average daily feed intake; FCR: feed conversion ratio.

3.3. Egg Quality

Dietary supplementation with EOs increased the ET, ES, AH, HA, YC, and YI ($p < 0.05$; Table 3). However, the dietary inclusion of EOs decreased the MDA content in the yolk ($p < 0.001$).

Table 3. Egg quality of laying hens supplemented with essential oils.

Item	N (NC)				Heterogeneity		Egger Test [1]	Begg Test [2]
		Control Means (SD)	WMD (95% CI)	*p*-Value	*p*-Value	I^2 (%)	*p*-Value	*p*-Value
Eggshell thickness (ET), mm	30 (106)	365.44 (68.85)	14.262 (10.811; 17.712)	<0.001	0.110	44.17	0.135	0.083
Eggshell strength (ES), kg/cm^2	16 (66)	3.71 (1.04)	0.080 (0.052; 0.109)	<0.001	0.177	13.80	0.363	0.458
Albumen height (AH), mm	14 (44)	6.96 (0.76)	0.201 (0.115; 0.287)	<0.001	0.121	42.56	0.490	0.699
Haugh unit (HU)	29 (105)	76.85 (18.55)	1.102 (0.774; 1.431)	<0.001	0.209	42.18	0.109	0.230
Yolk color (YC)	21 (62)	7.37 (1.66)	0.071 (0.017; 0.124)	0.010	0.144	48.80	0.776	0.697
Yolk index (YI)	9 (32)	0.42 (0.04)	0.007 (0.001; 0.014)	0.030	<0.001	69.69	0.401	0.064
MDA in yolk, ng/g	5 (16)	2.11 (1.05)	−0.573 (−0.831; −0.315)	<0.001	<0.001	98.34	0.179	0.302

N: the number of studies; NC: the number of comparisons between essential oil treatment and the control treatment; SD: standard deviation; WMD: weighted mean differences between control and treatments with microalgae; CI: confidence interval of WMD; *p*-Value to χ^2 (Q) test of heterogeneity; I^2: proportion of total variation of size effect estimates that is due to heterogeneity; [1]: Egger's regression asymmetry test; [2]: Begg's adjusted rank correlation; MDA: malondialdehyde.

3.4. Antioxidant Status

Table 4 shows that EO supplementation increased the serum levels of SOD, GPx, and TAC ($p < 0.05$). In contrast, the dietary inclusion of EOs decreased the MDA content in blood serum ($p = 0.001$).

Table 4. Antioxidant status in the blood serum of laying hens supplemented with essential oils.

Item	N (NC)				Heterogeneity		Egger Test [1]	Begg Test [2]
		Control Means (SD)	WMD (95% CI)	*p*-Value	*p*-Value	I^2 (%)	*p*-Value	*p*-Value
SOD, U/mL	5 (17)	64.74 (17.93)	1.147 (0.041; 2.253)	0.042	<0.001	84.66	0.064	0.121
GPx, U/mL	6 (20)	4283.00 (3183.00)	879.553 (506.015; 1253.091)	<0.001	<0.001	88.49	0.136	0.714
TAC, U/mL	4 (13)	5.17 (2.00)	1.163 (0.277; 2.049)	0.010	<0.001	87.71	0.639	0.418
MDA, nmol/mL	7 (20)	5.76 (2.689)	−0.324 (−0.523; −0.124)	0.001	0.231	47.66	0.554	0.189

N: the number of studies; NC: the number of comparisons between the essential oil treatment and control treatment; SD: standard deviation; WMD: weighted mean differences between control and treatments with microalgae; CI: confidence interval of WMD; *p*-Value to χ^2 (Q) test of heterogeneity; I^2: proportion of total variation of size effect estimates that is due to heterogeneity; [1]: Egger's regression asymmetry test; [2]: Begg's adjusted rank correlation; SOD: superoxide dismutase; GPx: glutathione peroxidase; TAC: total antioxidant capacity; MDA: malondialdehyde.

3.5. Intestinal Morphology

Table 5 shows that dietary supplementation with EOs increased ($p < 0.05$) the VH, CD, VW, and VH/CD ratio in the duodenum, jejunum, and ileum.

Table 5. Intestinal morphology of the laying hens supplemented with essential oils.

Item	N (NC)				Heterogeneity		Egger Test [1]	Begg Test [2]
		Control Mean (SD)	WMD (95% CI)	*p*-Value	*p*-Value	I^2 (%)	*p*-Value	*p*-Value
Duodenum								
Villus height (VH), µm	7 (20)	856.20 (295.90)	135.40 (62.57; 208.20)	<0.001	<0.001	99.11	0.329	0.413
Crypt depth (CD), µm	7 (20)	149.5 (83.50)	−12.63 (−19.22 −6.04)	<0.001	<0.001	97.69	0.428	0.330
Villus width (VW), µm	5 (15)	126.00 (56.90)	6.91 (−0.84; 14.66)	0.048	<0.001	84.39	0.348	0.324
VH/CD ratio	5 (14)	6.66 (2.97)	0.81 (0.56; 1.05)	<0.001	0.116	33.24	0.350	0.361
Jejunum								
Villus height (VH), µm	6 (17)	656.30 (218.60)	112.95 (20.13; 205.77)	0.017	<0.001	99.42	0.367	0.065
Crypt depth (CD), µm	6 (17)	11.40 (59.90)	−3.97 (−7.46; −0.48)	0.026	<0.001	74.53	0.101	0.283
Villus width (VW), µm	4 (12)	88.10 (37.20)	11.48 (6.51; 16.45)	<0.001	<0.001	81.33	0.749	0.802
VH/CD ratio	5 (14)	7.47 (3.84)	1.72 (0.66; 2.78)	0.002	0.129	42.88	0.877	0.998
Ileum								
Villus height (VH), µm	5 (15)	476.60 (90.60)	49.05 (21.27; 76.83)	<0.001	<0.001	93.91	0.101	0.170
Crypt depth (CD), µm	4 (12)	136.60 (23.16)	−16.91 (−24.55; −9.26)	<0.001	<0.001	68.83	0.727	0.073
Villus width (VW), µm	4 (12)	71.79 (18.01)	13.68 (8.83; 18.52)	<0.001	<0.001	76.95	0.100	0.431
VH/CD ratio	4 (12)	3.90 (1.07)	0.96 (0.62; 1.31)	<0.001	0.175	28.35	0.273	0.284

N: the number of studies; NC: the number of comparisons between the essential oil treatment and control treatment; SD: standard deviation; WMD: weighted mean differences between control and treatments with microalgae; CI: confidence interval of WMD; *p*-Value to χ^2 (Q) test of heterogeneity; I^2: proportion of total variation of size effect estimates that is due to heterogeneity; [1]: Egger's regression asymmetry test; [2]: Begg's adjusted rank correlation.

3.6. Meta-Regression and Publication Bias

Tables 2–5 show that the Egger regression asymmetry test was not significant ($p > 0.05$) for any of the response variables tested, indicating no publication bias.

On the other hand, Table 2 shows that there was heterogeneity (Q) ($p \leq 0.10$) in the ADFI, EP, EW, and FCR. Likewise, a significant Q ($p \leq 0.10$) was observed in YI and MDA in the egg (Table 3). In the blood serum, Table 4 shows a significant Q ($p \leq 0.10$) in the concentration of SOD, GPx, and TAC. Finally, significant Q ($p \leq 0.10$) was observed in the VH, CD, and VW in the duodenum, jejunum, and ileum (Table 5). However, Littell et al. [65] indicate that meta-regression analysis should only be applied when the variable of interest is reported in ten or more studies. Therefore, the meta-regression was only applied to the following response variables: ADFI, EP, EW, and FCR.

Table 6 shows no significant relationship between the covariates hen's age, supplementation period, and EO dose with any of the response variables tested. The breed/strain of the hens explained between 12.95 and 50.26% of the observed heterogeneity in the ADFI, EP, EW, and FCR. The primary bioactive metabolite covariate explained 37.99, 32.02, and 29.99% of the observed heterogeneity in the EP, EW, and FCR, respectively.

Table 6. Meta-regression of the effects of dietary essential oil supplementation on egg production and quality in laying hens.

Parameter		Hen's Age	Breed/Strain	Supplementation Period	EOs Dose	Primary Bioactive Compound
Average daily feed intake (ADFI)	QM	0.006	41.842	0.614	1.422	9.509
	Df	1	7	1	1	9
	p-Value	0.939	<0.001	0.433	0.233	0.392
	R^2 (%)	7.84	50.26	0.85	3.73	7.05
Egg production (EP)	QM	0.146	24.678	4.777	0.076	88.696
	Df	1	8	1	1	10
	p-Value	0.702	0.002	0.811	0.783	<0.001
	R^2 (%)	0.00	12.95	7.05	0.00	37.99
Egg weight (EW)	QM	8.441	45.57	2.608	0.316	25.645
	Df	1	8	1	1	8
	p-Value	0.074	<0.001	0.106	0.574	0.001
	R^2 (%)	3.41	34.42	0.00	0.00	32.02
Feed conversion ratio (FCR)	QM	0.005	56.77	3.452	0.891	99.485
	Df	1	8	1	1	10
	p-Value	0.943	<0.001	0.244	0.345	<0.001
	R^2 (%)	0.00	14.81	0.00	0.00	29.99

QM: coefficient of moderators; QM is considered significant at $p \leq 0.05$; df: degree of freedom; R^2: the amount of heterogeneity accounted for; EOs: essential oils.

3.7. Subgroup Analysis

Figure 2a shows that the ADFI decreased ($p < 0.01$) when the laying hen breeds/strains used were Hy-Line White (WMD = −2.163 g/d) and Super Nick H&N (WMD = −2.590 g/d). In contrast, the ADFI increased when the breeds/strains of laying hens used were Hy-Line Brown (WMD = 0.631 g/d) and Bovans Brown (WMD = 3.081 g/d) and was not affected when other breeds/strains of laying hens were used ($p > 0.05$). On the other hand, the EP increased ($p < 0.05$; Figure 2b) when the breeds/strains of laying hens used were Hy-Line White (WMD = 2.526%), Hy-Line Brown (WMD = 1.766%), Lohmann Brown (WMD = 4.028%), ISA Brown (WMD = 5.196%), and Bovans Brown (WMD = 3.542%). However, the EP was not affected when other breeds/strains of laying hens were used ($p > 0.05$). Similarly, Figure 2c shows that the EW increased when the laying hen breeds/strains used were Hy-Line Brown (WMD = 0.287 g), Lohmann Brown (WMD = 0.688 g), Lohmann White (WMD = 0.561 g), ISA Brown (WMD = 1.561 g), and Bovans Brown (WMD = 3.449%). However, the EW was not affected when other breeds/strains of laying hens were used ($p > 0.05$). The FCR decreased ($p < 0.01$) when the breeds/strains of laying hens used were Hy-Line White (WMD = −0.085 g/g), Hy-Line Brown (WMD = −0.045 g/g), Super Nick H&N (WMD = −0.069 g/g), Lohmann Brown (WMD = −0.068 g/g), ISA Brown (WMD = −0.269 g/g), and Bovans Brown (WMD = −0.122 g/g). However, the FCR was not affected when other breeds/strains of laying hens were used ($p > 0.05$).

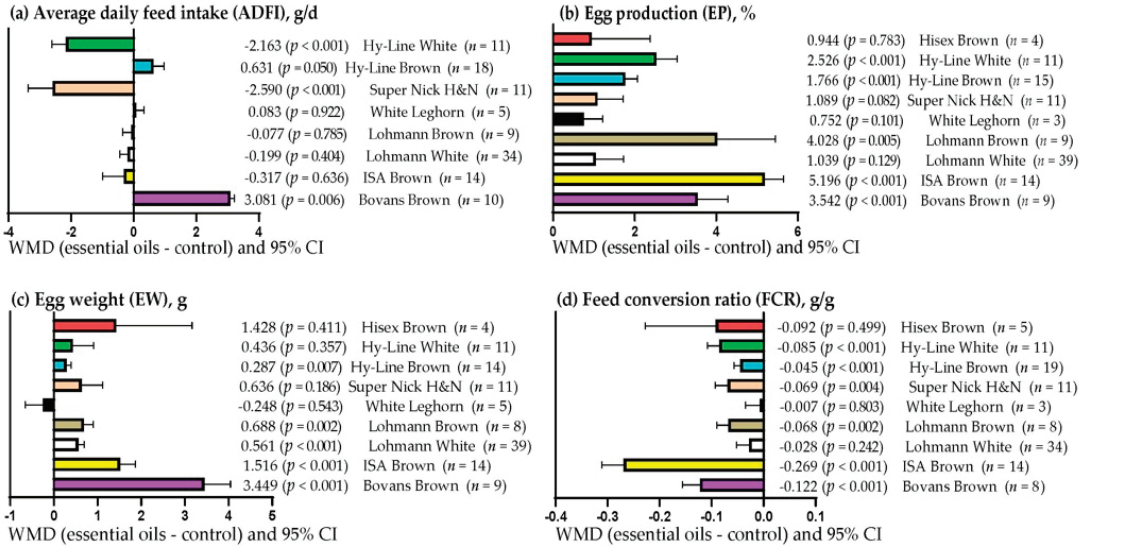

Figure 2. Subgroup analysis (subgroup = breed/strain of laying hens) of the effect of essential oil supplementation on the diets of laying hens; WMD = weighted mean differences between the essential oil treatments and control.

Figure 3a shows that the EP was increased ($p < 0.01$) when the primary bioactive metabolites of EOs were trans-anethole (WMD = 0.808%), terpinene-4-ol (WMD = 2.768%), cinnamaldehyde (WMD = 8.268%), blend (WMD = 2.650%), and menthol (WMD = 3.055%). However, when EOs were used with other primary bioactive metabolites, the EP was not affected ($p > 0.05$). On the other hand, Figure 3b shows that the EW increased ($p < 0.05$) when the primary bioactive metabolites of the EOs were trans-anethole (WMD = 0.122 g), cinnamaldehyde (WMD = 1.718 g), thymol (WMD = 0.282 g), blend (WMD = 0.677 g), and menthol (WMD = 1.671 g). However, the EW was not affected when EOs were used with other primary bioactive metabolites ($p > 0.05$). Figure 3c shows that the FCR decreased ($p < 0.05$) when the primary bioactive metabolites of the EOs were terpinene-4-ol

(WMD = −0.100 g/g), blend (WMD = −0.097 g/g), and menthol (WMD = −0.074 g/g). However, when EOs were used with other primary bioactive metabolites, the FCR was not affected ($p > 0.05$).

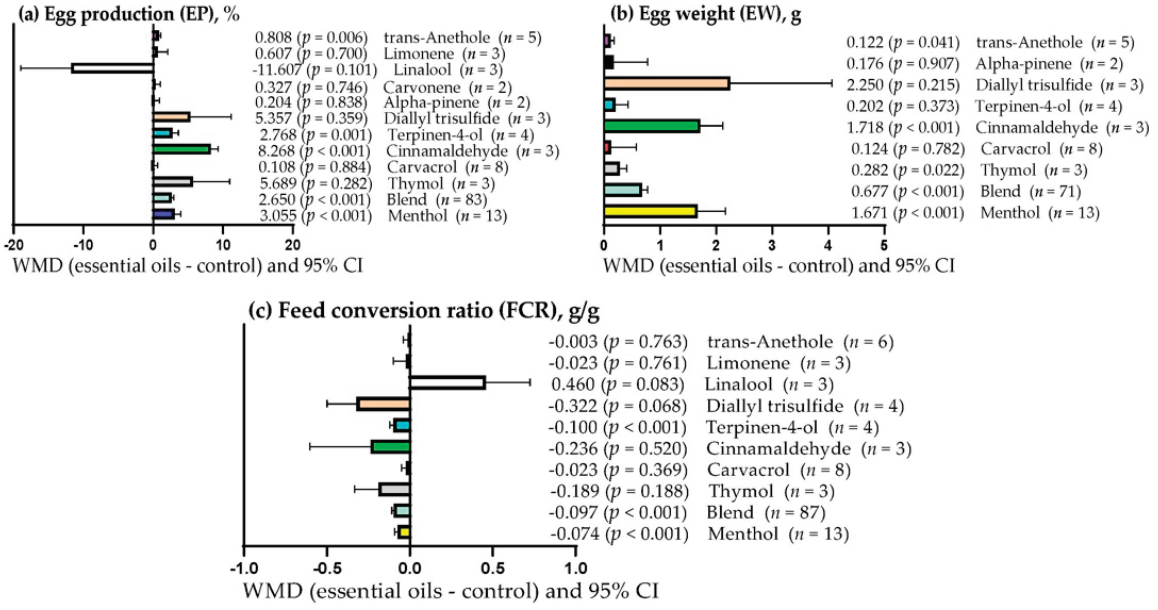

Figure 3. Subgroup analysis (subgroup = primary bioactive compound) of the effect of essential oil supplementation on the diets of laying hens; WMD = weighted mean differences between the essential oil treatments and control.

4. Discussion

4.1. Performance

Some authors [11–13] have indicated that EOs can be used as additives to improve the flavor and palatability of feed for farm animals. However, in the present meta-analysis, EO supplementation did not affect the ADFI. In a previous meta-analysis, Irawan et al. [15] also did not observe changes in ADFI in broilers supplemented with EOs. On the other hand, higher EP, EW, EM, and lower FCR were observed in response to supplementation with EOs. In the present study, EO supplementation increased the SOD, GPx, and TAC serum levels. Furthermore, Cheng et al. [10] reported up to 18 and 20% higher serum concentrations of immunoglobulin G and interleukin-2 in laying hens supplemented with EOs. Likewise, previous studies [2,49] also detected that dietary supplementation with EOs decreased the count of bacteria *Salmonella* sp. and *Escherichia coli* in the small intestine and cecum of laying hens between 17 and 26%. These effects of EOs could improve the health status of laying hens and positively modify the EP, EW, EM, and FCR.

In laying hens, it has been reported that the dietary inclusion of EOs increases the secretion of digestive enzymes (chymotrypsin, lipase, and α-amylase) [2,24] and the digestibility of dry matter, crude protein, and ether extract [23,38]. In the present study, increased VH was observed in the duodenum, jejunum, and ileum in response to EO supplementation. Furthermore, He et al. [42] detected that EO supplementation increased the expression of glucose transporters (GLUT2) and peptides (PEPT1) in the duodenum and jejunum of laying hens. These effects of EOs could increase the absorption and metabolic availability of nutrients and benefit the EP, EW, EM, and FCR. On the other hand, previous studies [35,42] have indicated that in laying hens, the dietary inclusion of EOs (between 24 and 100 mg/kg DM) increases the cecal abundance of the genus microbial *Lactobacillus*, which has a nega-

tive correlation with FCR in laying hens [39]. Furthermore, recent studies [1,39] detected that, in laying hens, supplementation with EOs increased the relative cecal abundance of *Anaerofilum*, *Fusobacterium*, and *Sutterella* bacteria, which have positively correlated with EP in laying hens [39]. Consequently, similar effects of the consumption of EOs in the present study partially explain the positive effects observed in EP and FCR.

4.2. Egg Quality

Egg quality is important in laying hen production systems because it relates to consumer acceptability and preference [59] as well as economic profitability [18]. In particular, ET and ES are used as indicators to evaluate the quality of the eggshell [10], which influences the transport and storage of eggs [66]. In the present study, ET and ES increased in response to EO supplementation. These results are positive, since higher ET and ES could result in a lower rate of broken eggs [59]. In laying hens, recent studies [10,23] have reported that low doses (between 50 and 75 mg/kg feed) of EO mixtures increase intestinal calcium absorption by up to 14.7%. Similar effects of the consumption of EOs in the present study partially explain the observed increase in ET since, in laying hens, ET increases linearly with the increase in the metabolic availability of calcium. On the other hand, the higher ES observed in the present study could be related to the observed increase in ET since there was a positive correlation (r = 0.64) between ES and ET in eggshells from laying hens [67].

Malfatti et al. [66] indicated that AH, HU, YC, and YI are important parameters that serve as indicators of the internal quality of the egg. Particularly, AH, HU, and YI are indicators commonly used to assess egg freshness [66,68] because they have a negative correlation (r between 0.82 and 0.89) with their storage time [69]. Likewise, YC is an important parameter because most consumers prefer eggs with darker yolks [70]. In the present study, EO supplementation increased the AH, HU, YC, and YI, indicating that EOs can be used as a nutritional strategy to improve the color and internal quality in the eggs of laying hens. EOs contain terpenoids such as thymol and carvacrol [9,71], which have been reported to stimulate ovomucin synthesis in laying hens [72]. Similar effects of the consumption of EOs in the present meta-analysis would partly explain the increases observed in the AH, HU, and YI because, in laying hens, the AH, HU, and YI are positively correlated (r between 0.69 and 0.71) with the ovomucin concentration in egg [73]. On the other hand, Kavan et al. [43] mentioned that dietary pigments are the main factors influencing YC changes. Therefore, the higher YC observed in the present study suggests that the EOs used probably contained pigments.

There is limited information on the use of EOs to increase the oxidative stability in eggs from laying hens [33]. In the present study, a lower MDA content was observed in the egg yolk in response to dietary supplementation with EOs. This result indicates that EOs decrease yolk lipid peroxidation [54], which could increase the shelf life of eggs. Previous studies [74,75] have indicated that EOs contain various bioactive compounds with antioxidant properties that can be absorbed in the laying hens' intestines and subsequently enter the egg yolk through blood circulation. This mechanism of absorption of the antioxidant compounds in the EOs partially explains the lower content of MDA in the yolk observed in the present study.

4.3. Antioxidant Status

In laying hens, ROS are continuously produced as a consequence of normal biological processes [22]. However, excessive accumulation of ROS can lead to OS [3,11]. According to Gholami-Ahangaran et al. [74], EOs can be used as natural antioxidants in poultry diets since they contain several bioactive metabolites with antioxidant activity. Serum levels of SOD, GPx, TAC, and MDA are essential to assess the antioxidant status in laying hens [10]. In the present study, EO supplementation increased the serum levels of SOD and GPx. According to Surai et al. [3], an increase in the serum levels of SOD and GPx decreases the oxidative damage caused by ROS in poultry cell membranes. To our knowledge, the

mechanism of action of EOs or their bioactive metabolites on the serum levels of SOD and GPx has not been studied in laying hens. However, terpenoid consumption has been documented to increase the rodents' expression of genes encoding SOD and GPx [76]. EOs contain a wide variety of terpenoids (e.g., linalool, carvonene, and 1,8-cineole) [71]. Therefore, similar effects of EO consumption in the present meta-analysis could explain the observed increase in SOD and GPx.

On the other hand, a higher TAC was observed in response to supplementation with EOs. This effect indicates that EOs improve the overall antioxidant status in laying hens. In poultry, most of the antioxidant metabolites of EOs are bioavailable, since after being ingested, they can be absorbed in the intestine and later transferred to the bloodstream [74,75]. Therefore, this effect could be related to the higher TAC detected in the present meta-analysis, since the TAC values in blood serum increase when the absorption and bioavailability of the antioxidants consumed are high [77].

In the present meta-analysis, the inclusion of EOs in the diets of laying hens decreased the serum MDA levels. This effect indicates that EOs can be used successfully to decrease lipid peroxidation in the blood of laying hens because, according to Nielsen et al. [78], the serum concentration of MDA decreases when lipid peroxidation is low. In addition, EOs contain several monoterpenes (e.g., p-cymene, limonene, and α-pinene) [71] that can be absorbed and transferred to the blood, where they can subsequently act by eliminating ROS [74]. This mechanism of action partially explains the lower serum concentration of MDA observed in the present study because lipid peroxidation decreases when the presence of ROS is low [79].

4.4. Intestinal Morphology

In laying hens, small intestine morphology characteristics such as the VH, VW, CD, and VH/CD ratio are used to assess the health and nutrient absorptive capacity [10]. The present study showed that the dietary addition of EOs increased the VH, VW, and VH/CD ratio and decreased the CD in the duodenum, jejunum, and ileum of laying hens. In a previous meta-analysis, Irawan et al. [15] also observed a higher VH and lower CD in broilers supplemented with EOs. A higher VH is related to a higher enterocyte count and production of digestive enzymes [49]. Likewise, an increase in VH and VW indicates a greater nutrient absorption surface in the intestine [23]. On the other hand, intestinal crypts contain cells that produce mucus and replace damaged and aged cells [49]. Some authors [23,52] have mentioned that the activity and depth of the crypt increase when cell detachment in the intestine is high due to inflammation induced by pathogens and their toxins. In addition, a high VH/CD ratio is associated with better structural integrity of the intestinal epithelium and a greater area of nutrient absorption [24]. Therefore, the changes in the VH, VW, CD, and VH/CD ratio observed in the present study suggest that EOs improve the integrity and absorptive capacity of the intestinal epithelium in laying hens.

In poultry, the consumption of EOs increases the mRNA expression of tight junction proteins (occludins and cadherins) [59] and decreases the count of pathogenic bacteria (*E. coli* and *Salmonella* sp.) in the small intestine [15,42]. According to Mousavi et al. [49] and Wang et al. [59], these effects reduce pathogen damage to intestinal epithelium cells and improve their integrity, which could partially explain the positive effects observed in the present study for the VH, VW, CD, and VH/CD ratio.

5. Conclusions

The results of this meta-analysis indicate that the inclusion of essential oils in the diets of laying hens can be used as a nutritional strategy to improve the productive performance, egg quality, antioxidant status in blood serum, and intestinal morphology. The best production and egg weight results were obtained with Lohmann Brown, ISA Brown, or Bovans Brown laying hens and when the primary bioactive compound of the essential oils was menthol, cinnamaldehyde, or mixtures of bioactive compounds. Likewise, the best feed conversion ratio was obtained with ISA Brown or Bovans Brown laying hens and when the

primary bioactive compound of the essential oils was menthol, terpinen-4-ol, or mixtures of bioactive compounds.

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Impacts of *Nigella sativa* Inclusion during Gestation and Lactation on Ovarian Follicle Development, as Well as the Blood and Metabolic Profiles of Ardi Goats in Subtropics

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Abstract: The present study aimed to alleviate the negative effects of the peripartum and postpartum periods on the timing of ovarian follicle development, milk composition, as well as blood and metabolic profiles due to *Nigella sativa* (*N. sativa*) supplementation. Twenty-seven pregnant Ardi goats were classified using a randomized complete design into three groups: a control group and two *N. sativa* groups (10.0 and 20.0 g *N. sativa* seeds per kg diet). Productive and reproductive performances, in addition to blood and metabolic profiles, were investigated and compared using Duncan's multiple test. *N. sativa* supplementation increased dry matter intake and body weight. Ruminal pH and total bacterial counts were increased versus a decreased total protozoal count due to *N. sativa* inclusion. Additionally, *N. sativa* supplementation increased the concentration of protein, lactose, solids not fat, fat, and ash in milk. Pulse rates were the lowest ($p < 0.05$) in the *N. sativa* group and the partial pressure of oxygen was the lowest in the control group. Red and white blood cells and their related parameters (hemoglobin, hematocrit, neutrophils, and lymphocytes) showed significant increases due to *N. sativa* inclusion. Total protein, albumin, globulin, glucose, and minerals (calcium, phosphorus, and magnesium) values were higher ($p < 0.05$) in the *N. sativa* group. Lower concentrations of blood urea nitrogen were found in *N. sativa* groups compared to control one. In conclusion, *N. sativa* inclusion from 4 weeks prepartum to 4 weeks postpartum of Ardi goats modified productive and reproductive performances without any adverse effects on blood and metabolic profiles.

Keywords: *Nigella sativa*; follicles; growth; blood; metabolites

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1. Introduction

The peripartum or transition period is the most critical for the productive and reproductive performances of mammalian pregnant species and their resulting offspring. It extends from 3 weeks prepartum to 3 weeks postpartum in small ruminants [1]. During this period, disruptions were observed in ovarian follicle development and the quality of the resulting oocytes and embryos, feed utilization and growth performance, milk production and composition, and blood metabolites [2–8]. The decrease in feed intake can reach 30–35%, especially during the summer season in subtropics where temperatures exceed 45.0 °C during the day. The decrease in feed intake or the lack of management resulted in severe deficiencies of productive and reproductive performances under such circumstances [2,5,6,9]. Therefore, the restoration of disruptions during the prepartum and postpartum periods might improve reproductive and productive performances of pregnant species.

Several approaches can be used to mitigate the disruptive effects of prepartum and postpartum periods in pregnant animals, such as diet composition and supplementation [2,4–7]. Because of the negative effects on animals' health and the fertility of increased protein in the diet [10,11], it is important to find beneficial and non-traditional supplements for

improving productive and reproductive performances [12]. There is an increased interest in using *N. sativa* as a diet supplement for ruminants and humans [4,13–18].

N. sativa or black seeds are obtained from an annual plant cultivated in Asia and the Middle East. The *N. sativa* seeds contain protein (20.0–27.0%), carbohydrates (23.50–33.20%), fat (34.5–38.7%), crude fiber (8.4%), and ash (4.8%), in addition to vitamins, minerals, and carotene [4]. In addition, the *N. sativa* seeds contain active materials known as thymohydroquinone, thymoquinome, and nogelleone, which are known to give antitoxic, antimicrobial, and pharmacological properties that improve the defense system [4]. The *N. sativa* seed and their purified constituents have been widely used in the treatment of different diseases. Because *N. sativa* plays an important role as a natural antioxidant and immune stimulant [4,13], it might be used for anti-stress during the elevation of ambient temperature and humidity.

Several studies have been designed using different animal species to explore the effect of *N. sativa* on growth and reproductive performance [19–21]. The supplementation of animals with black seeds or black seed oil resulted in the improvement of growth performance and milk production [22], blood profiles, plasma metabolites [20,23], and reproductive performance [21,24,25]. There is inadequate information on the effects of *N. sativa* on ovarian follicle development, growth and feed utilization, as well as the biochemical profiles of Ardi goats during the peripartum period in subtropics. Therefore, our hypothesis is that the supplementation of *N. sativa* seeds would alleviate the negative effects of the peripartum and postpartum periods through improving feed utilization, blood and metabolic profiles, ovarian follicles' development, and milk composition.

2. Materials and Methods

2.1. Animals' Diets, Management, and Experimental Management

This study was carried out in the Research and Training Station of King Faisal University and approved by the scientific research ethical committee (Ref. No. KFU-REC-2022-JAN-EA000130). Twenty-one healthy pregnant Ardi goats of 101.66 ± 0.4 kg body weight, aged 2.0–2.5 years, were allocated using complete random design to three groups: a control group and two *N. sativa* groups (10 and 20 g/kg diet). The goats were given the routine vaccination of the farm station. The goats of each group during the experimental period were kept free in pens at a stocking rate of 2.00 m^2/head, and they were fed individually. The average ambient temperature ranged between 34.0 to 45.0 °C, and the relative humidity ranged between 29.5 and 40.0%. The goats were offered daily 2.0 kg concentrate diet for the control group (Table 1) and concentrate diet supplemented with the recommended doses of 10.0 and 20.0 g *N. sativa* seeds/kg diet per head in addition to ad libitum berseem hay [4].

Table 1. Chemical composition of experimental diets on dry matter basis (%).

Parameters	Dry Matter	Organic Matter	Crude Protein	Ether Extract	Crude Fiber	Nitrogen-Free Extract	Ash
Concentrate feed mixture	90.10	90.41	14.30	3.90	8.51	63.70	9.59
Alfalfa hay	90.76	87.90	20.63	1.46	23.83	41.98	12.10

The goats were randomly assigned into three groups: two control groups and 1.0% and 2.0% *N. sativa* groups. The periods for the study included approximately 4 weeks prepartum and continued to 4 weeks postpartum with *N. sativa* supplementation. The doses of *N. sativa* seeds were mixed with the concentrate diet and given daily at 08:00 a.m. to each goat. The diet was prepared according to the guidelines from the National Research Council for goats to meet the requirements [26]. Fresh water was available ad libitum. The recorded feed intake was calculated through the difference between the daily offered diet and its respective ort. The diet and ort samples were collected daily, transformed into

composites, and stored for chemical analysis at the end of the experiment. The body weight gain (kg) of the goats was recorded 4 weeks prepartum and 4 weeks postpartum.

2.2. Collection of Ruminal Fluid

A stomach tube with sufficient diameter and length was used to collect ruminal fluid from the control and *N. sativa* (1.0 and 2.0 g/kg diet) animal groups [27]. The tube was inserted into the rumen to suck out the ruminal fluid. The tube was inserted into the mouth to the pharynx and enabled the animal to swallow the tube. The ruminal samples were aspirated 4 h after morning feeding. The ruminal samples were filtered through two layers of cheesecloth to count the protozoal [28] and bacterial numbers [29]. The filtrate portion was immediately used for pH measurement using a digital pH meter (Eacam, China) [30].

2.3. Rectal Body Temperature (RT), Heart Rate (HR), and Partial Pressure of Oxygen (SPO2)

The physiological parameters (RT, HR, and SPO2) of *N. sativa* (1.0 and 2.0 g/kg diet) and control groups were recorded biweekly. Rectal body temperature was recorded using a digital thermometer (Citizen Flex Digital Thermometer CTA303, Citizen, Stuttgart, Germany). The partial pressure of oxygen (PO2) and pulse rate were recorded using a pulse oximetry apparatus (CMS60D-VET, Contec Medical Systems Co., Ltd., Qinhuangdao (Hebei), China). The goats were kept in a pen, restrained, and the heart rate and PO2 were recorded by the proper sensor that was put on the upper lip of the goat [6].

2.4. Ovarian Follicle Development

Goats were investigated postpartum by a real-time B-mode ultrasound scanner (ContecTM B-Ultrasound Diagnostic System Model CMS 600 P2VET, Qinhuangdao (Hebei), China) with a 3.5 MHz transducer (C3.5-80R20-A16A IPX7). Ovaries of *N. sativa* and control goats were examined at days 3, 6, 9, 12, 15, and 18 postpartum to record the numbers and sizes of ovarian follicles. The ovarian follicles were categorized according to diameter into small—(diameter 2–2.9 mm), medium—(diameter 3–4.9 mm), and large-sized (diameter $\geq$ 5 mm) follicles [6].

2.5. Milk Chemical Analyses

One hundred milliliters of milk (three samples) were collected through hand milking from the control and *N. sativa* (1.0% and 2.0%) animal groups weekly (weeks 2, 3, and 4 postpartum) in flasks for the chemical analysis of protein, lactose, solids not fat, fat, and ash (MilkoScan™ Mars, Hilleroed, Denmark). Milk energy was calculated as described by Economides [31].

2.6. Blood Samples' Collection and Analysis

Blood samples were collected biweekly through jugular vein puncture from each goat of *N. sativa* (10.0 and 20 g/kg diet) and control groups in a sterile tube containing anticoagulant (EDTA K3 Australia). The collected blood samples were analyzed for hematological profiles using a hematology analyzer (Abaxis Vetscan HM5, Union City, CA, United States) and biochemistry parameters using a chemistry analyzer (Skyla VB1, Hsinchu, Taiwan). The measured hematological parameters included red blood cells, hematocrit, and hemoglobin values, in addition to white blood cells and their differentiation and platelets. The measured biochemistry plasma parameters included total protein, glucose, urea, liver functions, and mineral values.

2.7. Statistical Analysis

The statistical analysis of variances was conducted through the general linear model of the Proc Mixed SAS package version 9.2 [32]. Differences between *N. sativa* (1.0 and 2.0 g/kg diet) and control groups were tested for body weight, rumen parameters, milk traits, ovarian follicle development, as well as blood and metabolic profiles by one-way ANOVA. A comparison between the means of *N. sativa* and control groups and the level

of significance ($p < 0.05$) was set using Duncan's test [33]. The statistical model was Yij = μ + Ti + Eij, where Yij = the observation ij, μ = the overall mean, Ti = the effect due to *N. sativa* supplementation (10.0 and 20 g/kg diet), and Eij = the experimental error.

3. Results

3.1. Feed Intake, Body Weight, Ruminal Parameters, and Physiological Parameters

Feed intake, body weight, ruminal traits, and physiological parameters are shown in Table 2. Treatments with *N. sativa* (10.0 and 20 g/kg diet) resulted in higher ($p > 0.05$) dry matter intake and body weight (kg) 4 weeks postpartum. Regarding ruminal parameters, ruminal pH and total bacterial count increased, whereas the total protozoal count decreased in *N. sativa* groups compared to the control group. The highest values were recorded in the 2.0% *N. sativa* group followed by 1.0% *N. sativa* and control groups, respectively. The body temperature and partial pressure of oxygen were insignificantly increased in the 2.0% *N. sativa* when compared to 1.0% *N. sativa* and control groups. The lowest pulse rate (beats/min) was recorded in 2.0% *N. sativa* group ($p < 0.05$) compared to other groups.

Table 2. Effects of *N. sativa* (10.0 and 20 g/kg diet) on body weight, ruminal, and physiological parameters of Ardi goats in subtropics.

Parameters	Treatments			SEM	*p*-Value
	Control	*N. sativa* 1 g/kg Diet	*N. sativa* 2 g/kg Diet		
Body weight 4 weeks prepartum, kg	101.42	102.85	100.71	0.5	0.08
Body weight 4 weeks postpartum, kg	109.28 ^{c}	112.28 ^{b}	114.14 ^{a}	0.53	<0.0001
Dry matter intake (kg/d)	1.62 ^{b}	1.71 ^{a}	1.78 ^{a}	0.02	0.005
Ruminal parameters					
pH	6.30 ^{b}	6.40 ^{ab}	6.46 ^{a}	0.03	0.043
Total protozoa, $\times 10^5$/mL	3.16 ^{a}	3.06 ^{ab}	3.01 ^{b}	0.03	0.030
Total bacteria, $\times 10^{11}$/mL	6.20 ^{c}	6.76 ^{b}	7.10 ^{a}	0.09	<0.0001
Physiological parameters					
Body temperature, °C	37.3	37.3	37.4	0.03	0.526
Pulse rate	125.5 ^{a}	122.6 ^{a}	115.5 ^{b}	1.16	0.0005
Partial pressure of oxygen	92.5 ^{b}	94.4 ^{b}	96.8 ^{a}	0.54	0.0071

$^{a, b, c}$ Values with different superscripts between groups significantly differ at $p < 0.05$.

3.2. Ovarian Follicle Development and Milk Composition

The results of small, medium, and large ovarian follicles' development recorded 3, 6, 9, 12, 15, and 18 days postpartum indicated earlier ovarian follicle resumption in *N. sativa* groups compared to the control group (Figure 1). The numbers of small, medium, and large follicles were higher ($p < 0.05$) in the *N. sativa* groups during the postpartum periods (Figure 1A–C). *N. sativa* effects (10.0 and 20 g/kg diet) on the milk composition of Ardi goats after kidding are presented in Table 3. The *N. sativa* (10.0 and 20 g/kg diet) supplementation increased ($p < 0.05$) solids not fat, protein, lactose, and ash compared to the control group.

Table 3. Effects of *N. sativa* (10.0 and 20 g/kg diet) on milk constituents of Ardi goats in subtropics.

Parameters	Treatments			SEM	*p*-Value
	Control	*N. sativa* 1 g/kg Diet	*N. sativa* 2 g/kg Diet		
Solids not fat, %	8.83 ^{c}	9.57 ^{b}	9.84 ^{a}	0.10	<0.0001
Fat, %	3.11	3.18	3.13	0.02	0.14
Protein, %	3.11 ^{b}	3.62 ^{a}	3.69 ^{a}	0.06	<0.0001
Lactose, %	4.73 ^{b}	5.28 ^{a}	5.39 ^{a}	0.07	<0.0001
Ash, %	0.31 ^{b}	0.31 ^{b}	0.38 ^{a}	0.01	<0.0001
Density	1.026	1.029	1.030	0.00	0.052
Milk energy content, MJ/kg	3.27 ^{b}	3.31 ^{a}	3.28 ^{b}	0.01	0.0097

$^{a, b, c}$ Values with different superscripts between groups significantly differ at $p < 0.05$.

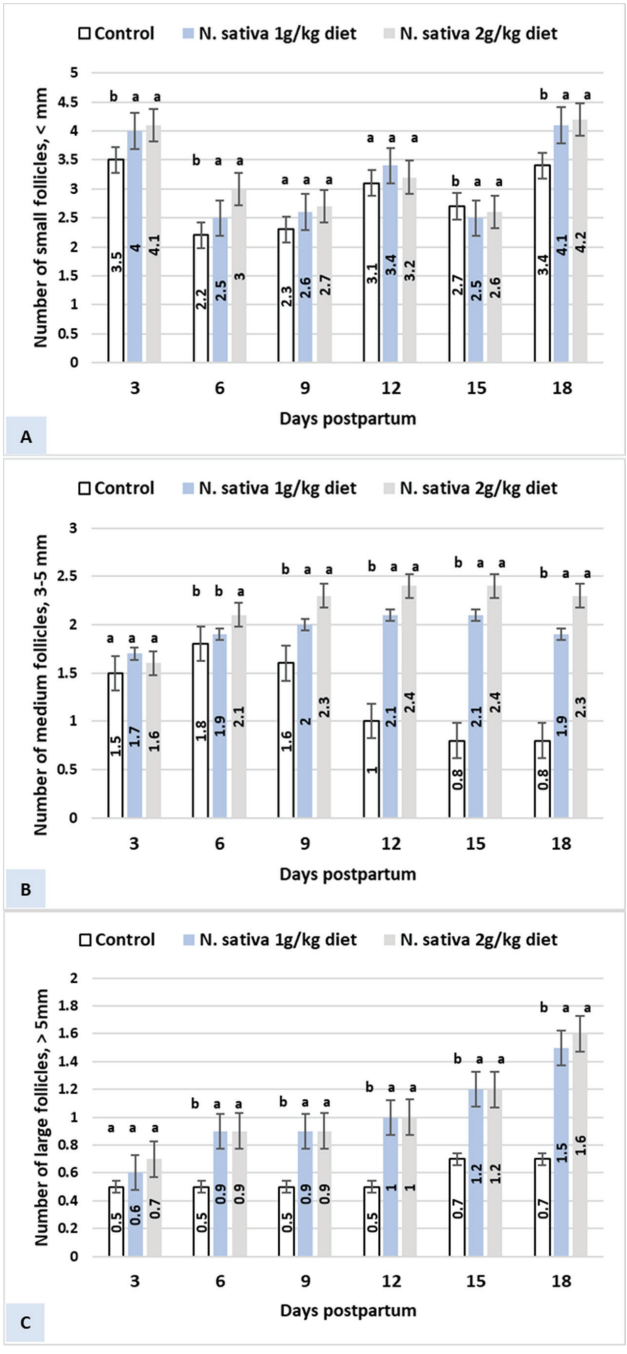

Figure 1. Effects of *N. sativa* (10.0 and 20 g/kg diet) on ovarian follicle development postpartum of Ardi goats. [a, b] Values with different superscripts between groups significantly differ at $p < 0.05$. (**A**) Number of small follicles < 3 mm; (**B**) Number of medium follicles 3–5 mm; (**C**) Number of large follicles > 5 mm.

3.3. Hematological and Biochemistry Profiles

Hematological indices are presented in Table 4. Hematological indices showed a significant increase in the values of RBCs (106/μL) and Hb (g/dL), in addition to WBCs (103/μL), lymphocytes, and neutrophils (103/μL) in *N. sativa* groups (10.0 and 20 g/kg diet), when compared to the control one. The highest values were observed in 2.0% *N. sativa* group when compared to 1.0% *N. sativa* and control groups, respectively. The blood biochemistry indices of *N. sativa* (10.0 and 20 g/kg diet) and control groups are presented in Table 5. The results indicated the highest values ($p < 0.05$) of total protein (g/dL), albumin (g/dL), globulin (g/dL), and glucose (mg/dL) recorded in the 2.0% *N. sativa* group when compared to the other groups. Urea nitrogen ($p < 0.05$) and liver enzymes ($p > 0.05$) were lowered due to 2.0% *N. sativa* supplementation when compared to 1.0% *N. sativa* and control feeding, respectively. Furthermore, the mineral concentrations (calcium, phosphorus, and magnesium) were improved ($p < 0.05$) due to *N. sativa* supplementation when compared to the control one.

Table 4. Effects of *N. sativa* (10.0 and 20 g/kg diet) on blood indices of Ardi goats in the subtropics.

Parameters	Treatments			SEM	*p*-Value
	Control	*N. sativa* 1 g/kg Diet	*N. Sativa* 2 g/kg Diet		
Red blood cells, 10^{12}/L	10.97 ^{b}	12.72 ^{a}	12.53 ^{a}	0.19	<0.0001
Hemoglobin, g/dL	12.80 ^{c}	13.40 ^{b}	15.30 ^{a}	0.28	<0.0001
Hematocrit, %	35.15 ^{b}	36.19 ^{ab}	41.22 ^{a}	0.68	<0.0001
MCV, fl or μm^3	32.00 ^{a}	28.00 ^{b}	33.00 ^{a}	0.57	0.0001
MCH, pg/cell	11.70 ^{b}	10.60 ^{c}	12.20 ^{a}	0.17	<0.0001
MCHC, g/dL or %	36.60	37.20	37.10	0.59	0.8284
RDWc, %	22.40 ^{c}	27.00 ^{a}	24.00 ^{b}	0.46	<0.0001
RDWs, fl	31.20 ^{b}	32.00 ^{ab}	32.80 ^{a}	0.25	0.0096
White blood cells, 10^{9}/L	9.36 ^{b}	11.66 ^{a}	12.0 ^{a}	0.29	<0.0001
Lymphocytes, 10^{9}/L	5.47 ^{b}	5.72 ^{b}	6.31 ^{a}	0.10	<0.0001
Monocytes, 10^{9}/L	0.05 ^{b}	0.06 ^{a}	0.06 ^{a}	0.00	<0.0001
Neutrophils, 10^{9}/L	2.39 ^{b}	4.55 ^{a}	4.47 ^{a}	0.23	<0.0001
Eosinophils, 10^{9}/L	1.27 ^{a}	1.20 ^{a}	1.05 ^{b}	0.03	0.0063
Basophils, 10^{9}/L	0.18 ^{a}	0.13 ^{b}	0.12 ^{b}	0.01	<0.0001
Platelet, 10^{9}/L	153.0 ^{b}	161.0 ^{a}	165.0 ^{a}	1.67	0.0100
Mean platelet volume, fl	6.13 ^{b}	6.50 ^{a}	6.10 ^{b}	0.06	0.0176
Platelet distribution width PDWc, %	29.50 ^{c}	27.90 ^{b}	32.30 ^{a}	0.44	<0.0001
Platelet distribution width PDWs, fl	7.00 ^{b}	6.80 ^{c}	7.30 ^{a}	0.06	<0.0001

$^{a, b, c}$ Values with different superscripts between groups significantly differ at $p < 0.05$. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

Table 5. Effects of *N. sativa* (10.0 and 20 g/kg diet) on blood biochemistry of Ardi goats in subtropics.

Parameters	Treatments			SEM	*p*-Value
	Control	*N. sativa* 1 g/kg Diet	*N. Sativa* 2 g/kg Diet		
Total protein, g/dL	7.00 ^{b}	7.20 ^{a}	8.00 ^{a}	0.11	<0.0001
Albumin g/dL	3.20 ^{b}	3.30 ^{ab}	3.50 ^{a}	0.05	0.05
Globulin g/dL	3.80 ^{b}	3.90 ^{b}	4.50 ^{a}	0.08	0.0006
Blood urea nitrogen, mg/dL	17.20 ^{a}	15.60 ^{b}	13.50 ^{c}	0.40	0.0003
Urea, mg/dL	36.80 ^{a}	33.40 ^{b}	28.90 ^{c}	0.85	0.0003
Glucose mg/dL	60.00 ^{b}	61.00 ^{b}	75.33 ^{a}	1.88	<0.0001
Alkaline phosphatase, U/L	82.23	80.37	80.0	0.65	0.271

Table 5. *Cont.*

Parameters	Treatments			SEM	*p*-Value
	Control	*N. sativa* 1 g/kg Diet	*N. Sativa* 2 g/kg Diet		
Aspartate aminotransferase, U/L	98.13 [a]	97.27 [a]	94.27 [b]	0.56	0.008
Gamma–glutamyl transferase, U/L	40.34	39.27	38.35	0.39	0.055
Creatine Phosphokinase, U/L	114.54	115.65	112.23	0.80	0.0221
Calcium, mg/dL	08.70 [c]	09.80 [b]	10.20 [a]	0.15	<0.0001
Phosphorus, mg/dL	3.87 [c]	4.10 [b]	5.53 [a]	0.17	<0.0001
Magnesium, mg/dL	0.95 [b]	1.03 [a]	1.03 [a]	0.01	<0.0001
Sodium, mmol/L	136.61 [b]	141.31 [a]	138.17 [b]	0.56	<0.0001
Potassium, mmol/L	04.30 [b]	04.60 [a]	04.50 [a]	0.05	0.0166
Chloride, mmol/L	107.0	109.21	107.31	0.51	0.26

[a, b, c] Values with different superscripts between groups significantly differ at $p < 0.05$.

4. Discussion

The beneficial effects of feed additives, especially *N. sativa* seeds on feed utilization, metabolic conditions, and reproductive functions, have been reported in several studies [13,21,34–36]. Our present study was designed to restore the negative effects of the peripartum period on feed intake and rumen parameters, milk traits, postpartum ovarian follicle development, and the related blood and plasma metabolites of Ardi goats in the Eastern subtropical area of KSA. The significant effects of *N. sativa* seeds and extract on body health status, as well as productive and reproductive performances, were confirmed earlier in several studies [13,21,25]. In the current study, the improvement in body health, as well as productive and reproductive performances, was the highest in the *N. sativa* (20 g/kg diet) group when compared with the *N. sativa* (10.0 g/kg diet) and control groups.

4.1. Body Weight, Ruminal, and Physiological Parameters

Body weight gain was improved in *N. sativa* groups (10.0 and 20 g/kg diet) when compared to the control group, as indicated in previous studies [12,13,16,19]. This might be attributed to increased feed intake, in addition to significant changes in rumen microbes, including increased bacteria and decreased protozoa values (Table 2), which might improve digestibility coefficients. It was found that *N. sativa* inclusion (12 g/day) significantly improved the digestibility coefficients of dry matter, organic matter, crude protein, and crude fiber [12]. Collectively, earlier reports concluded that *N. sativa* seeds or their extract have positive effects on body weight gain and nutrient digestibility [12–16,19]. The stimulation of appetite and increased peristaltic action of the stomach and bowels have been recorded due to *N. sativa* actions [37,38].

Ruminal pH and total bacterial counts increased, while total protozoal counts decreased in *N. sativa* groups (10.0 and 20 g/kg diet) when compared to the control group (Table 2). The changes in the rumen environment due to the 10.0 and 20 g/kg diet *N. sativa* treatments might be favorable for bacterial species growth [12,39]. It is reported that nitrogen retention and ruminal ammonia nitrogen values were improved ($p < 0.05$) due to *N. sativa* supplementation [12]. In addition, the pulse rate decreased in *N. sativa* groups, as previously indicated [40], and this might be consequently useful in the treatment of hypertension [41]. The higher partial pressure of oxygen (PO2) in *N. sativa* groups could be attributed to the increase in RBCs in those groups compared to control one (Table 4) [42].

4.2. Ovarian Follicle Development and Milk Composition

Feed additives must be safe for the health and well-being of pregnant and lactating goats to support their ovarian structures' development, milk production, and milk quality [5–8,34]. *N. sativa* seed supplementation (10.0 and 20 g/kg diet) resulted in an increase in small, medium, and large ovarian follicles compared to the control diet. This might be attributed to the significant increase in glucose ($p < 0.05$) and the decrease in urea ($p < 0.05$)

levels in *N. sativa* groups (Table 5). The positive energy balance in the *N. sativa* group might lead to an increase in insulin concentration and glucose uptake [43] (Nielsen and Ingvartsen, 2004). This change appears to stimulate the ovary and is associated with increased folliculogenesis. This explanation could be confirmed through our supplementation of *N. sativa* oil to female mice upon ovarian transplantation. Our results indicated an increased number of aspirated oocytes and quality from ovarian follicles [21]. Furthermore, the decreased level of blood urea nitrogen is associated with increased fertility [10,11]. The other metabolic factors attributed to the beneficial effects of *N. sativa* seeds and their extracts on ovarian follicle development and reproductive performance include essential amino and fatty acids and reproductive hormone values [4,11,13,21,24,25,36,44–49]. The improvement in metabolic factors was due to the effects of *N. sativa* seeds on the digestive system, including nutrient digestibility, better absorption, and body weight gain [13,19,20,45], as indicated in this study through higher total protein and body weight gain.

The *N. sativa* inclusion in the diets (10.0 and 20 g/kg diet) significantly improved milk constituents. Earlier studies coinciding with our study indicated the significant effects of *N. sativa* on improving milk production and composition [13,16]. The main factors of *N. sativa* that are involved in milk traits' improvement are the improvement in nutrient digestibility and blood metabolic profiles [12,16,50] (Tables 3 and 4), which lead to the availability of nutrients required for milk secretion. Additionally, *N. sativa* contains several nutrients, which might be attributed to the observed increase in milk production and composition [13–15].

4.3. Hematological and Biochemistry Indices

Of note, in the current experiment, the changes in not only the blood indices were obtained by the supplementation of *N. sativa* to pregnant or lactating goats' feed, but also in the blood plasma composition. Hematological profiles (RBCs, Hb, and PCV) showed significant beneficial changes between the *N. sativa* and control groups, as previously indicated [13,21,51]. Blood profiles are indicative of the body's health status. The immune stimulation of *N. sativa* seeds recorded in earlier reports [52], in addition to antioxidant activity [53–56], plays a crucial role in the protection of the body against inflammation or infection. Therefore, the improvement in blood profiles in *N. sativa* groups (10.0 and 20 g/kg diet) compared to the control might be attributed to the increase in feed conversion and body weight gain [4].

Metabolic profiles were improved in *N. sativa* groups if compared to control one. The increased activity of hepatic function is suggested when *N. sativa* seeds were fed [16], which resulted in a higher concentration of total proteins as recorded in the present study. Furthermore, supplementation with *N. sativa* seeds enhanced glucose concentration as a result of improved nutrient digestibility and greater total volatile fatty acids production [16]. Propionate is considered as the primary gluconeogenic volatile fatty acid used for glucose biosynthesis [57]. The present data of lower blood urea nitrogen and creatinine in *N. sativa*-treated groups were the same as previous reports [13,16]. Therefore, it can be assumed that *N. sativa* supplementation might improve the protein balance in goats.

Measurements of AST, GGT, and ALP hepatic enzymes are considered reliable indicators of liver function in ruminant animals [58,59], and the liver AST enzyme values were lowered ($p < 0.05$) due to *N. sativa* inclusion in the diet of goats. Moreover, CK, which has been used as a screening diagnostic parameter for endometritis muscular damage or hypocalcemia in dairy cattle [60], was unchanged due to *N. sativa* supplementation. There were lower values ($p > 0.05$) of liver enzymes with feeding *N. sativa* seeds to calves and goats [20], indicating their probable protective roles against liver dysfunction [61–63] or renal tissue damage [64].

The calcium, phosphorus, magnesium, and potassium values were increased in *N. sativa* groups when compared to control one, as indicated in several previous studies [13]. Calcium, phosphorus, and magnesium minerals in the blood provide an indication of the animals' health, and they are important for animals' production [65]. Herein, the

aforementioned mineral values were in the normal range and reflected the adequacy of minerals of in the *N. sativa* and control diets [66]. The increase in aforementioned minerals in *N. sativa* groups could be attributed to their presence in *N. sativa* seeds [4]. Calcium, phosphorus, and magnesium minerals are essential elements for muscle contraction, skeletal building, the production of energy, and anti-viral and anti-inflammatory agents [67,68]. Collectively, *N. sativa* seed components lead to a significant improvement in the functions of the gastrointestinal tract and liver, leading to an increase in body health, body weight gain, milk production, and ovarian structures' development during gestation and lactation periods of Ardi goats in subtropics.

5. Conclusions

The supplementation of pregnant Ardi goats with *N. sativa* seeds (1.0 and 2.0 g/kg diet) during the peripartum period is an effective strategy for improving feed utilization, milk traits, and blood and metabolic profiles, in addition to higher ovarian follicle development in subtropics. Further studies may be designed to explore the effects of *N. sativa* bioactive compounds to give proof of their possible applications for treatments of metabolic dysfunctions.

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Improving the Use of White Lupine in the Laying Quail Feeding by Enzymes Addition: Effects on Productive Performances, Digestion, Blood Biochemical Indices and Eggs Quality

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Abstract: Lupine seeds are a valuable nutritive source for animal feeding, but for poultry nutrition, the content in crude fiber and non-starch polysaccharides (NSP) have an antinutritional factor. The aim of this research was to highlight the effect of partial soybean meal replacement with *L. albus* seeds and enzyme addition in the laying quail diets on productive performance, digestion, blood biochemical indices and egg quality. A total of 210 homogenous female Japanese quails (*Coturnix japonica*) at 24 week of age were randomly assigned to 6 dietary treatments, with the standard diet based on soybean meal unsupplemented (−) and supplemented with enzyme (+) (S−/S+) and the experimental diets on which the soybean meal was based partially substituted by including lupine in the amount of 200 g/kg and 250 g/kg, unsupplemented and supplemented with enzymes ($L_{20}-/L_{20}+$; $L_{25}-/L_{25}+$).The use of enzymes in the lupine-based diets allowed increasing the proportion of lupine in the diet of laying quails by up to 25% (% of feed) without changing egg production, egg weight, feed conversion rate and physical–chemical quality parameters of the eggs. In addition, the use of lupine (−/+) improved ($p < 0.001$) the carotenoid content of the egg yolk, as well as the quality of the yolk fats by decreasing the cholesterol content and the level of fatty acids (FA) with an atherogenic effect, in favor of omega-3 FA. Enzyme supplementation of the lupine-based diets had a negative effect on the health lipid indices of the fats in the yolk (ratio of the hypocholesterolemic/Hypercholesterolemic FA—h/H, atherogenic index—AI, thrombogenic index—TI and health promotion index—HPI). The use of exogenous enzymes increased the nutrients' efficiency of the quails' feed, which is supported by the improvement of the blood metabolic indices and a decrease of intestinal digesta viscosity and feces moisture. In conclusion, white lupine can be used up to 25% in the laying quail feed in association with specific enzymes without affecting the productive performance and egg quality; moreover, lupine use has improved the quality of the eggs, increasing humans' health.

Keywords: white lupine; non-starch polysaccharides; omega-3 FA; carotenoids; cholesterol

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1. Introduction

At the present time, the high protein requirements for human and animal nutrition determine the protein sources which have become progressively more limited and expensive [1]. In the field of animal feeding, in the past 10 years, research attention has focused years on the detection and validation of alternative protein sources for soybean meal, considered the most important vegetable protein source. In this context, the seed of *Lupinus* spp. represent a potential alternative for soybean meal [2,3].

L. albus from the low-alkaloid varieties is important for monogastric animals feeding, due to their high content in crude protein (CP, 35–43%) and crude fat (EE, 8–12%) [4,5].

The amino acid content of proteins is also appreciable, and the FA profile of fats is well represented in unsaturated fatty acids (UFA), at approximately 70–75% of FAME [6–8]. However, the high content in crude fiber (12–15%), oligosaccharides from the raffinose family and non-starch polysaccharides (arabinose, xylose, rhamnose, mannose, galactose) reduce the potential of white lupine to substitute for soybean meal in high proportions in the feed structure of monogastrics, especially for poultry [9,10]. Among NSP compounds, attention is directed toward soluble compounds, whose antinutritional effect in poultry consists in the binding of high amounts of water giving a gelatinous consistency to the digesta, which contributes to the increase of intestinal tract viscosity and to the reduction of nutrients' absorption [11,12]. It is well known that poultry do not possess specific endogenous enzymes for the efficient use of these compounds [13,14].

A common practice worldwide is the use of carbohydrase enzymes to improve feed energy [15]. Supplementing the poultry diets with multi-carbohydrase can improve nutrient digestibility and growth rates [16–18]. The main mechanism behind this consists of improving nutrient availability through the NSP degradation [19,20]. Moreover, it can degrade the cell walls, enhancing the access of endogenous enzymes [20–22].

Available research in the field has revealed that the use of exogenous multi-enzymes combinations containing cellulase, β-glucanase, xylanase, hemicellulase, pentosanase and pectinase in broiler feeds has allowed an increase of *L. albus* seeds' inclusion by up to 35% in the diet, without influencing productive performance, because the digestibility of proteins, fats and NSP from feed has improved, while the viscosity of the intestinal digesta has decreased [23–26]. Moreover, *L. luteus* seeds were efficiently used up to 40% in the diet of broilers from 1 to 25 days age old when a mix of enzymes (β-glucanase, hemicellulase, pectinase, endoglucanase, cellulase, β-xylanase and protease) was used, due to the feed conversion improvement as a result of a better NSP valorization [11,27].

To our knowledge, research about the addition of specific enzymes in the lupine-based diets for laying hens is limited and has only partially examined the effects on productive performance or egg quality. In this regard, the use of enzymes derived from a natural culture of *Aspergillus Niger* in the laying hens' diet which contains 150 g/kg of blue lupine has led to obtaining similar productive performances to the use of only soybean meal [28]. Research regarding the effects of exogenous enzyme use in poultry diets containing lupine seeds is relatively limited.

Therefore, considering this background, as well as a lack of information related to improving lupine use efficiency in the diets of poultry for egg production, we aimed to investigate the possibility of increasing the use of lupine in quail feeding, by the addition of specific exogenous enzymes in relation to productive performance, quality of the eggs and physiological status of the birds.

In this research, the following hypotheses were evaluated: (1) an increase proportion of soybean meal substitution with lupine seeds by supplementing the laying quail diets with exogenous enzyme, without affecting the productive performance and health status; (2) substantial changes in egg quality are expected (egg weight, fatty acid profile and health lipid indices of yolk, and yolk content in carotenoids) after the inclusion of lupine and exogenous enzyme in the diets.

2. Materials and Methods

2.1. Animal Ethics

The experimental procedures were reviewed and approved by the ethical committee of the University of Agricultural Science and Veterinary Medicine of Cluj-Napoca, number 291/22/11/2021.

2.2. Birds and Experimental Design

A total of 210 homogenous laying Japanese quails at 24 weeks (wk) of age were distributed in a completely randomized design of a 3 × 2 factorial arrangement. The dietary factors were: (1) three sources for ensuring the protein requirements (soybean meal

and two levels of lupine seed: 20% and 25% in feed); (2) the enzymes addition (commercial product Hostazym® X: 0 and 0.02%—in feed). Hostazym® X is a non-GMO enzyme complex consisting of endo-1.4-β-xylanase (EC 3.2.1.8), endo-1.4-β-glucanase (cellulase; EC 3.2.1.4), 1.3(4)-α-glucanase (EC 3.2.1.59), α-amylase (EC 3.2.1.1) and protease. The primary enzymatic activity is endo-1.4-β-xylanase (minimum enzyme activity: 6000 EPU/g produs) and the secondary is that of endo-1.4-β-glucanase (200 units/g produs), 1.3(4)-α-glucanase (60 units/g), α-amylase (120 units/g) and protease (traces). The additive Hostazym® X micro-granulate was introduced into quail feed in a quantity of 0.2 g/kg, ensuring an enzyme activity of 1200 EPU/kg feed for β-xylanase; 40 units/kg feed for β-glucanase; 12 units/kg feed for α-glucanase and 24 units/kg feed for α-amylase. The quails were randomly assigned to 6 dietary treatments (experimental groups), with each treatment consisting of 5 replicates with 7 birds/unit (35/group). The quails were kept in cages with an equal surface area of 337.5 cm^2/bird. The trial period lasted for 8 wk (age of the birds: 24–32 weeks) after an initial adaptation period of 2 wk. During the trial, all technological parameters specific to laying quails were ensured (temperature 22 °C, humidity 65%, ventilation 0.2 m/s and 18 h light/day).

2.3. Experimental Diets

All groups of birds were randomly assigned to the tested diets. The ingredients and nutrient levels of laying quail diets are listed in Table 1.

Table 1. Ingredients and nutritional characteristics of diets used in the laying quails' feeding experimental procedure (as-fed basis, %).

Specification	Treatments					
	S−	S+	L_{20-}	L_{20}+	L_{25-}	L_{25}+
Ingredients						
Maize	46.03	46.01	41.85	41.83	41.10	41.08
Triticale	10.00	10.00	10.00	10.00	10.00	10.00
Soybean meal	33.00	33.00	16.50	16.50	12.30	12.30
White lupine	**-**	**-**	**20.00**	**20.00**	**25.00**	**25.00**
Sunflower oil	3.20	3.20	3.85	3.85	3.80	3.80
DL-methionine	0.02	0.02	0.05	0.05	0.05	0.05
L-lysine HCL	-	-	-	-	-	-
Limestone	5.25	5.25	5.25	5.25	5.25	5.25
Vit.-min Premix [1]	2.50	2.50	2.50	2.50	2.50	2.50
Enzyme (Hostazyme X)	-	*0.02*	-	*0.02*	-	*0.02*
TOTAL	100.0	100.0	100.0	100.0	100.0	100.0
Nutritional characteristics (calculated values)						
ME (kcal/kg)	2901	2901	2906	2906	2904	2904
Crude protein (%)	20.02	20.02	20.04	20.04	20.03	20.03
Crude fat (%)	5.83	5.83	7.90	7.90	8.23	8.23
Crude fiber (%)	2.88	2.88	4.55	4.55	4.98	4.98
Lysine (%)	1.02	1.02	1.03	1.03	1.01	1.01
Methionine (%)	0.45	0.45	0.45	0.45	0.45	0.45
Methionine + cisteine (%)	0.80	0.80	0.81	0.81	0.81	0.81
Calcium (%)	2.50	2.50	2.50	2.50	2.50	2.50
Available phosphorus (%)	0.43	0.43	0.44	0.44	0.44	0.44
ME/CP ratio	144.90	144.90	145.00	145.00	144.98	144.98

[1] Provided per kg of diet: 12,000 IU of vitamin A, 420 mg of vitamin B_4, 5 mg of vitamin B_2, 1.5 mg of vitamin B_1, 11 mg of vitamin B_5, 5 mg of vitamin B_6, 1.5 mg of vitamin B_9, 0.02 mg of vitamin B_{12}, 2375 UI of vitamin D_3, 30 UI of vitamin E, 60 mg of vitamin PP, 2.5 mg of vitamin K_3, 0.1 mg of vitamin H, 54 mg of iron, 15 mg of coper, 93 mg of manganese, 71 mg of zinc, 1.2 mg of iodine, 0.15 mg of selenium, 3.6 g of calcium, 3 g of phosphorus, chlor 7.1%, sodium 5.5%, 1.37 g of DL-methionine, antioxidant (BHT), etoxiquin. ME = metabolizable energy; S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes; L_{25-}: experimental diet (250 g/kg lupine) without enzymes; L_{25}+: experimental diet (250 g/kg lupine) with enzymes.

All diets ensured the nutritional requirements of quails [29]. The main protein source (soybean meal) of standard diet (S) was partially substituted by including the lupine without enzymes (−): L_{20-} = group with lupine (200 g/kg of feed) and L_{25-} =group with lupine (250 g/kg). The treatments with enzymes were marked with + (C+, L_{20}+, L_{25}+). The quails feeding was ad libitum.

2.4. Ouails' Performance Evaluation

The birds were weighed individually at the start and end of the trial. The following parameters were determined on a weekly basis: feed intake, laying rate, egg weight and feed conversion rate. The birds' health and behavior were observed daily.

2.5. Egg Collection and Evaluation

The egg morphological structure (albumen, yolk and eggshell-% of the whole egg) from a number of 25 eggs/treatment (5 eggs/replicate) were assessed bi-weekly (at 2, 4, 6, and 8 wk).

The physical characteristics of the eggs were assessed based on: AI (albumen index = equatorial diameter ÷ height × 100), YI (yolk index = equatorial diameter ÷ height × 100), and HU (albumen Haugh Unit = 100 log (height − 1.7) × egg weight$^{0.37}$ + 7.57) [30].

The eggshell thickness was measured at three different points (sharp end, equator and air cell) without internal membrane and after they were dried in the oven [14].

The values of yolk color were performed by the "La Roché scale", with color samples corresponding to values from 1 to 15. Egg quality evaluations (chemical content of the albumen and yolk, egg yolk fatty acids, cholesterol, egg yolk carotenoids and health lipid indices) were performed only for the eggs provided from groups with best performance responses: the birds fed with standard diets (S− and S+) and of which the lupine seed were used at a level of 20% in feed (L_{20-} and L_{20}+).

2.6. Feed and Egg Chemical Analyses

2.6.1. Feed and Egg Composition

The white lupine seeds (cv. Amiga; low-alkaloid variety) were provided by a local farmer from Romania (Transylvanian area). From the collected seeds, the following analyses of samples were performed in the laboratory: raw chemical composition, amino acids (AA) and fatty acid (FA) content (n = 5).

The raw chemical composition was performed according to AOAC International [31] and the nitrogen free extract (N-FE) by difference (100% − CP% + CA% + EE% + CF%). Nitrogen-corrected metabolizable energy (AME_N) of lupine was calculated according to Sibbald [32]:

$$AME_N = 3951 + 54.4\ MG - 88.7\ CB - 40.8\ Ce, \quad (1)$$

where MG = crude fat, CB = crude fiber and Ce = crude ash.

The AA determination from lupine proteins (n = 5) was performed by HPLC (high performance liquid chromatography) according to the SR EN ISO 13903: 2005 standard and the method performed by Struți et al. [14]. All amino acids (AA) were determinated in the presence of ninhydrin and the AA identification was performed by comparing the retention times of each AA resulting from lupine samples with those of the standard (Santa Cruz Biotechnology standard).

Fatty acids from lupine fats were identified as FAME (methyl esters of fatty acids; g/100 g of total) using the technique of GC-MS (gas chromatography with mass spectrometry detection), according to the standards: SR EN ISO/TS 17764-2: 2008 and ISO 5508: 2002. The steps of the method followed are described by Struți at al. [14]. In the lupine fats, the major fatty acids identified were: palmitic (C16:0); stearic (C18:0); oleic (C18:1 n-9); eicosenoic acid (C20:1 n-9); linoleic acid (C18:2 n-6); α-linolenic acid (C18:3 n-3). The identification of FA peaks was realized by comparing the FAME relative retention time with that of the standard (Mix FAME Supelco 37).

The raw chemical content of albumen and yolk was analyzed according to the methods of AOAC International [31] (albumen: CP and CA and yolk: EE, CP and CA).

2.6.2. Egg Yolk Fatty Acid Analysis

The FA profile of egg yolk was performed from a number of 5 eggs randomly selected from each replicate and group: miristic (C14:0); miristoleic (C14:1); pentadecanoic (C15:0); pentadecenoic (C15:1); palmitic (C16:0); palmitoleic (C16:1); heptadecanoic (C17:0); heptadecenoic (C17:1); stearic (C18:0); oleic (C18:1 n-9); linoleic (C18:2 n-6); γ-linolenic (C18:3 n-6); α-linolenic (C18:3 n-3); eicosadienoic (C20:2 n-6); eicosatrienoic (C20:3 n-6); eicosapentaenoic (C20:3 n-3); erucic (C22:1 n-9); arachidonic (C20:4 n-6); nervonic (C24:1 n-9); docosatetraenoic (C22:4 n-6); docosapentaenoic (C22:5 n-3) and docosahexaenoic (C22:6 n-3). The fatty acids analysis was carried out in three steps: extraction with chloroform–methanol [33], methylation [34] and identification of fatty acids by gas chromatography analysis according to Struți et al. [14].

2.6.3. Egg Yolk Cholesterol Analysis

The cholesterol content from egg yolk was performed according to AOAC International [35] and Method no. 994.10 and 976.26. The principle consists of the saponification of lupine samples, followed by petroleum ether extraction. The concentration extract was resumed in chloroform and standard solution of cholesterol in chloroform 10 mg/mL was used [36,37]. The Perkin Elmer-Clarus 500 GC used was set up according to Struți et al. [14].

2.6.4. Egg Yolk Carotenoid Analysis

The carotenoids were extracted with a mixture of methanol/ethyl acetate/petroleum ether (1:1:1) according to the procedure described by Schlatterer et al. [38]. A total of 5 g of yolk/sample was used (5 samples/group). The LGC Standards (UK) were used for the identification of zeaxanthin, lutein and β-cryptoxanthin.

2.6.5. Nutritional Indices and FA Ratios

In order to highlight the influence of the dietary treatments on the nutritional qualities of yolk fats, it was considered optimal to calculate some health lipid indices (n = 5), as follows:

Ratio of n-6/n-3 FA.

Polyunsaturation index [39]:

$$PI = C18{:}\ 2\ n\text{-}6 + (C18{:}\ 3\ n\text{-}3 \times 2) \quad (2)$$

Atherogenic index [40]:

$$AI = (C12{:}0 + C16{:}0 + 4 \times C14{:}0) \div [\Sigma MUFA + \Sigma(n-6) + \Sigma(n-3)] \quad (3)$$

Thrombogenic index [40]:

$$TI = (C14{:}0 + C16{:}0 + C18{:}0) \div [0.5 \times \Sigma MUFA + 0.5 \times \Sigma(n-6) + 3 \times \Sigma(n-3) + \Sigma(n-3) \div \Sigma(n-6)] \quad (4)$$

Hypocholesterolemic/Hypercholesterolemic FA ratio [41]:

$$h/H = (C18{:}1 + PUFA) \div (C12{:}0 + C14{:}0 + C16{:}0) \quad (5)$$

Health promotion index [42]:

$$HPI = UFA \div [C12{:}0 + (4 \times C14{:}0) + C16{:}0)] \quad (6)$$

2.7. Measurements of the Excreta Dry Matter and Digesta Viscosity

The dry matter of excreta was evaluated every 7 days, from the fresh feces that resulted in 60 min (n = 5/group). The samples were cleaned of impurities and dried in an oven (105.5 °C).

The intestinal content viscosity was determined on the last day of the experimental period (n = 5/group).

The samples were collected from the digestive content of ileon and prepared according to Konieczka and Smulikowska [43]. The Brookfield viscometer (model LVDV E, Brookfield Engineering Laboratories, Middleboro, MA, USA) was used to determine the viscosity in cP (centipoise).

2.8. Blood Parameters

Samples (n = 5) of fresh blood were collected in vacutainers on lithium–heparin medium after 5 h of no feed. At the laboratory, the samples were centrifuged for 30 min at 3000 rotations/min to separate the blood plasma and were then stored at −20 °C. The following were determined: hemoglobin (Hb, g/dL); hematocrit (Hct, %); erythrocytes (Ery, mL/mm^3); total proteins (Prot, g/dL); total lipids (Lip, mg/dL); albumins (Alb, g/dL); γ-globulins (g/dL); aspartate aminotransferase (AST, U/L); alanine aminotransferase (ALAT, U/L); urea (mg/dL); creatinine (Creat., mg/dL); glutathione peroxidase (GPx, U/gHb); superoxide dismutase (SOD, U/gHb); cholesterol (Chol, mg/dL); triglycerides (Try, mg/dL) [44].

2.9. Statistical Analysis

All data were analyzed using the GLM procedure, software system ver. 10.0 (StatSoft Inc., Tulsa, OK, USA, 2011). The experiment was a completely randomized 3 × 2 factorial design, with a two-way ANOVA performed to assess the main effects of dietary lupine seed level (20 and 25%) compared with the standard diet, without and with enzymes supplementation (−/+), as well as the interaction with these factors (lupine level and enzymes). The two-way ANOVA was performed to assess the effects of dietary treatments on the excreta dry matter content and intestinal viscosity, blood biochemical parameters and egg quality (raw chemical composition, fatty acids, nutritional indices, carotenoids, cholesterol). The Tukey multiple-range test was used to compare the differences between the mean values of applied treatments. Differences were considered significant when $p < 0.05$. All data were presented as means with a pooled standard error of the mean estimates.

3. Results

3.1. Chemical Composition of the Lupine Seeds

In addition to the high content in crude protein (CP, 43.11% of DM) and fat (CF, 10.55% of DM), lupine seeds are also characterized by a high level of essential amino acids (e.g., lysine—4.98 g/16 g N; arginine—9.42 g/16 g N) but also in UFA, of which the oleic acid (C18:1 cis-9; OA), linoleic acid (C18:2 n-6; LA) and α-linolenic acid (C18:3 n-3; ALA) are well represented (Table 2).

Table 2. Chemical composition, major amino acids and fatty acids of white lupine from low-alkloid varieties (cv. Amiga).

Raw Chemical Composition (% of DM)		Amino Acids (g/16 g N)		Fatty Acids (% of FAME)	
Dry matter	92.41	Lysine	4.98	Palmitic acid (C16:0)	10.70
Crude protein	43.11	Methionine	0.49	Stearic acid (C18:0)	2.71
Crude fat	10.55	Tryptophan	0.46	Oleic acid (C18:1 n-9)	50.65
Crude fiber	14.18	Arginine	9.42	Eicosenoic acid (C20:1 n-9)	3.01
Crude ash	3.93	Glutamine	31.64	Linoleic acid (C18:2 n-6)	14.55
N-FE extract	28.23			α-linolenic acid (C18:3 n-3)	12.75
AME_N kcal/kg DM	3112.2			SFA	16.96
				MUFA	55.15
				PUFA	27.84
				n-6/n-3 FA	1.14

N-FE extract: nitrogen-free extract; AME_N: nitrogen-corrected metabolizable energy, calculated according to Sibbald [32]; SFA: saturated FA; UFA: unsaturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; n-6: omega-6 FA; n-3: omega-3 FA.

3.2. Quails' Performance

Feeding quails with diets containing 20% and 25% of white lupine with the enzymes did not influence the weight of the birds ($p > 0.05$) (Table 3).

Table 3. Influence of enzymes addition in lupine-based diets on the productive performance of quails (mean ± std. error).

Specification	Body Weight (g/quail)		Laying Rate (%)	Feed Intake (g/Quail/Day)	Feed Conversion Ratio	
	Initial	Final			g Feed/Egg	kg Feed/ kg Egg Mass
Without enzyme						
S−	299.95 ± 3.87	302.33 ± 4.47	88.67 ± 0.62 ^{b}	32.52 ± 0.27	36.53 ± 0.33 ^{a}	2.77 ± 0.03 ^{a}
L_{20}−	299.03 ± 3.75	299.49 ± 4.48	88.32 ± 0.60 ^{bc}	32.27 ± 0.31	36.82 ± 0.30 ^{a}	2.87 ± 0.03 ^{ab}
L_{25}−	300.14 ± 4.06	306.61 ± 4.32	87.42 ± 0.64 ^{d}	32.78 ± 0.24	37.75 ± 0.40 ^{b}	2.91 ± 0.03 ^{b}
With enzyme						
S+	299.85 ± 3.51	303.58 ± 3.90	89.90 ± 0.60 ^{a}	32.34 ± 0.38	36.34 ± 0.41 ^{a}	2.75 ± 0.04 ^{a}
L_{20}+	300.7 ± 3.47	306.52 ± 4.01	91.12 ± 0.63 ^{a}	31.96 ± 0.31	35.95 ± 0.33 ^{a}	2.77 ± 0.03 ^{a}
L_{25}+	300.15 ± 4.14	308.37 ± 4.88	89.19 ± 0.63 ^{b}	32.34 ± 0.33	36.62 ± 0.37 ^{ab}	2.79 ± 0.03 ^{ab}
p-value						
Lupine	0.997	0.490	0.001	0.100	0.014	0.001
Enzymes	0.865	0.347	0.001	0.127	0.075	0.063
Lupine x enzymes	0.967	0.763	0.285	0.236	0.908	0.520

$^{a–b}$ Within a column, values with no common superscripts differ significantly ($p < 0.05$). S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes; L_{25-}: experimental diet (250 g/kg lupine) without enzymes; L_{25}+: experimental diet (250 g/kg lupine) with enzymes.

The laying rate (%) was influenced by the use of lupine in the diets, with the lowest laying intensity recorded when the lupine was added in an amount of 250 g/kg in the feed (L_{25-}) without enzymes ($p < 0.001$) (Table 3). The use of exogenous enzymes in lupine-based diets led to an increase in egg production ($p < 0.001$). The use of lupine in the amount of 200 g/kg in the diet with enzymes (L_{20}+) resulted in the highest egg production.

The feed intake (g feed/bird/day) was not influenced ($p > 0.05$) by the applied treatments (Table 3). The quails fed with lupine in the amount of 250 g/kg in their diets (L_{25-} and L_{25}+) had similar feed intakes to quails with standard diets (S− and S+) (32.78 and 32.34 vs. 32.52 and 32.34 g of feed/quail/day) (Table 3).

The feed conversion ratio (FCR) was influenced by the applied treatments (Table 3). The use of lupine in the amount of 250 g/kg in the diet without enzymes led to the FCR depreciation ($p < 0.05$). Enzyme inclusion in the diet did not affect the FCR (Table 3).

In the trial period, no specific symptoms of a pathological condition or a nutritional deficiency were observed.

3.3. Excreta Dry Matter Content and Intestinal Viscosity

The feces moisture of the quails fed with lupine based-diets was higher, while the enzymes addition had a positive effect, as it ensured a level of moisture close to that obtained by quails fed with standard diets (Table 4). Large amounts of lupine in the feed, without enzymes (L_{25-}), led to the highest level of excreta moisture.

The use of lupine without enzymes (L_{20-}; L_{25-}) in the diets of laying quails increased the viscosity of the intestinal digesta ($p < 0.001$), compared to the standard diet (S−) and also with the diets with enzymes. When the enzymes were used (L_{20}+ and L_{25}+), a decrease ($p < 0.001$) in the intestinal digesta viscosity of the quails was achieved (Table 4).

Table 4. Influence of enzymes addition in lupine-based diets on the excreta moisture and intestinal viscosity of the quails (mean ± std. error).

Specification	Excreta Moisture (%)	Intestinal Viscosity (cP)
Without enzyme		
S-	78.06 ± 0.27 ^{b}	4.22 ± 0.16 ^{c}
L_{20}-	78.13 ± 0.24 ^{ab}	5.96 ± 0.36 ^{b}
L_{25}-	78.77 ± 0.27 ^{a}	7.02 ± 0.13 ^{a}
With enzyme		
S+	77.47 ± 0.22 ^{b}	3.45 ± 0.37 ^{c}
L_{20}+	77.33 ± 0.34 ^{b}	3.35 ± 0.38 ^{c}
L_{25}+	77.75 ± 0.30 ^{b}	4.83 ± 0.14 ^{c}
p-value		
Lupine	0.033	0.001
Enzymes	0.005	0.001
Lupine x enzymes	0.940	0.008

$^{a–b}$ Within a column, values with no common superscripts differ significantly ($p < 0.05$). L = lupine effect; E = enzyme effect. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes; L_{25-}: experimental diet (250 g/kg lupine) without enzymes; L_{25}+: experimental diet (250 g/kg lupine) with enzymes.

3.4. Blood Hematological and Biochemical Parameters

The use of lupine and the enzymes in the laying quail feeding did not affect ($p > 0.05$) the hematological parameters: Hct (hematocrit %); Hb (hemoglobin g/dL); Ery (erythrocytes mL/mm^3); Alb (albumins g/dL); γ-globulins (g/dL) (Table 5).

Table 5. Influence of enzymes addition in lupine-based diets on the blood biochemical parameters of quails.

Indicators	*Without Enzyme*			*With Enzyme*			*p-Value*		
	S−	**L_{20-}**	**L_{25-}**	**S+**	**L_{20}+**	**L_{25}+**	***L***	***E***	***L* × *E***
Hematocrit (%)	39.25	35.80	39.75	39.75	39.00	40.25	0.091	0.165	0.440
Hemoglobin (g/dL)	11.51	12.01	10.99	11.29	11.78	11.05	0.052	0.637	0.889
Ery (mL/mm^3)	3.41	3.22	2.96	3.23	2.52	3.07	0.124	0.153	0.187
SOD (U/gHb)	614.50 ^{b}	669.08 ^{a}	683.25 ^{a}	608.50 ^{b}	649.25 ^{ab}	676.25 ^{ab}	0.026	0.064	0.320
GPx (U/gHb)	91.65 ^{b}	99.78 ^{a}	102.18 ^{a}	90.10 ^{b}	97.40 ^{a}	100.85 ^{a}	0.021	0.545	0.125
Proteins (g/dL)	4.67	3.67	3.95	4.91	4.18	4.48	0.057	0.133	0.887
Albumin (g/dL)	1.64	1.48	1.59	1.71	1.53	1.67	0.263	0.191	0.483
γ-Globulin (g/dL)	0.56	0.49	0.49	0.42	0.53	0.66	0.709	0.802	0.346
Urea (mg/dL)	35.53 ^{b}	38.00 ^{ab}	42.28 ^{a}	23.80 ^{c}	19.80 ^{c}	23.53 ^{c}	0.038	0.001	0.738
Creatinine (mg/dL)	0.58 ^{a}	0.58 ^{a}	0.63 ^{a}	0.32 ^{c}	0.49 ^{b}	0.47 ^{b}	0.084	0.001	0.025
Total lipids (mg/dL)	758.60 ^{a}	723.75 ^{ab}	647.83 ^{c}	747.00 ^{a}	686.98 ^{cb}	518.95 ^{cd}	0.011	0.018	0.517
Cholesterol (mg/dL)	152.41 ^{a}	145.53 ^{ab}	136.55 ^{bc}	153.55 ^{a}	143.00 ^{ab}	122.78 ^{c}	0.028	0.041	0.866
Triglyceride (mg/dL)	502.10 ^{a}	457.55 ^{ab}	371.00 ^{c}	477.33 ^{a}	430.18 ^{b}	279.83 ^{d}	0.004	0.032	0.413
ALT (U/L)	14.83 ^{d}	22.48 ^{c}	34.95 ^{a}	10.79 ^{d}	19.13 ^{c}	29.80 ^{b}	0.008	0.038	0.988
AST (U/L)	99.60 ^{a}	91.30 ^{ab}	91.05 ^{ab}	96.25 ^{a}	84.80 ^{c}	70.20 ^{d}	0.212	0.020	0.625

$^{a–d}$ Within a row, values with no common superscripts differ significantly ($p < 0.05$). L = lupine effect; E = enzyme effect. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes; L_{25-}: experimental diet (250 g/kg lupine) without enzymes; L_{25}+: experimental diet (250 g/kg lupine) with enzymes.

The highest value of plasmatic urea and creatinine was recorded when the lupine was used at 250 g/kg in feed, but the use of enzymes led to lower values ($p < 0.05$) at a level similar to that of quails fed with standard diets (Table 5).

The indice values of the lipid profile have a decreasing trend as a result of the enzymes addition, as well as with the lupine dose increase in the feed (Table 5). Compared to the groups without lupine, the birds from L_{25}+ recorded the lowest values ($p < 0.05$) of plasmatic Lip, Try and Chol.

Moreover, the presence of lupine in the diets led to an increase ($p < 0.05$) in the SOD and GPx values (Table 5).

3.5. Physico-Chemical Traits of the Eggs

The lupine-based diets without enzymes (L_{20-} and L_{25-}) led to obtaining eggs with a lower weight ($p < 0.05$) (Table 6). An egg weight improvement was achieved when the lupine-based diets were supplemented with specific enzymes (L_{20}+ and L_{25}+), with the values similar to the weight of eggs obtained from the birds fed with standard diets (S− and S+) (Table 6).

Table 6. Influence of enzymes addition in lupine-based diets on the physical quality indices of fresh eggs (mean ± std. error).

Specification	Egg Weight (g/egg)	Morphological Components (%)			Physical Quality Indices of Eggs (%)			Shell Thickness (mm)
		Albumen	Yolk	Shell	Albumen Index	Yolk Index	Haugh Unit	
Without enzyme								
S−	13.20 ± 0.04 [a]	56.90 ± 0.12 [a]	30.69 ± 0.09 [a]	12.42 ± 0.09 [a]	17.40 ± 0.34	50.27 ± 0.76	95.26 ± 0.42	0.229 ± 0.01 [a]
L_{20-}	12.91 ± 0.04 [b]	58.17 ± 0.14 [b]	30.07 ± 0.13 [b]	11.76 ± 0.06 [b]	17.86 ± 0.35	51.00 ± 0.71	96.72 ± 0.38	0.213 ± 0.01 [b]
L_{25-}	12.96 ± 0.05 [b]	58.42 ± 0.13 [b]	29.88 ± 0.12 [b]	11.72 ± 0.07 [b]	17.61 ± 0.42	50.97 ± 0.64	96.20 ± 0.49	0.196 ± 0.01 [c]
With enzyme								
S+	13.11 ± 0.04 [a]	57.50 ± 0.17 [a]	30.58 ± 0.12 [a]	12.18 ± 0.06 [a]	17.57 ± 0.19	50.10 ± 0.98	95.92 ± 0.24	0.227 ± 0.01 [a]
L_{20}+	13.08 ± 0.03 [a]	58.09 ± 0.12 [a]	30.23 ± 0.10 [a]	11.68 ± 0.06 [b]	17.76 ± 0.24	51.78 ± 0.52	96.55 ± 0.35	0.219 ± 0.01 [a]
L_{25}+	13.06 ± 0.04 [a]	58.04 ± 0.13 [a]	30.23 ± 0.11 [a]	11.72 ± 0.06 [b]	17.83 ± 0.21	49.92 ± 0.44	96.81 ± 0.16	0.209 ± 0.01 [b]
p-value								
Lupine	0.001	0.001	0.001	0.001	0.795	0.097	0.934	0.001
Enzymes	0.044	0.163	0.045	0.055	0.809	0.409	0.213	0.033
Lupine x enzymes	0.001	0.001	0.129	0.234	0.584	0.131	0.434	0.009

[a–c] Within a column, values with no common superscripts differ significantly ($p < 0.05$). S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes; L_{25-}: experimental diet (250 g/kg lupine) without enzymes; L_{25}+: experimental diet (250 g/kg lupine) with enzymes.

The proportion of the egg's morphological components, namely the albumen (%), yolk (%) and shell (%), were influenced by the applied treatments (Table 6). The use of lupine in the quail diets (200 and 250 g/kg) led to an increase ($p < 0.05$) in the albumen weight.

The proportion of yolk and eggshell decreased ($p < 0.001$) when the lupine was included in the diets (without enzymes) (Table 6). The presence of enzymes in diets led to an increase in yolk weight, but the weight of the eggshell did not change (Table 6). The decrease in eggshell weight at the same time as the lupine inclusion in diets is associated with the reduction in eggshell thickness ($p < 0.05$) (Table 6).

The use of lupine in the diets (−/+) did not influence ($p > 0.05$) the physical quality indices of the eggs, namely, AI, YI and HU (Table 6).

The partial substitution of soybean meal with lupine (−/+) did not affect ($p > 0.05$) the CP and CA in albumen, nor the CF, CP and CA of yolk (Table 7).

Table 7. Influence of enzymes addition in lupine-based diets on the chemical composition of quail eggs (% of DM).

Egg Component	Chemical Content	Without Enzyme		With Enzyme		*p*-Value		
		S−	L_{20-}	S+	L_{20}+	*L*	*E*	*L* × *E*
Albumen	DM %	12.37 ± 0.39	12.09 ± 0.12	11.61 ± 0.15	11.83 ± 0.17	0.903	0.183	0.299
	OM %	8.60 ± 0.36	8.07 ± 0.25	7.76 ± 0.89	7.92 ± 0.67	0.539	0.104	0.783
	CP %	85.03 ± 0.41	85.87 ± 0.47	84.26 ± 0.55	84.42 ± 0.49	0.312	0.272	0.491
	CA %	3.77 ± 0.12	4.02 ± 0.41	3.85 ± 0.23	3.91 ± 0.11	0.294	0.114	0.598
Yolk	DM %	53.41 ± 0.12	53.22 ± 0.21	52.68 ± 0.11	52.67 ± 0.09	0.259	0.478	0.528
	OM %	49.82 ± 0.89	49.51 ± 0.93	49.23 ± 0.59	49.04 ± 0.78	0.627	0.294	0.673
	CP %	31.73 ± 0.22	31.21 ± 0.65	32.66 ± 0.45	31.90 ± 0.17	0.146	0.070	0.785
	EE %	56.51 ± 0.41	57.43 ± 0.88	56.39 ± 0.94	55.56 ± 1.67	0.970	0.366	0.425
	CA %	3.59 ± 0.15	3.71 ± 0.19	3.45 ± 0.14	3.63 ± 0.22	0.252	0.135	0.832

L = lupine effect; E = enzyme effect. DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; CA: crude ash. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes.

3.6. Egg Yolk Fatty Acids and Cholesterol Content

The fats from the yolk are rich in several fatty acids such as oleic acid (31.67–34.00% of FAME), palmitic acid (19.04–23.21% of FAME), linoleic acid (16.92–18.52% of FAME) and stearic acid 13.85–14.38% of FAME) (Table 8).

Table 8. Influence of enzymes addition in lupine-based diets on FAs' profile of fats in the yolk of quail eggs (% of FAME).

Indicators		*Without Enzyme*		*With Enzymes*		*p-Value*		
		S−	L_{20-}	S+	L_{20}+	*L*	*E*	*L* × *E*
Miristic Acid	C14:0	0.32 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.30 ± 0.01	0.728	0.169	0.089
Miristoleic Acid	C14:1	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.271	0.512	0.127
Pentadecanoic Acid	C15:0	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.078	0.095	0.885
Pentadecenoic Acid	C15:1	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	0.155	0.806	0.079
Palmitic Acid	C16:0	23.21 ± 0.15 ^{a}	19.04 ± 1.65 ^{b}	23.17 ± 0.16 ^{a}	22.35 ± 0.24 ^{ab}	0.009	0.020	0.064
Palmitoleic Acid	C16:1	2.88 ± 0.12	2.79 ± 0.10	2.95 ± 0.16	2.85 ± 0.12	0.488	0.633	0.959
Heptadecanoic Acid	C17:0	0.10 ± 0.01 ^{a}	0.14 ± 0.01 ^{b}	0.10 ± 0.01 ^{a}	0.10 ± 0.01 ^{a}	0.016	0.034	0.009
Heptadecenoic Acid	C17:1	0.18 ± 0.02 ^{ab}	0.19 ± 0.01 ^{a}	0.16 ± 0.01 ^{ab}	0.11 ± 0.02 ^{b}	0.249	0.012	0.055
Stearic Acid	C18:0	13.98 ± 0.30	14.38 ± 0.50	13.85 ± 0.19	13.87 ± 0.18	0.527	0.336	0.563
Oleic Acid	C18:1 n-9	31.67 ± 0.33 ^{b}	34.00 ± 0.31 ^{a}	32.09 ± 0.22 ^{bc}	32.99 ± 0.39 ^{ac}	0.001	0.361	0.037
Linoleic Acid (LA)	C18:2 n-6	16.92 ± 0.43 ^{b}	18.52 ± 0.80 ^{a}	17.05 ± 0.22 ^{b}	17.85 ± 0.29 ^{ab}	0.026	0.585	0.424
γ-Linolenic Acid	C18:3 n-6	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.452	0.495	0.327
α-Linolenic Acid	C18:3 n-3	0.13 ± 0.01 ^{b}	0.30 ± 0.03 ^{a}	0.11 ± 0.0 ^{b}	0.29 ± 0.01 ^{a}	0.001	0.255	0.713
Eicosadienoic Acid	C20:2 n-6	0.12 ± 0.01 ^{b}	0.20 ± 0.01 ^{a}	0.20 ± 0.01 ^{a}	0.22 ± 0.02 ^{a}	0.002	0.004	0.069
Eicosatrienoic Acid	C20:3 n-6	0.20 ± 0.01 ^{a}	0.18 ± 0.01 ^{b}	0.19 ± 0.02 ^{a}	0.16 ± 0.01 ^{b}	0.041	0.235	0.653
Eicosapentaenoic Acid	C20:3 n-3	0.21 ± 0.01 ^{a}	0.20 ± 0.01 ^{ab}	0.21 ± 0.01 ^{a}	0.18 ± 0.01 ^{b}	0.010	0.205	0.132
Erucic Acid	C22:1 n-9	0.04 ± 0.01 ^{b}	0.05 ± 0.01 ^{a}	0.02 ± 0.01 ^{c}	0.04 ± 0.01 ^{b}	0.022	0.016	0.021
Arachidonic Acid	C20:4 n-6	6.03 ± 0.10 ^{a}	5.52 ± 0.20 ^{b}	5.77 ± 0.07 ^{b}	4.92 ± 0.13 ^{b}	0.001	0.007	0.232
Nervonic Acid	C24:1 n-9	0.34 ± 0.02 ^{a}	0.27 ± 0.02 ^{bc}	0.32 ± 0.01 ^{ab}	0.25 ± 0.01 ^{c}	0.001	0.352	0.962
Docosatetraenoic Acid	C22:4 n-6	1.49 ± 0.12 ^{a}	0.98 ± 0.06 ^{b}	1.38 ± 0.02 ^{a}	0.89 ± 0.08 ^{b}	0.001	0.189	0.900
Docosapentaenoic Acid	C22:5 n-3	0.14 ± 0.01 ^{b}	0.26 ± 0.02 ^{a}	0.14 ± 0.01 ^{b}	0.26 ± 0.01 ^{a}	0.001	0.981	0.753
Docosahexaenoic Acid	C22:6 n-3	0.86 ± 0.02 ^{b}	1.55 ± 0.08 ^{a}	0.77 ± 0.01 ^{b}	1.36 ± 0.03 ^{c}	0.001	0.006	0.322
Other FA		0.66 ± 0.12	0.67 ± 0.04	0.74 ± 0.04	0.55 ± 0.09	0.263	0.706	0.249
Σ SFA		37.96 ± 0.24 ^{a}	34.21 ± 1.14 ^{b}	37.76 ± 0.04 ^{a}	36.90 ± 1.16 ^{ab}	0.001	0.051	0.026
Σ UFA		61.37 ± 0.30 ^{b}	65.11 ± 1.11 ^{a}	61.51 ± 0.06 ^{b}	62.56 ± 0.19 ^{ab}	0.002	0.507	0.087
Σ MUFA		34.97 ± 0.38 ^{b}	37.14 ± 0.35 ^{a}	35.41 ± 0.35 ^{b}	36.17 ± 0.46 ^{a}	0.008	0.195	0.376
Σ PUFA		26.40 ± 0.48 ^{b}	27.98 ± 1.17 ^{a}	26.09 ± 0.29 ^{b}	26.39 ± 0.51 ^{b}	0.001	0.045	0.035

$^{a–c}$ Within a row, values with no common superscripts differ significantly ($p < 0.05$). L = lupine effect; E = enzyme effect. SFA: saturated FA; UFA: unsaturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes.

The use of lupine white in the laying quail diets led to an improvement in the fatty acid profile of yolk fats, as a result of the decrease in the SFA proportion (mainly palmitic acid; $p < 0.01$) and an increase in the PUFA proportion ($p < 0.001$) (mainly α-linolenic acid—ALA, eicosapentaenoic acid—EPA and docosahexaenoic acid—DHA) (Table 8).The use of enzymes in quail diets did not influence the SFA and cholesterol level in the yolk fats ($p > 0.05$) (Figure 1). The addition of enzymes in the lupin-based diets of quails led to a decrease in the PUFA level (27.98 vs. 26.39% of FAME) ($p < 0.05$) of yolk (Table 8).

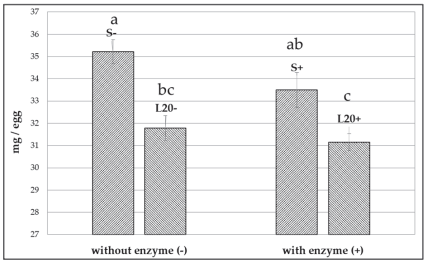

Figure 1. Influence of enzymes addition in lupine-based diets on the cholesterol content of quail eggs. a–c: bars with no common letters differ significantly ($p < 0.05$). S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes.

3.7. Egg Yolk Carotenoids Content

The carotenoids content of egg yolk increased ($p < 0.001$) after lupine and also enzymes were used in the laying quail diets (Table 9).

Table 9. Influence of enzymes addition in lupine-based diets on the carotenoids content (μg/g yolk) and the color of the yolk.

Indicators	Without Enzyme		With Enzymes		*p*-Value		
	S−	L_{20-}	S+	L_{20}+	*L*	*E*	*L* × *E*
Lutein	3.32 ± 0.29 [b]	7.08 ± 0.89 [a]	4.41 ± 0.34 [b]	6.58 ± 0.2 [a]	0.001	0.001	0.003
Zeaxanthin	4.54 ± 0.10 [b]	9.47 ± 0.13 [a]	6.20 ± 0.12 [b]	11.95 ± 0.27 [a]	0.001	0.001	0.254
Canthaxanthin	3.71 ± 0.16 [b]	5.11 ± 0.89 [a]	2.97 ± 0.10 [b]	4.31 ± 0.56 [a]	0.001	0.004	0.242
ß-cryptoxanine	0.58 ± 0.03 [b]	0.84 ± 0.08 [a]	0.50 ± 0.05 [b]	0.77 ± 0.61 [a]	0.038	0.004	0.001
Total Carotenoids	12.15 ± 0.23 [b]	22.51 ± 0.44 [a]	14.07 ± 0.18 [b]	23.61 ± 0.31 [a]	0.001	0.001	0.001
La Roché scale points	9.12 ± 0.73 [b]	12.78 ± 0.54 [a]	9.53 ± 0.82 [b]	13.23 ± 0.68 [a]	0.001	0.073	0.089

[a–b] Within a row, values with no common superscripts differ significantly ($p < 0.05$). L = lupine effect; E = enzyme effect. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes.

Lupine use in the diets of laying quails led to an increase ($p < 0.05$) in the yolk color intensity, but the enzymes addition did not influence this parameter ($p > 0.05$).

3.8. Nutritional Indices for Assessing the Fatty Acids of Egg Yolks

The use of lupine in quail diets led to an improvement in the health lipid indices, except for n-6 FA and PUFA/SFA (Table 10). The enzymes addition in the quail diets had a negative effect on the value of the health lipid indices of yolk fats (Table 10).

Table 10. Influence of enzymes addition in lupine-based diets on the lipid quality indices of the fats from egg yolk.

Indicators	Without Enzyme		With Enzymes		*p*-Value		
	S−	L_{20-}	S+	L_{20}+	*L*	*E*	*L x E*
n-3 FA	1.35 ± 0.03 [b]	2.30 ± 0.12 [a]	1.23 ± 0.01 [b]	2.08 ± 0.04 [a]	0.001	0.069	0.001
n-6 FA	25.05 ± 0.48	25.68 ± 1.06	24.86 ± 0.28	24.31 ± 0.48	0.957	0.244	0.374
HFA	23.53 ± 0.14 [a]	19.32 ± 1.65 [b]	23.44 ± 0.17 [a]	22.65 ± 0.24 [a]	0.009	0.017	0.059
hFA	58.07 ± 0.24 [b]	61.98 ± 1.18 [a]	58.19 ± 0.11 [b]	59.38 ± 0.29 [b]	0.001	0.033	0.045
PUFA/SFA	0.70 ± 0.02 [ab]	0.84 ± 0.06 [a]	0.69 ± 0.01 [b]	0.72 ± 0.01 [ab]	0.030	0.087	0.115
MUFA/SFA	0.92 ± 0.01 [b]	1.10 ± 0.04 [a]	0.94 ± 0.01 [b]	0.98 ± 0.02 [b]	0.001	0.042	0.009
UFA/SFA	1.62 ± 0.02 [b]	1.93 ± 0.10 [a]	1.63 ± 0.01 [b]	1.70 ± 0.01 [b]	0.002	0.049	0.032
n-6/n-3 FA	18.65 ± 0.58 [b]	11.22 ± 0.16 [c]	20.23 ± 0.04 [a]	11.67 ± 0.04 [c]	0.001	0.004	0.079
h/H	2.47 ± 0.02 [b]	3.49 ± 0.42 [a]	2.48 ± 0.02 [b]	2.62 ± 0.04 [b]	0.014	0.016	0.054
PI	17.18 ± 0.45 [b]	19.12 ± 0.85 [a]	17.27 ± 0.23 [b]	18.42 ± 0.29 [a]	0.008	0.559	0.457
AI	0.61 ± 0.01 [a]	0.52 ± 0.03 [b]	0.60 ± 0.01 [a]	0.58 ± 0.01 [a]	0.050	0.001	0.027
TI	1.10 ± 0.01 [a]	0.89 ± 0.05 [b]	1.10 ± 0.01 [a]	1.00 ± 0.01 [c]	0.037	0.001	0.040
HPI	2.51 ± 0.01 [b]	3.49 ± 0.04 [a]	2.54 ± 0.02 [b]	2.66 ± 0.04 [b]	0.042	0.014	0.047

[a–c] Within a row, values with no common superscripts differ significantly ($p < 0.05$). SFA: saturated FA; UFA: unsaturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; h/H: hypocholesterolemic/Hypercholesterolemic FA; n-3: omega 3 FA; n-6: omega 6 FA; PI: polyunsaturated index; TI: thrombogenic index; AI: atherogenic index; HPI: health promotion index. S−: standard diet without enzymes; S+: standard diet with enzymes; L_{20-}: experimental diet (200 g/kg lupine) without enzymes; L_{20}+: experimental diet (200 g/kg lupine) with enzymes.

The use of lupine in the quail diets led to obtaining eggs with health lipid indices favorable to the consumer's health (Table 10). As a result, compared to the eggs provided from quails fed with standard diets, the proportion of FA with a hypercholesterolemic (HPA) effect decreased and the content of FA with a hypocholesterolemic (hFA) effect increased ($p < 0.05$) (Table 10).

Moreover, the n-6/n-3 ratio decreased ($p < 0.001$), but the n-3 FA content and h/H FA ratio increased ($p < 0.05$) (Table 10). In addition, the presence of quality fats in lupine-based diets caused a decrease of the atherogenic (AI) and thrombogenic index (TI) values, as well as an increase of the health promotion index (HPI) ($p < 0.05$) values of the fats (Table 10).

The addition of enzymes in the diets containing lupine had a negative effect on the quality of the fats in the egg yolk, producing an increase in the HFA proportion and a decrease in the hFA content, which had a negative effect on the value of the health lipid indices (n-6/n-3 FA, h/H, AI, TI and HPI) (Table 10).

4. Discussion

The purpose of this research was to increase the efficiency of lupine use in quail nutrition by adding enzymes to improve the digestion and nutrients utilization, especially non-starch polysaccharides. As a result, the productive performances of the quails fed with lupine-based diets and enzymes were similar to those fed with standard diets. The egg quality was improved by introducing lupine into the diets, due to the increase in the n-3 FA proportion in yolk fats.

4.1. Productive Performance

The use of enzymes in the lupine-based diets allowed a higher nutrients' utilization in quails due to the minimization of the antinutritional effect of NSP from lupine; therefore, the body weight was not affected. Similarly, to our findings, Lee et al. [28] showed that the weight of laying hens was not influenced by the use of enzymes (Allzyme–*Aspergillus niger*) in a diet which contained 150 g/ kg blue lupine.

The enzymes addition in lupine-based diets led to an egg production comparable to the group without lupine, where the main protein source was soybean meal. These findings can be justified by the efficient activity of the enzymes which diminished the negative effects of NSP in the gastrointestinal tract of birds and improved the digestion processes and nutrients absorption from feed [27]. Similar findings were previously reported by Lee et al. [28], with laying hens fed a lower content of blue lupine (150 g/kg) in their diet.

In our research, a trend of improving the daily feed intake of quails was observed when the enzymes were included in the lupine-based diets. Similar findings have been reported by Brenes et al. [45], who revealed that the inclusion of enzymes (β-glucanases, hemicellulases, pectinases, endoglucanases, proteases and α-galactosidases) in the lupine-based diets (350 g/kg white lupine in feed) of broilers improved their daily feed intake (by 3.8%), being close to that of a standard diet. Quails fed with lupine based-diets with enzymes have a similar FCR (kg feed/kg egg mass) to those of standard diets ($p > 0.05$). Based on these results, the addition of enzymes improved the feed efficiency for quails and contributed to the successful partial substitution of soybean meal with lupine in diets. Similar findings with broilers highlight an improvement in feed utilization by 9% as a result of enzymes addition in the lupine-based diets, due to the increase of digestion and nutrient absorption [11,23,24].

The soluble NSP from lupine seeds bind large amounts of water and increases the viscosity of the intestinal digesta, thus reducing the nutrient digestion and absorption [13,27]. In our research, the antinutritional effects of NSP were reduced when the exogenous enzymes were included in the diets of quails, causing an increase in the feed energy availability and increasing the efficiency of nitrogen assimilation, which led to the improvement of productive performances [13,18,20].

4.2. Excreta Moisture and Intestinal Viscosity

The specific enzymatic activity of endo-1.4 β-xylanases, endo-1.3(4) β-glucanases and galactosidases contributed to the degradation of soluble NSP from lupine, which has the ability to retain high amounts of water [9,46]. This led to a decrease in the excreta moisture of quails from enzyme-supplemented diets ($p < 0.01$).

The role of the soluble NSP to increase the intestinal transit time at the area of the small intestine is well known, due to the modification of useful microbiota and the resultant viscous consistency [43,47]. The addition of enzymes into lupine-based diets reduced the viscosity of the quails' intestinal chyle, contributing to the increase in digestibility and absorption of nutrients [48,49]. The decrease of intestinal viscosity of broiler chickens was reported by Kocher et al. [23], when the exogenous enzymes (endo-1.4-β-xylanase, endo-1.3-β-glucanase, pectinases, cellulases, hemicellulases) were included in the lupine-based diets (350 g/kg).

4.3. Biochemical Blood Parameters

The enzymes addition into quail diets improved the NSP digestion which led to an increase in the available energy in the organism, ensuring a better use of proteins from feed in the synthesis processes. Therefore, there was a resultant decrease in plasmatic urea and creatinine levels, which are the final compounds of proteins catabolism (Table 5). The use of multi-enzymatic complex for NSP degradation in broilers was previously shown to decrease uric acid, which suggests an improvement in nutrients' use from feed [50].

The use of white lupine in quail diets and the addition of enzymes had a beneficial effect on lipid metabolism, which consisted of a decrease in the total level of Lip, Chol and plasma Try ($p < 0.05$). This is due to a higher PUFA content from lupine and to the improvement in nutrients' digestibility realized by enzymes. In this way, Konca et al. [51] concluded that raw materials rich in PUFA can reduce blood cholesterol levels of quails. Similar to our results, Straková et al. [52] showed that plasma triglycerides and cholesterol levels in laying hens decreased significantly when 50% of soybean meal from the diet was substituted with white lupine.

The lower values of ALT and AST in the blood of quails fed with diets supplemented with enzymes may suppose a higher level of protein use from the feed. This may be due to the protease content of the enzymes complex; therefore, a lower quantity of AA was deaminated [53].

The use of lupine up to 250 g/kg in the quail's diet (without enzymes) led to increased values of SOD and GPx, being consistent with our previous findings in quails [44]. These two enzymatic biomarkers of antioxidant activity in the organism increase when oxidative stress is induced by the increase of UFA in the blood [53,54].

It can be considered that enzymes addition improves the use efficiency of lupine and ensures a successfully inclusion at a level of 25% in the laying quails' feeding without affecting their physiological and health status.

4.4. Egg Quality

The use of white lupine without enzymes in the diet of quails led to obtaining eggs with a lower weight compared to the standard diet. Previous studies have reported a reduction in egg weight when *L. albus* seeds in amounts of 180–300 g/kg were included in the laying hens' feed [55], or *L. luteus* seeds in amounts of 250 g/kg [56]. Previously, Hammershøj and Steenfeldt [57] reported a significant decrease in egg weight when lupine was used in feed at a level of 250 g/kg. However, some of the research revealed that supplementation of up to 22% lupine did not exert a deleterious effect on egg weight [58,59].

The addition of exogenous enzymes contributed to an increase in egg weight ($p < 0.05$) (case of L_{20}+), respectively, to obtaining eggs with a similar weight ($p > 0.05$) to those of quails fed with soybean meal. These findings are in agreement with research conducted by Lee et al. [28], which used an enzyme complex in the diet of hens with blue lupine (150 g/kg in feed) and obtained eggs with a similar weight ($p > 0.05$) to those of hens fed a standard diet.

Similar to our results, a reduction in hens' eggshell weight ($p < 0.05$) was also found by Lee et al. [28], even though the lupine-based diet was supplemented with enzymes. However, Nguyen et al. [13] revealed that xylanase inclusion in the wheat-based diets

(higher in soluble NSP) improved the shell thickness of eggs, due to increased digestion and absorption of feed minerals, but this effect was not confirmed in our research.

The yolk index is an indicator of the spherical nature of the egg yolk and can be used to reflect freshness [60]. A yolk index of 48.9–51.7% is considered optimal for a fresh egg and the decrease occurs over time as a result of the perivitelline membrane alteration, following the hydrolytic processes [61]. In the present research, there were no differences regarding the yolk index, which means that lupine do not influence this physical parameter of egg quality.

The Haugh Unit (HU) is frequently used as a standard indicator for assessing the quality of albumen proteins. The HU values are influenced by the albumen content in ovomucin, which is a glycoprotein that gives the albumen thick its viscous property [61,62]. The ovomucin is composed of α-ovomucin and β-ovomucin, and the α part is composed of acidic amino acids such as glutamic acid and aspartic acid, while the β part is predominantly composed of hydroxyl amino acids such as threonine and serine [63,64]. Therefore, the content of the diets in these amino acids influences the ovomucin composition, and the lupine seeds are rich in glutamine and aspartic acid, which can explain the slightly higher values of HU values obtained for the eggs provided from quails fed with lupine-based diets. A high value (HU: 85–98%) corresponds to a very good quality of egg albumen [65].

The applied treatments in quail's feed did not influence the chemical composition of the albumen and yolk ($p > 0.05$). Similar findings have been reported by Lee et al. [28] in a study conducted on laying hens.

The differences between the FAs' content of the fats from the egg yolk of quails fed with lupine-based diets and those fed with standard diets are due to the high amount of UFA in the lupine seeds which are rich in oleic, linoleic and α-linolenic acid. Thus, the fatty acid profile of yolk fats has improved ($p < 0.05$) as a result of the increase in the FA proportion from omega-3 series (ALA, EPA, DHA), considered important for human health [66–68]. Some researchers consider the current Western diets as being generally deficient in n-3 FA compared to the diets of their human ancestors [69]. Therefore, one way to increase the intake of n-3 FA is by consuming natural sources rich in these FA, such as fish or linseed [70], or by consuming functional foods enriched in n-3 FA such as eggs [71–73].

The enzymes addition in the diets did not affect the fatty acid profile of fats from the egg yolk, with the exception of PUFA which decreased, being in line with some previous reports [74,75]. These studies reveal that no significant differences were found regarding the FA profile of egg yolk after feeding laying hens with diets based on different proportions of corn–wheat–soybean meal and multienzymes consisting of xylanase, protease and amylase. In another study, Westbrook et al. [76] reported a decrease in PUFA content (especially linoleic and arachidonic acid) after the addition of xylanase in diets based on corn and flaxseed. In the current research, the enzymes addition did not affect the levels of n-3 FA. Contrary to our findings, research carried out by Jia et al. [77] showed that the use of multicarbohydrase in the diets of hens which contain flaxseed can result in an increase ($p < 0.05$) in the n-3 FA content of yolk fats. The authors attribute these findings to a depolymerization of the polysaccharides cell wall due to the enzymatic action, which increase the availability of fats in the intestine and favor a better action of digestive enzymes.

The lower cholesterol content in the eggs of quails fed with lupine is due to the high quality of lupine fats, which is characterized by a high level in PUFA [8]. Our results are not confirmed by the data reported by Krawczyk et al. [78], where the cholesterol content did not change in the eggs of laying hens fed with diets containing yellow lupine in amounts of 300 g/kg compared with a standard diet.

It is known that carotenoids are fat-soluble pigments which are absorbed by passive diffusion together with dietary lipids by forming mixed micelles. Papadopoulos et al. [79] claimed the possibility that an improvement in the nutrients' digestion and absorption by xylanase supplementation could be reflected in the carotenoid content of egg yolks. Their research showed that the properties of xylanase NSP enzyme improved the digestibility

and absorption of specific dietary nutrients such as carotenoids from wheat-based diets in laying hens [79]. In our research, the high content of carotenoids in the yolk of quail eggs is because of the presence of lutein, zeaxanthin and canthaxanthin in the lupine seeds [80]. Increasing the concentration of carotenoids in egg yolk by using lupine in the diet of birds has also been reported in other studies [56,80].

Egg yolk color is influenced by the natural pigments from the feed [81,82]. The yolk enrichment in carotenoids led to a higher yolk color intensity ($p < 0.05$) in quail groups fed lupine-based diets. Canthaxanthin is a red carotenoid which convert the typical yellow–orange color of the yolk into an orange–red color [79]. In our research, canthaxanthin has the highest level in the egg yolk from quails fed with lupine-based diets. The final yolk color is given by the content in yellow and red carotenoids, because the yellow base is necessary to establish a good saturation, while the red carotenoids act additively to establish the final orange–reddish color [79]. The addition of enzymes in the quail diets did not influence ($p > 0.05$) the yolk color intensity, being in agreement with the report by Lee et al. [28] that showed a similar color intensity of the yolks, even if the enzymes were added in the lupine-based diet of laying hens. However, recently, Nguyen et al. [13] showed an improvement in the egg yolk color following xylanase supplementation of the wheat-based diets in laying hens.

5. Conclusions

This research reveals that lupine seeds can be used in laying quail diets up to 20% (% of feed), as an alternative source to proteins of soybean meal, without any negative effect on productive performance. The addition of commercial enzymes (Hostazyme®) in diets containing white lupine has proved to be a feasible nutritional strategy, which allows an increase in the lupine proportion in quail diets by up to 25% (% of feed) without negatively influencing egg production, egg weight and FCR.

White lupine can be used as part of a global strategy to improve the nutritional quality of egg yolk fats (by increasing the omega-3 FA and carotenoid content and decreasing cholesterol concentration), although data have shown a negative effect on eggshell thickness. The FA profile and health lipid indices of the yolk fats were negatively influenced by the presence of enzymes in the laying quail diets.

It is important to continue the research in order to improve the use efficiency of lupine in quails feed by using enzymes or a mix of enzymes adapted to the chemical components of lupine, but also by associating with a source of polyunsaturated FA (e.g., hemp seeds, flax seeds, camelina seeds) to contribute to the yolk enrichment in FA with a health effect and bioactive compounds with an antioxidant role.

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Impact of Different Levels of Crude Protein on Production Performance and Meat Quality in Broiler Selected for Slow Growth

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Abstract: The production performance and meat quality of the slow-growing hybrid Hubbard JA757 were monitored under conditions of diets differentiated by crude protein content. A total of 1200 as-hatched day-old chickens were equally and randomly allotted into two treatments (T-1 and T-2), with six replicates provided for each treatment (100 chickens/replicate). T-1 chickens received standard diets (according to Hubbard Company recommendations), and those in T-2 were fed diets supplemented with crude protein (+0.5% CP in the growing phase and +1.0% CP in the finishing phase). At the end of the investigations (age 56 days), the T-2 chickens performed better than the T-1 chickens for growth traits (+2.72% body weight; +2.77% daily growth gain; −0.34% mortality; and −4.15% feed conversion ratio); for slaughtering (+0.66% dressed yield; +1.10% breast weight; and +1.25% thigh weight); and for quality meat (+0.55% dry matter in thigh muscles and +1.52% dry matter in breast muscles) ($p > 0.05$). Statistically significant differences ($p < 0.05$) occurred between treatments for body weight, daily weight gain, and feed conversion ratio due to the 0.5% CP feed supplementation during the 15–28 day age period, justifying the usefulness of the CP increasing throughout the grower diet only and not during the finishing period.

Keywords: Hubbard; slow growth; protein level; performance; meat yield

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1. Introduction

Although the world consumption of poultry meat is following an upward trend, its production according to industrial principles has generated negative reactions from those interested in animal welfare (too-high brooding densities, lack of access to the external environment, incidence of specific diseases, etc.) [1], but also from consumers who have complained about poor meat sensory properties and the nutritional value of the meat from the current chicken broilers selected for rapid growth [2]. To respond to the new preferences of the market [3], new genetic resources are being sought to replace the industrial broiler, such as nonimproved local breeds, certified Label Rouge chickens, purebred fowl [4], and special genotypes selected for low growth speeds (slow-growing chickens), a solution increasingly accepted by both meat producers [5,6] and particularly by consumers, due to the quality of the meat, which is perceived as similar to that from traditionally reared fowl [7,8].

The biggest challenge in fowl farming is to provide proper nutrition that is perfectly adapted both to the performance potential and physiological requirements of each category of poultry [9], while ensuring the financial profitability; hence, 70–75% of production costs are generated by feeding [10]. Among the dietary ingredients, the most expensive are those providing the desired level of protein [11]. They differ greatly in terms of quality

parameters and especially of purchasing price, related to a multitude of conjunctural factors and even geopolitics applied at regional levels [12]; i.e., the production cost of 1 kg of chicken meat is about 45% higher in EU countries than in Brazil, one of the world's largest soybean producers [13]. In broiler chickens, the dietary protein is extremely important because it serves as the raw matter for structural body protein neosynthesis [14]. Artificial poultry selection was conducted to increase the muscle mass in breast and leg meat, which implies a high protein intake [15]. As for the effectiveness of the use of proteins by the body of chickens, the studies carried out have shown that this is influenced by various factors, such as their content of amino acids and the bioavailability of the amino acids provided [16]; the existence of some antinutritional factors and the ratio to other nutrients [17]; and the microclimate factors and especially the ambient temperature, feeding technique, consumption level, etc. [18]. When enough protein is provided, normal productive results are obtained [19], while too-low protein levels or the use of proteins of low biological value reduce growth gains and increase feed conversion [20] and depreciate the quality of the carcasses and negatively affect fowl health [21]. The demand for meat obtained from broilers selected for slow growth has increased as well in Romania, but most of the poultry farms are experiencing productive levels below the theoretical performance of the biological material used due to climate and socioeconomic peculiarities specific to the area, but also to the lack of experience on this production system [22].

Under these conditions, we aimed to study the effect of feeding diets supplemented with crude proteins on the growth and slaughter performances, including meat quality, in a well-rated slow-growing chicken hybrid in Romania, namely the Hubbard JA757. In establishing the experimental plan, it was considered that the nutrients in the feed are mainly directed to the function of thermoregulation in the first days of life [15]; consequently, the diets supplemented with crude proteins were fed after the age of 14 days (when enzymatic equipment is fully functional and can efficiently absorb proteins and transaminate the proteins) until the moment of slaughter, in order to achieve higher quantitative and quality performances of the meat. When formulating the experimental diets, it was taken into account that the proportion of inclusion of different ingredients should not affect the nutritional features (especially the metabolizable energy content) and that their manufacturing cost should be similar to that of the standard diets, in order to avoid interferences when calculating the economic profitability applied to the experimental variant proposed by us.

2. Materials and Methods

2.1. Ethics Statement

All experimental procedures complied with the specifications of the Code of Ethics in Scientific Research of the University of Life Sciences of Iasi, Romania (statement no. 26, issued on 17 April 2022 by the Bioethics Committee of Faculty of Food and Animal Sciences, Iasi University of Life Sciences), as well as with those comprised in the Procedures Manual for slow-growth chicken broilers applied in the poultry unit where the investigations were carried out, validated by the International GC-Mark System.

2.2. Biological Material and Management Measures

Studies were carried out on 1200-day-old Hubbard JA757 hybrid chickens in their natural sex ratio (slow-growing genotype, https://www.hubbardbreeders.com/media/leaflet-premium-tradition-en-20220706-ld.pdf (accessed on 14 August 2022)) that were brooded as-hatched and studied until slaughtering (56 days). To achieve the proposed goal, 2 experimental treatments were designed: T-1 treatment = fed standard diets (according to Hubbard recommendations) and T-2 treatment = fed diets supplemented with crude proteins throughout the growing period (+0.5% CP) and the finishing period (+1.0% CP) (Table 1).

Table 1. Experimental protocol.

Treatment	Experimental Factors			
	Hybrid	Protein Level (%)		
		Starter Diet (1–14 Days)	Grower Diet (15–28 Days)	Finisher Diet (29–56 Days)
T-1	Hubbard JA757	21.5	19.0	17.0
T-2	Hubbard JA757		19.5	18.0

Broilers in both experimental treatments were reared on permanent litter, in 2 halls identical in size and technological systems, with uniformly provided microclimate at the levels recommended by the hybrid producer company. In each hall, 6 rearing pens (3 m × 2.25 m) were set up, and then 100-day-old broilers (density = 15 heads/m^2) were randomly brooded in each one, ensuring 6 replicates for each experimental treatment; the rearing pens were arranged in pairs at the front end of the hall, in the central area, and at the rear end of the hall.

Assessment of growth performance was carried out on a total of 1200 chicken broilers, equally allotted in the two experimental treatments (600 heads/treatment); for each experimental treatment, 6 replicates were organized (100 heads/replicate), corresponding to the 6 rearing pens deployed per hall.

Evaluation of the quantitative meat production was applied on a total of 120 chicken broilers (60 heads/treatment, half males and half females), slaughtered at 56 days. To achieve the number of slaughtered individuals per treatment, 5 males and 5 females were extracted from each rearing pen (replicate) (10 heads/pen × 6 pens/treatment = 60 heads/treatment) in such a way that their body weight fell within the average weight of the respective treatment.

Meat quality analyses were performed on samples taken from 24 carcasses (12 carcasses/treatment), both from the breast and thigh regions. Meat samples used in the sensory examination were taken in whole pieces of approx. 100 g each, and those for the assessment of chemical composition were mixed and chopped (breast separated from thighs) and then subjected to laboratory investigations. Ten analytical repetitions/sensory tests were carried out for each investigated trait.

The studied broilers were given combined feed, as Starter diets (1–14 days), Grower diets (15–28 days), and Finisher diets (29–56 days) made of cereals and other feedstuffs, at different inclusion rates (Table 2).

Standard diets were fed to T-1 broilers, formulated based on the Hubbard hybrid guide, following different crude protein levels: 21.5% in Starter diet; 19.0% in Grower diet; and 17.0% in Finisher diet. Supplemented crude protein diets were provided to the T-2 broilers at the beginning of the growing phase (19.5% CP) and all through the finishing phase (18.0% CP). In terms of metabolizable energy contents, there were no differences between the T-1 and T-2 treatments (Starter diet = 2952 kcal/kg; Grower diet = 2950 kcal/kg; and Finisher diet = 3000 kcal/kg). Crude protein supplementation in T-2 treatment also increased essential amino acid dietary levels by 10.38–11.21% for lysine; by 10.87–11.36% for methionine; by 11.9–12.36% for methionine + cystine; and by 13.0–13.9% for threonine.

2.3. Growth Performances

Chicken broilers were individually weighted before feeding and at the same hour in the morning of days 1, 15, 29, and 56 using an electronic scale with an error of ±5 g. Values measured outside the standard deviation of the average weight specific to the respective age were excluded from the analysis.

Weight gain was calculated as the difference between the average weight of the broilers at the beginning of the period and their weight at the end of the analyzed period.

Mortality rate was derived from daily registration of the dead chickens in each hall that accumulated for each age period and overall production series, and their number was reported to the initial flock.

Table 2. Diet composition and nutritional values.

Feedstuffs	Standard Diets (T-1)			Crude Protein-Supplemented Diets (T-2)		
	Starter (1–14 Days)	**Grower (15–28 Days)**	**Finisher (29–56 Days)**	**Starter (1–14 Days)**	**Grower (15–28 Days)**	**Finisher (29–56 Days)**
Maize %	42.84	39.59	46.95	42.84	46.46	67.04
Wheat %	20.00	30.00	20.00	20.00	20.00	7.94
Soybean meal %	25.00	11.26	9.20	25.00	23.09	12.70
Full-fat soybean %	-	15.23	-	-	-	
Sunflower meal %	-	-	8.00	-	-	
Brewer's yeast %	3.00	-	4.00	3.00	6.00	6.00
Soybean oil %	2.06	0.49	4.80	2.06	1.20	0.51
Calcium carbonate %	1.56	0.91	1.60	1.56	1.30	1.14
Monocalcium phosphate %	1.03	0.89	1.03	1.03	0.71	0.49
Vit–min. premix %	0.68	0.69	0.43	0.68	0.59	0.58
Salt %	0.34	0.40	0.33	0.34	0.37	0.23
L-lysin %	0.26	0.31	0.40	0.26	0.14	0.20
Dl-metihonine %	0.17	0.23	0.17	0.17	0.03	
L-Threonine %	0.06	-	0.09	0.06	0.11	0.03
PL68 * %	3.00	-	3.00	3.00	-	3.00
Sodium bicarbonate %	-	-	-	-	-	0.14
Nutritional features						
ME (kcal/kg)	2952	2950	3000	2952	2950	3000
Crude protein %	21.50	19.00	17.00	21.50	19.50	18.00
Lysine %	1.28	1.16	1.06	1.28	1.16	1.06
Methionine %	0.50	0.46	0.44	0.50	0.46	0.44
Meth. and Cyst. %	0.95	0.89	0.84	0.95	0.89	0.84
Valine %	0.98	0.90	0.84	0.98	0.90	0.84
Isoleucine %	0.84	0.77	0.72	0.84	0.77	0.72
Arginine %	1.36	1.24	1.17	1.36	1.24	1.17
Tryptophan %	0.21	0.19	0.19	0.21	0.19	0.19
Threonine %	0.84	0.77	0.72	0.84	0.77	0.72
Lysine dig. %	1.15	1.03	0.95	1.15	1.03	0.95
Methionine dig. %	0.45	0.41	0.39	0.45	0.41	0.39
Meth. and Cyst. dig. %	0.85	0.78	0.74	0.85	0.78	0.74
Valine dig. %	0.86	0.78	0.73	0.86	0.78	0.73
Isoleucine dig. %	0.73	0.67	0.62	0.73	0.67	0.62
Arginine dig. %	1.19	1.08	1.02	1.19	1.08	1.02
Tryptophan dig. %	0.18	0.17	0.16	0.18	0.17	0.16
Threonine dig. %	0.73	0.67	0.62	0.73	0.67	0.62
EAA	6.01	5.49	5.14	6.01	5.49	5.14
NEAA	15.49	13.51	11.86	15.49	14.01	12.86
EAA % of CP	28.0	28.9	30.2	28.0	28.2	28.6
NEAA % of CP	72.0	71.1	69.8	72.0	71.8	71.4
EAA/NEAA ratio	0.39	0.41	0.43	0.39	0.39	0.40
Calcium %	1.03	0.84	0.99	1.03	0.95	0.75
Crude fat %	4.13	6.55	7.02	4.13	3.50	2.91
ME/CP ratio	137.30	155.26	176.47	137.30	151.28	166.66
Cost (EUR/kg)	0.66	0.43	0.64	0.66	0.44	0.63

* Non-GMO bacterial SCP concentrate based on microbial fermentation of vegetal raw materials from crop origin (Intraco Ltd., Antwerp, Belgium). Composition: protein—68%; fat—3.25%; crude fiber—1%; ash—10%; and moisture—10%. Amino acid profile: lysine—2.35%; methionine + cysteine—1.15%; isoleucine—2.45%; tryptophan—0.60%; threonine—3.0%; arginine—2.95%; valine—3.25%; alanine—5.02%; and glutamic acid—18.1%.

Feed intake was calculated based on the feed quantity supplied to each hall and the existing average flock. The individual average daily feed intake (g feed/head/day) and the total individual consumption (g feed/head/period) were calculated for the three stages of feeding (starter, grower, and finisher diets) as well as for the total studied period (1–56 days). The feed conversion ratio (FCR) was calculated by dividing the individual total feed intake to the individual weight gain.

The European Production Efficiency Factor (EPEF) was calculated as the percentage ratio between livability (L, %) and body weight at slaughter (LW, kg) and slaughter age (SA, days) and FCR (kg feed/kg weight gain), according to Equation (1):

$$\text{EPEF} = \frac{\text{L} \times \text{BW}}{\text{SA} \times \text{FCR}} \times 100 \quad (1)$$

Using the unitary cost to manufacture 1 kg of feed (Table 2) from each type of formulation, the data related to total weight gain, to total feed intake per technological period (starter, grower, and finisher), to flock size per period, and to production costs generated by feeding were calculated and were considered as expenses (Es). Total produced body weight at slaughter, multiplied by the unitary selling price per kg meat (EUR 1.67) resulted in the income (I) values. Revenue (R) was calculated using Equation (2), while the profitability rate (PR), as % revenue generated by each EUR 100 invested expenses, was calculated using Equation (3).

$$R\ (EUR) = I\ (EUR) - E\ (EUR) \quad (2)$$

$$PR\ (\%) = R\ (EUR) \times 100/E\ (EUR) \quad (3)$$

2.4. Performances at Slaughter

From each experimental group, 60 broilers (30 males + 30 females), whose weight fell within the average of the respective group, were selected and were slaughtered (after prior electrical stunning) in accordance with the technological stages specific to the poultry slaughterhouse of the farm where the research was conducted.

At the end of the slaughter, the resulting carcasses (with head and claws) were chilled for 24 h, at a temperature of +3 °C, and then the dressed yield was calculated based on the individual body weight of the broilers prior to slaughter (LW, kg), and the weight of the corresponding individual refrigerated carcasses (CW) was calculated according to Equation (4):

$$\text{Dressed yield } (\%) = \frac{CW}{LW} \times 100 \quad (4)$$

Each obtained carcass was manually cut by a single operator into four anatomical pieces that included the related musculature, bone base, and skin (breast, legs, wings, and remnants); each cut portion was weighed using a precision electronic scale, and the values obtained were calculated as a percentage of the originating carcasses.

2.5. Assessment of Meat Quality

Meat sensorial exams were carried out by 5 trained evaluators, using a hedonic scale with 5 graduations: (1 = very disagreeable; 2 = moderately disagreeable; 3 = slightly disagreeable; 4 = agreeable; and 5 = very agreeable). Each evaluator received whole meat samples (taken from the pectoral and thigh muscles), precooked in an oven until +80 °C was achieved in the thermal center and then brought to an acceptable temperature for tasting (ISO 11136:2014, updated 2020 [23]).

Meat chemical composition was investigated by standard protocols, in accordance with the Romanian standards in force applicable for meat and meat preparations: the content in dry matter by the oven-drying method at +105 °C (ISO 1442:1997, updated 2018) [24]; crude protein by the Kjeldahl method (SR ISO 937:1978, updated 2018) [25]; crude fat by the Soxhlet method (SR ISO 1443:1973, updated 2018) [26]; and crude ash by the calcination method at +550 °C (SR ISO 936:1998, updated 2018) [27]. Nine analytical repetitions were carried out for each sample (breast and thighs).

2.6. Data Treatment

Obtained data were input into a database grouped by columns with individual values, and then they were statistically treated to compute the main statistical descriptors (mean, standard error of mean) and to analyze the variance in one-to-one comparisons via the unpaired *t*-test with Welch correction, assuming not equal standard deviations, using the GraphPad Prism 9 software (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Productive Performances

Body weight dynamics. The day-old chicks came from a specialized hatchery and were homogenous in terms of body weight (41.38 g for broilers in T-1 treatment and 41.39 g for those in T-2 treatment). At 14 days old, the average weight of the T-1 broilers was 312.86 g, while T-2 reached 315.69 g, with no statistical differences. At 28 days, significant differences ($p < 0.05$) were found between the broilers fed the 0.5%-supplemented CP diet (T-2986.52 g) and those fed the standard diet (T-1935.39 g). At the slaughter moment (day 56), the body weight reached 2359.27 g (standard diet) and 2425.36 g (diet supplemented with CP), with no statistical difference (Table 3).

Table 3. Dynamics of body weight, daily weight gain, and mortality.

Broilers Age (Days)	Statistics	Body Weight (g)		Daily Weight Gain (g/day)		Mortality (%)	
		T-1	T-2	T-1	T-2	T-1	T-2
1	Mean ± SEM	41.38 ± 0.09	41.39 ± 0.11	-	-		
	t test *p* values	0.9916		-		-	
14	Mean ± SEM	312.86 ± 1.07	315.69 ± 1.25	19.39 ± 0.04	19.59 ± 0.05	1.00 ± 0.01	1.00 ± 0.01
	t test *p* values	0.6973		0.6650		0.9899	
28	Mean ± SEM	935.39 ± 3.51	986.52 ± 3.94	44.47 ± 0.29	47.92 ± 0.17	0.84 ± 0.02	0.67 ± 0.01
	t test *p* values	0.0464 *		0.0146 *		0.0024 **	
56	Mean ± SEM	2359.27 ± 9.52	2425.36 ± 10.19	50.85 ± 0.15	51.39 ± 0.20	1.70 ± 0.05	1.53 ± 0.04
	t test *p* values	0.2518		0.6502		0.0191 *	
1–56	Mean ± SEM			41.39 ± 0.15	42.57 ± 0.18	3.54 ± 0.12	3.20 ± 0.10
	t test *p* values	-		0.2461		0.0171 *	

SEM—standard error of mean. * significant differences for $p < 0.05$. ** significant differences for $p < 0.01$.

Weight gain dynamics. From the analysis of the data related to the growth rate of the studied chickens, it emerged that it was influenced by the nutritional characteristics of the provided feed, according to the data presented in Table 3. For example, in the first period (1–14 days), the chickens in both treatments were fed diets with identical protein levels (Starter), and their growth rate varied similarly (19.39 g/head/day in T-1 and 19.59 g/head/day in T-2). Throughout the next age period (15–28 days), significant differences ($p < 0.05$) occurred between the treatments (47.92 g/head/day in T-2 vs. 44.47 g/head/day in T-1). In the last studied period (29–56 days), chickens from T-2 (+1.0% CP diet) achieved a daily weight gain of 51.39 g/head, compared to 50.85 g/head in T-1 (standard diet). Throughout the whole series, the dynamics of weight gain was at an average level of 41.39 g/head/day in chickens fed the standard diet (T-1) and 42.57 g/head/day in chickens fed the diet supplemented with CP (T-2).

Mortality dynamics. In the first 14 days, the mortality was similar in the two treatments at 1.0% of the initial flock (Table 3). In the next age period (15–28 days), the mortality rate decreased to 0.84% in T-1 and to 0.67% in T-2, and then it increased to 1.70% and 1.53%, respectively, throughout the last period (29–56 days). Over the total period studied (1–56 days), the broilers fed standard diets (T-1 treatment) reached a mortality of 3.54%, while those fed CP-supplemented diets (T-2 treatment) reached 3.20%.

Feed intake and conversion. Table 4 shows the data related to the average daily feed intake and to the feed conversion ratio for the three dietary stages (starter, grower, and finisher) and for the entire growth period as well (1–56 days).

Table 4. Feed intake and feed conversion rates.

Broilers' Age and Dietary Periods (Days)	Statistics	Average Feed Intake (g feed/head/day)		Feed Conversion Ratio (kg feed/kg Gain)	
		T-1	T-2	T-1	T-2
1–14 (starter)	Mean ± SEM	40.68 ± 0.70	40.56 ± 0.63	2.098 ± 0.07	2.070 ± 0.07
	t test *p* values	0.8853		0.5914	
15–28 (grower)	Mean ± SEM	56.88 ± 1.37	56.35 ± 1.27	1.279 ± 0.05	1.176 ± 0.05
	t test *p* values	0.6707		0.0125 *	
29–56 (finisher)	Mean ± SEM	118.39 ± 3.48	116.57 ± 3.33	2.245 ± 0.11	2.187 ± 0.09
	t test *p* values	0.5124		0.3677	
1–56 (overall)	Mean ± SEM	83.21 ± 2.99	82.16 ± 2.85	2.010 ± 0.11	1.930 ± 0.10
	t test *p* values	0.6157		0.2061	

SEM—standard error of mean. * significant differences for $p < 0.05$.

The obtained data show that chickens fed CP-supplemented diets (T-2) consumed slightly less feed, leading to better feed conversion rates than chickens fed standard diets (T-1), both by diet type and by the total studied period.

European Production Efficiency Factor and Economic Profitability. Based on the data obtained during the growth of the studied chickens, the European Performance Efficiency Factor values were calculated and are presented in Table 5.

Table 5. European Production Efficiency Factor.

Traits	Statistics	Treatments	
		T-1	T-2
Survival rate (%)		96.46 ± 3.27	96.80 ± 3.08
Body weight (kg)		2.36 ± 0.009	2.43 ± 0.010
Age at slaughter (days)		56	56
FCR (kg feed/kg gain)		2.01 ± 0.11	1.93 ± 0.10
EPEF (European Production Efficiency Factor)	Mean ± SEM	202.25 ± 0.44	217.63 ± 0.59
	t test *p* values	0.0038 *	

SEM—standard error of mean. * significant differences for $p < 0.01$.

The European Production Efficiency Factor had a lower value in the T-1 treatment (202.25 points), compared to that calculated for the T-2 broilers (217.63 points) ($p < 0.05$).

A lower income was obtained by the end of the experiment in the standard-fed broilers (EUR 615.02) compared to those fed the CP-supplemented diet (719.76), hence the difference in the profitability rate (36.92% in T-1 and 44.07% in T-2).

Table 6 depicts the economic results of each tested diet (standard or supplemented for crude protein level).

3.2. Meat Yield

Dressed yield. At the end of the production series, the chickens were slaughtered in order to establish the yield at slaughter (Table 7).

The body weight at slaughter in the chickens fed the standard diet (T-1) was 2365.18 g and that of the carcasses reached 1712.63 g, resulting in a dressed weight of 72.41%. In the chickens fed diets supplemented with CP, the body weight at slaughter was 2428.19 g, and the carcass weight reached 1774.28 g; therefore, the dressed weight was 73.07%. No statistical differences occurred between treatments.

Proportion of cut parts. The meat production was also evaluated through the proportion of the four main anatomical regions (breast, thighs, wings, and remnants) in the structure of the carcasses (Table 8).

Table 6. Economic efficacy indices (Expense, Incomes, Revenue, and Rate of Profitability).

Dietary Periods	Traits	Treatments	
		T-1	**T-2**
Starter	Total feed intake (kg)	340	339
	Unitary diet cost (EUR/kg)	0.66	0.66
	Feeding expenses (EUR)	224.40	223.74
Grower	Total feed intake (kg)	471	467
	Unitary diet cost (EUR/kg)	0.43	0.44
	Feeding expenses (EUR)	202.53	205.48
Finisher	Total feed intake (kg)	1936	1911
	Unitary diet cost (EUR/kg)	0.64	0.63
	Feeding expenses (EUR)	1239.04	1203.93
Overall (1–56 days) feeding expenses (E) (EUR)		1665.97	1633.15
Flock size sent to slaughterhouse (heads)		579	581
Average individual weight (kg)		2.359	2.425
Total body weight at slaughter/group (kg)		1365.86	1408.93
Meat unitary selling price (EUR/kg)		1.67	1.67
Income (I) (EUR)		2280.99	2352.91
Revenue (R) (EUR)		615.02	719.76
Profitability rate (PR) (%)		36.92	44.07

Table 7. Dressed yield.

Traits	Statistics	Treatments	
		T-1	**T-2**
Body weight (g)	Mean ± SEM	2365.18 ± 24.12	2428.19 ± 21.63
	t test *p* values	0.2735	
Carcass weight (g)	Mean ± SEM	1712.63 ± 18.35	1774.28 ± 14.66
	t test *p* values	0.1525	
Dressed yield (%)	Mean ± SEM	72.41 ± 0.76	73.07 ± 0.64
	t test *p* values	0.6960	

SEM—standard error of mean.

Table 8. Proportions of cut parts in carcasses.

Cut Parts	Statistics	Treatments	
		T-1	**T-2**
Breast (%)	Mean ± SEM	34.23 ± 0.39	34.61 ± 0.39
	t test *p* values	0.6370	
Thighs + drumsticks (%)	Mean ± SEM	33.95 ± 0.32	34.38 ± 0.33
	t test *p* values	0.5914	
Wings (%)	Mean ± SEM	12.26 ± 0.13	12.39 ± 0.14
	t test *p* values	0.6656	
Remnants (%)	Mean ± SEM	19.56 ± 0.17	18.62 ± 0.19
	t test *p* values	0.0633	

SEM—standard error of mean.

When cutting the carcasses, we found that those from chickens fed CP-supplemented diets (T-2) had a higher proportion of breast (34.61% vs. 34.23% in T-1), thighs (34.38% vs. 33.95% in T-1), and wings (12.39% vs. 12.26% in T-1). A higher proportion of remnants

was found (19.56% vs. 18.62%) in T-1 carcasses (standard diets). No statistically significant differences occurred between the treatments in any of the compared regions.

3.3. Meat Quality

This was evaluated through the lens of sensory properties and chemical composition on samples taken both from the leg and breast muscles.

Sensory assessment of meat. There were certain differences between the experimental treatments in favor of the meat obtained from chickens that received diets supplemented with crude protein (T-2). In thigh muscles, it was found that the most appreciated attribute was flavor + savoriness (T-1 = 4.84 points and T-2 = 4.90 points), followed by tenderness (T-1 = 4.65 points and T-2 = 4.72 points) and consistency (T-1 = 4.62 points and T-2 = 4.70 points); the least-valued trait in thigh meat was juiciness, for which only 3.84 points (T-1) and 3.89 points (T-2) were awarded (Table 9).

Table 9. Sensory scores of thigh and breast meat.

Traits	Statistics	Thigh Meat		Breast Meat	
		T-1	T-2	T-1	T-2
Tenderness	Mean ± SEM	4.65 ± 0.12	4.72 ± 0.13	4.80 ± 0.14	4.85 ± 0.15
	t test *p* values	0.2582		0.7305	
Juiciness	Mean ± SEM	3.84 ± 0.13	3.89 ± 0.13	3.52 ± 0.11	3.61 ± 0.11
	t test *p* values	0.6982		0.4741	
Flavor and savoriness	Mean ± SEM	4.84 ± 0.16	4.90 ± 0.14	4.25 ± 0.14	4.32 ± 0.13
	t test *p* values	0.6815		0.6078	
Firmness	Mean ± SEM	4.62 ± 0.14	4.70 ± 0.14	4.74 ± 0.14	4.82 ± 0.13
	t test *p* values	0.5764		0.5817	

SEM—standard error of mean.

In the case of the pectoral muscles, the sensory examination revealed that the best-rated quality was tenderness (4.80 points for the T-1 treatment and 4.85 points for the T-2 treatment), and the lowest-rated was juiciness (3.52 points for T-1 and 3.61 points at T-2); intermediate scores were recorded for consistency (4.74 points for T-1 and 4.82 points for T-2) and for flavor + savoriness (4.25 points for T-1 and 4.32 points for T-2) (Table 9).

Chemical composition of meat. A higher proportion of dry matter (29.33% vs. 29.17%) and crude protein (19.58% vs. 19.26%), but lower crude fat (6.34% vs. 6.46%) and crude ash (3.28% vs. 3.30%) were achieved in the T-2 treatment vs. the T-1 treatment (Table 10).

Table 10. Proximate composition of thigh and breast meat.

Traits	Statistics	Thigh Meat		Breast Meat	
		T-1	T-2	T-1	T-2
Dry matter (%)	Mean ± SEM	29.17 ± 0.38	29.33 ± 0.32	28.53 ± 0.35	28.97 ± 0.40
	t test *p* values	0.8146		0.5167	
Crude protein (%)	Mean ± SEM	19.26 ± 0.32	19.58 ± 0.29	22.85 ± 0.25	23.32 ± 0.35
	t test *p* values	0.4910		0.3959	
Crude fat (%)	Mean ± SEM	6.46 ± 0.20	6.34 ± 0.19	2.81 ± 0.11	2.74 ± 0.11
	t test *p* values	0.4972		0.5382	
Crude ash (%)	Mean ± SEM	3.30 ± 0.11	3.28 ± 0.14	2.73 ± 0.12	2.80 ± 0.10
	t test *p* values	0.5298		0.5377	

SEM—standard error of mean.

As for the breast muscles, the dry matter content was lower than that in the thighs in both experimental treatments (28.53% in T-1 and 28.97% in T-2). Higher levels were found for crude protein (22.85% in T-1 and 23.32% in T-2) and much lower for crude fat (2.81% for

T-1 and 2.74% for T-2). The crude ash in the meat was lower than in the thighs, within the range of 2.73–2.80% (Table 10).

4. Discussion

Producing poultry meat in farming systems that is different from the conventional industrial type has become a necessity at the present time, given the orientation of consumer preferences toward a meat with better-accentuated sensory features, characteristics resembling to wild or traditionally farmed fowl, and with less moisture content. To respond to this demand, farmers began to rear various slow-growth hybrids (broilers) or to limit the weight gain up to a maximum of 45 g/day, by reducing the crude protein level of the fed diets.

4.1. Productive Performances

Body weight dynamics. Body weight was directly influenced by the nutritional characteristics of the diets. After the first 14 days of life, the body weight was close in the chickens from the two experimental treatments (312.86 g in the T-1 treatment and 315.69 g in the T-2 treatment), because both groups received an identical diet of protein content, and the weight differences at the age of one day were insignificant (41.38 g vs. 41.39 g). During the period of 15–28 days, a diet supplemented with 0.5% CP was used in the T-2 treatment, so in the end, the chickens had a body weight that was higher by 5.18% compared to the chickens fed the standard diet (T-1) ($p < 0.05$). Although the highest CP supplementation was provided in the age period 29–56 days (+1.0% CP), the difference in body weight between the experimental treatments was only 2.74% in favor of the chickens in the T-2 group. The body weight at 56 days (2359.27 g in T-1 and 2425.36 g in T-2) can be considered as normal for a slow-growing hybrid, comparable to those obtained under similar experimental conditions. In the Hubbard broiler line JA957 (slow-growing), Mikulski et al., 2011 [28] reported a body weight of 1.94 kg (42 days) and of 3.64 kg (65 days), while in the F15 Hubbard line (rapid-growing), the average body weights reached at the same ages of slaughter were 2.39 kg and 4.40 kg. In addition, in the Hubbard ISA Red JA chicken broiler that reared free-range chickens (access to a grassy paddock since 14 days old until slaughtering at 84 days), there were reached body weights of 2.99 kg in the group fed the protein- and energy-rich diet, compared to a 2.27 kg body weight in the control group fed a diet restricted in energy and protein levels [29]. Other studies carried out on slow-growth hybrids (ISA J 457 and ISA J 257), fed differentially in the starter period (standard organic feed vs. feed supplemented with fish meal) and slaughtered at different ages (56 and 77 days), showed that the genetic variant ISA J 257 fed higher protein levels achieved a better growth performance and a higher quality of the carcass and of the yielded meat [30].

Weight gain. The average daily weight gain was correlated with the body weight dynamics, with certain differences given by the experimental factor. For example, in the age period 15–28 days, the weight gain rate of chickens in the T-2 treatment was higher by 7.20% than that of chickens in the T-1 treatment, while in the period 29–56 days, the difference between the treatments was only 1.05%. The data indicate that the tested hybrid (Hubbard JA757) utilizes the dietary proteins more efficiently during the growth period than during the finishing period. This statement is supported by the significant statistical differences ($p < 0.05$) between the two experimental treatments, but also by the specifications in the literature that show that the formation of muscle mass in chickens intensifies after the age of 14 days when their digestive enzymatic equipment becomes fully functional [15]. During the entire studied period (1–56 days), the average daily weight gain was 41.39 g/head/day in T-1 chickens and 42.57 g/head/day in T-2 chickens, attesting the fact that the Hubbard JA757 is a well-selected hybrid for slow growth, in both cases not exceeding the maximum threshold of 45 g/head/day as is specific for this category of broilers. For this trait, other studies conducted on slow-growth broilers did not underline the differences due to the dietary levels of energy and protein (low vs. high), but did underline the changes in feeding behavior of the fowl based on the feeding technique (ad libitum vs. restricted feeding) [30]. Weight gains of 50.48–53.20 g/head/day were

obtained in the ROSS-308 conventionally reared broilers, slaughtered at 42 days, due to the protein profile optimization of the diet, through supplementing different levels of lysine, methionine, and L-carnisine [31]. Weight gain could also be influenced by lighting schedule adjustments, such as reported for Beijing-You in a slow-growing chicken broiler, where the daily gain was 20.28–21.09 g/head, within the 1–90 day interval [32].

Mortality. A higher mortality rate occurred in the first 14 days (1.0% for each treatment) when mostly the low-viable chickens died, but also in the last 28 days of the study (1.70% in T-1 treatment and 1.53% in T-2 treatment) due to cardiovascular issues that occurred in specimens with a high body weight; the other mortality cases were mostly due to mechanical incidents (fracture, dislocation, suffocation, etc.), and no metabolic–nutritional morbidity was observed. Overall, the mortality levels throughout the whole period (1–56 days) fell within the normal limits (3.54% in T-1 and 3.20% in T-2) specific to slow-growing chickens designed to be reared in closed sheds, with no access to the outer environment. Significant differences between treatments occurred both in the grower diet phase (15–28 days, $p < 0.01$) and the finisher diet period (29–56 days), as well as in the total studied period (1–56 days) ($p < 0.05$), showing that crude protein supplementation ensured a better growth pace and a better immune response and therefore better resilience to morbidities. In other similar studies, feeding a high-energy, protein-rich diet to Hubbard ISA Red JA chickens significantly improved growth and feed intake compared to a low-energy, protein-poor diet, but with no differences between groups in the mortality rate [33]. Other studies reported that using high dietary energy levels (3000–3300 kcal ME/kg feed) in "Lingnan" slow-growing chickens induced a 4.17–6.67% mortality, compared to just a 3.33% flock loss in chickens fed 2900 kcal ME/kg feed, despite all groups receiving the same dietary crude protein content (16%) [34]. In addition, rearing different fowl genotypes (fast-growing, slow-growing, and Rhode Island chickens) in different systems (intensive and free-range) resulted in differences in body weight, growth rate, and feed conversion efficiency, but not in the rate of survival ($p > 0.05$) [35].

Feed intake and conversion. In all analyzed situations, the chickens from T-2 (diets supplemented with CP) had a lower average intake than those from T-1 (standard diets). In the age period 1–14 days, the difference between treatments was only 0.30% (40.68 g feed/head/day in T-1 and 40.56 g feed/head/day in T-2); in the period 15–28 days, the difference increased to 0.94% (56.88 g feed/head/day vs. 56.35 g feed/head/day); in the period 29–56 days, the difference increased to 1.56% (118.39 g feed/head/day vs. 116.57 g feed/head/day). Throughout the entire period (1–56 days), chickens fed diets supplemented with CP (T-2) had a more convenient average daily intake, lower by 1.28% than chickens fed standard diets (T-1). The results on feed intake reveal that the Hubbard JA757 is a hybrid that responds to dietary crude protein supplementation, well proven by the better values of the feed conversion ratio (FCR) that were 1.35% lower throughout the 1–14 day period, 8.76% lower throughout the 15–28 day period, and 2.65% lower throughout the 29–56 day period. For the entire period (1–56 days), a 4.15% lower FCR value occurred (1.93 kg feed/kg gain vs. 2.01 kg feed/kg gain) due to the better T-2 chicken performance throughout the 15–28 day period ($p < 0.05$). Other studies run on slow-growth chicken broilers, reared in similar conditions with rapid-growth ones, revealed an FCR of 2.54 in slow-growing fowl, compared to conventional ones (FCR of 2.47) [36]. In addition, regarding the thermal conditions provided, the "i657" slow-growing chicken hybrid achieved a daily feed intake of 57–73 g and an FCR of 2.15–2.49, while the ROSS-308 conventional broiler consumed 142–150 g feed/day to achieve eventually an FCR of 2.02–2.34 [37]. Providing access to slow-growth chicken broilers to feed resources in an outer hall environment (free-range rearing) allowed the FCR to decrease to 2.96, compared to the FCR 3.38 achieved by chickens exclusively maintained and fed indoors on deep litter [38]. In certain genetic strains with slow growth, different FCRs were gender-specific, reaching a 2.46–2.63 kg feed/kg gain in females and a 2.09–2.16 kg feed/kg gain in males [39].

European Production Efficiency Factor and Economic Profitability. The calculated values for the EPEF were related to the experimental variable applied in our study (different levels of CP) that influenced the performance of the Hubbard hybrid. The EPEF was 217.63 points

in T-2 and only 202.25 points in T-1 ($p < 0.05$). Expressed as a percentage, the difference reached 7.07% due to the higher body weight (+2.74%), better livability (+0.35%), and lower FCR (−4.15%) values in the T-2 chickens fed CP-supplemented diets. In other studies, providing low-protein feed caused a more severe decrease in the production performance in the fast-growth genotypes (Ross 308) and in medium-growth ones (JA757) compared to slow-growth ones (ISA Dual) and negatively impacted the European Production Efficiency Factor ($p < 0.001$) in fast-growth (−10%) and medium-growth (−6%) fowl, but not in slow-growth fowl [40]. Other authors reported that in slow-growing chickens obtained through Hubbard × Yellowleg Partridge crossings slaughtered at 56 days the EPEF reached a value of 111.9 due to a body weight of 1217 g, an FCR of 1.896, and a casualty rate of 2.92% [41].

Economic efficiency. The total revenues obtained at the end of the series were at a level of EUR 615.02 in T-1 and EUR 719.76 in T-2, corresponding to revenues of EUR 1.06 and EUR 1.24 for each chicken delivered to the slaughterhouse. The rate of profitability calculated for each EUR 100 invested was higher by 7.16% in the T-2 than in the T-1 group (44.07% vs. 36.92%). Of course, if we had conducted a total expense/income analysis, the profitability rates would be much lower, knowing that feeding costs usually account for up to 70–75% of the total production expenses. In other studies, in the case of chickens raised in closed shelters for 21 days and then in the free-range system until slaughter (56 days), in the fast-growing genetic variants, a profit of USD 3.54/head was obtained, and in the slow-growing hybrids, there was a profit of only USD 0.37/head [35]. With regard to the net income achieved from the sale of the carcasses of the broilers that received diets differentiated in terms of the energy and protein provided (high, medium, low, and very low levels), the best results were obtained in the males provided an average level of nutrients (EUR 1.68/carcass) and in females (EUR 1.32/carcass) where a low level was ensured [42].

4.2. Meat Yield

Dressed yield. Although the differences in body weight at slaughter were only 2.59% in favor of chickens fed CP-supplemented diets (T-2), the experimental factor influenced meat deposition during growth, so that the weight of the carcasses was higher by 3.47% than in the chickens fed standard diets (T-1). Under these conditions, the dressed yield was better with 0.66% in the T-2 chickens than those in the T-1 chickens (73.07% vs. 72.41%), despite the fact that no statistical significance was obtained. In other studies with slow-growth broilers receiving the same diet but reared in closed halls versus free-range, better results were obtained in free-range fowl (69.88% vs. 69.80% dressed yield; 20.17% vs. 18.20% breast fillet proportion; and 27.65% vs. 27.23% thigh proportion) [43]. Other authors [44] also reported that crude protein supplementation in the Hubbard JA757 chickens generated a 74.89% dressed yield, compared to 74.66% (same genotype fed standard diet). In "Lingnan" slow-growing chickens, providing 16% crude protein and 2900 kcal ME led to a 68.99% dressed yield or a 0.25–1.36% better value than those achieved by groups fed with 16% crude protein and higher ME values (3000–3300 kcal/kg feed) [45]. Other authors reported dressed yield levels at 69.90% (Gushi local Chinese chicken) [46], 68.63% (Indbro slow-growth experimental broiler obtained from White Plymouth Rock × Cornish strain crosses) [34], or slightly above 72% (novel Slow-Growing Broiler, Australia) [47]. Increasing the level of crude protein from 15% to 23% in Hubbard broiler diets during the 22–42 day period caused a linear increase in the values of dressed yield from 66.2% to 67.87% [14].

Proportion of cut parts. Carcasses with higher proportions of breast and legs are preferred in chicken broilers, because such anatomic parts could be better capitalized when marketed as separate products. The biological material used in our experiment (Hubbard JA757) came from genetic strains selected for the purpose of increasing the proportion of anatomical regions of commercial interest (breast and thighs). In our study, they reached together 68.18–68.99% of the carcass weight. However, the experimental factor led to certain differences in favor of the group fed CP-supplemented diets (T-2), where a higher weight was recorded for breast (+0.38%), thighs (+0.43%), and wings (+0.13%). Even if these differences were not statistically significant, it is most likely that they will be maintained in

industrial production conditions, and their cumulative effect would lead to the addition of the annual revenues to the respective farms. In similar studies, the proportion of thighs and drumsticks in the carcass structure was calculated at 29.69% in novel Slow-Growing Broiler, Australia [47], at 31% in the Hubbard commercial slow-growth broilers [48], and at 32.70% in the Hubbard S757 strain [49]. In other studies, the Hubbard slow-growth broilers aged 8 weeks at slaughter had a 30% breast proportion in the carcass structure, while wings had a 12% proportion [48], and up to 31.5% breast and 12.8% wings were obtained in the Hubbard S757 carcasses slaughtered at 9.5 weeks [49]. Other research reported a 45.26% thigh and shank proportion as well as a 36.45% breast proportion in slow-growing broilers reared free-range and slaughtered at 56 days, while in those reared on deep litter exclusively indoors the proportion quota reached 46.04% for legs and 34.78% for breast [50]. Almost at the same body weight at slaughter (2 kg), Vencobb fast-growing broilers reached 37.97% for breast, 33.52% for thighs and shanks, and 12.47% for wings, while Indbro slow-growing chickens achieved carcass proportion quotas of 29.95% for breast, 30.93% for thighs and shanks, and 11.17% for wings [34]. In COBB-500 broilers, conventionally reared and slaughtered at 42 days, the breast proportion in the carcass reached 33.5–35.2%, and that of the legs was reported at 27.9–28.7% in relation to the proportion of organic selenium added in diet the [51].

4.3. Meat Quality

Sensory assessment. Meat from chickens fed CP-supplemented diets was better sensory-wise, achieving average scores of 4.40 points (breast muscle) and 4.55 points (leg muscle), compared to only 4.33 points and 4.49 points, respectively, in chickens fed standard diets. In the case of the leg muscles, the scores were higher by 1.22–1.70% in chickens fed CP-supplemented diets than in chickens fed standard diets. The highest scores were awarded for flavor and savoriness and the lowest ones for juiciness. A similar situation was found in the case of the breast muscles, with differences between the two treatments reaching 1.03–2.49%. The most appreciated was tenderness, and the least appreciated was juiciness. In the meat of Hubbard Hi-Y chickens slaughtered at the age of 42 days, tenderness was appreciated with 4.4 points (breast muscles) and 4.55 points (leg muscles), juiciness with 4.3 points and 4.13 points, respectively, and aroma and taste with 4.22 points and 4.18 points, respectively [51]. The taste, flavor, juiciness, and overall acceptability of the breast meat were higher in day 21 open-air-reared broilers, but tenderness was higher in non-free-air broilers [52].

Chemical composition. Increasing the dietary crude protein influenced the chemical composition of the meat in the sense that it favored a better accumulation of proteins and a decrease in lipids. Muscles in the legs of the T-2 broilers (CP-supplemented diets) had a higher level of dry matter (+0.55%) and CP (+1.63%); meanwhile, they were lower in crude fat (−1.89%) compared to chickens in the T-1 (standard diets). The effect was more accentuated in the breast, where the DM was higher by 1.52% and CP by 2.02% than those found in standard diet-fed chicken; in the latter samples, the meat contained 2.55% more crude fat. The achieved data are similar to those in the literature, with certain differences given by the experimental factors and especially by the age at slaughter. For example, the addition of *Scutellaria baicalensis* radix (0.5%, 1.0%, and 1.5%) in the diet fed to Hubbard Hi-Y chickens determined the following values for the chemical composition of the meat (leg muscles/breast muscles): DM = 26.71%/26.82%; CP = 19.81%/22.94%; CF = 5.72%/2.37%; and crude ash = 0.96%/1.22% [53]. Pectoral muscles taken from Hubbard JA757 chickens slaughtered at 42 days (average body weight of 2.0 kg) contained 25.69% DM; 22.90% CP; 4.64% crude fat, and 1.17% crude ash [54]. In another study performed on two Hubbard strains slaughtered at 56 days, the leg muscles contained 31.78% DM, 20.14% CP, and 11.22% crude fat (Hubbard Classic) and 29.93% DM, 20.01% CP, and 9.44% crude fat (Hubbard Yield Color) [22]. In the meat of Hubbard JA 957 chickens slaughtered at the age of 63 days, the meat DM content was 25.57% (breast) and 27.37 (thighs); CP was 23.17% and 19.10%; crude fat was 0.88% and 6.91%, crude ash reached 1.18% and 1.04% [55].

5. Conclusions

Feeding crude protein-supplemented diets to the Hubbard JA757 hybrid led to a better performance compared to the nonsupplemented chickens. Most of the differences did not cross the statistical significance threshold, except for body weight, weight gain rate, and feed conversion ratio ($p < 0.05$) throughout the grower diet period (15–28 days, +0.5 CP supplementation). The obtained data suggest that dietary crude protein supplementation provides a good response when the chicken broiler has the most intense growth rate (after 14 days, after dressing in plumage), but on the condition of keeping it within normal limits, related to the other quality parameters of the combined feed, especially without increasing its price. However, considering that the price and quality of the ingredients used to ensure a certain protein level in broilers are extremely variable, it is necessary to continue investigations in this direction to establish to what extent crude protein could be increased without affecting economic profitability to ensure better meat yield and good quality.

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Effects of Supplementation of 25-Hydroxyvitamin D_3 as a Vitamin D_3 Substitute on Performance, Bone Traits, and Egg Quality of Laying Hens from 1 Day to 72 Weeks of Age

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Abstract: This experiment was conducted to explore the effect of long-term supplementation of 25-hydroxyvitamin D_3 (25-OHD) as a vitamin D_3 (VD_3) substitute on performance, bone traits, and egg quality of laying hens from 1 day to 72 weeks of age. In total, 900 one-day-old Lohman pullets were randomly allotted into three dietary groups (three treatments × 15 replicates × 20 birds per replicate): VD_3 2800 IU/kg; 25-OHD 69 μg/kg; 25-OHD 125 μg/kg. At the end of the 20th w, five replicates from each group were selected to feed on the same vitamin D diets, as used during the rearing stage (1–20 w) until 72 w. The result showed that the 25-OHD 125 μg/kg treatment had the lowest average daily feed intake (ADFI) at 1–8 or 1–19 w, body weight at 8 w, body weight gain between 1 and 8 w and shank length at 4 w ($p < 0.05$). The 25-OHD 125 μg/kg treatment had a lower shank length at 7 w, compared with the 25-OHD 69 μg/kg treatment. The shank length of the birds in each treatment reached the maximum (about 103 mm) at about 18 w of age. For the bone traits, the 25-OHD 125 μg/kg treatment had the lowest femur bone diameter at 20 w ($p < 0.001$) and femur bone plumpness at 20 w ($p = 0.002$). The 25-OHD 125 μg/kg treatment had a lower tibia strength at 10 w ($p = 0.023$) and keel length at 10 w ($p = 0.046$), compared with the 25-OHD 69 μg/kg treatment. However, both 25-OHD 69 and 125 μg/kg treatments had a greater femur strength at 72 w ($p = 0.006$), compared with the VD_3 2800 IU/kg treatment. No difference in laying performance was observed among all treatments. The overall (21–72 w) ADFI in the 25-OHD 125 μg/kg treatment was significantly lower than that in the 25-OHD 69 μg/kg treatment ($p = 0.030$). At 60 w, the 25-OHD 125 μg/kg treatment had a lower eggshell thickness ($p = 0.012$) and proportion of eggshell ($p = 0.022$), compared with the 25-OHD 69 μg/kg treatment. No significant differences in egg quality parameters were observed at 50 and 70 w among treatments. In general, supplementary 2800 IU/kg doses of VD_3 at the early stage were sufficient to maintain the bone quality and growth and development of pullets. Feeding birds at a higher 25-OHD level (125 μg/kg) resulted in the reduced ADFI and growth at the rearing period, but the long-term supplementation of 25-OHD as a VD_3 substitute improved the bone quality in the late laying period.

Keywords: 25-hydroxyvitamin D_3; bone traits; egg quality; laying performance; laying hen

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1. Introduction

Modern commercial laying hens are more productive and have a longer laying cycle. This is a huge challenge for the health of the hen's various tissues and organs, specifically in bone health. For hens, bone quality and eggshell quality are closely related. Medullary bone can provide 40% of the calcium in the eggshell during daily eggshell formation [1]. Furthermore, some studies have shown that keel fractures in birds had a negative effect on

the laying performance and egg quality [2,3]. Thus, mineral reservoirs, such as Ca in the bones, are important for maintaining healthy bones and the best eggshell quality.

In the poultry industry, vitamin D_3 (VD_3) has long been the most commonly used supplemental form of vitamin D in diets. Since 2006, 25-hydroxyvitamin D_3 (25-OHD), a metabolite of VD_3, has been allowed as an additional form of dietary vitamin D in the poultry industry [4,5]. Compared with VD_3, 25-OHD had a higher absorption efficiency and stronger affinity with vitamin D binding protein [6]. Due to functional defect and weakening of the liver and kidney in young pullets and old laying hens [7,8], the use of 25-OHD in the laying hen diet can be a good choice.

Vitamin D can maintain the homeostasis of blood calcium by regulating intestine calcium absorption, kidney calcium reabsorption, and bone mineralization and mobilization [9,10]. Vitamin D is essential for maintaining the laying performance, egg quality, and bone quality of laying hens [11]. Studies have shown that partial and complete replacement of VD_3 with 25-OHD in diets improved the eggshell quality [12–14] and the yolk color [15], and reduced dirty and broken egg rates [16] in breeder hens or laying hens. Otherwise, a previous study found that adding 25-OHD to a basal diet containing VD_3 increased the thickness of eggshells [17]. However, a lower daily feed intake and egg weight were reported, when VD_3 was completely substituted for 25OHD in laying hen diets [18]. Numerous studies have shown that the dietary addition of 25-OHD did not significantly improve the eggshell quality and egg production performance [5,19–21]. For bone health, the positive effects of 25-OHD on bone quality in laying hens were not observed in most of studies [5,6,12,19,20]. A study also showed that 25-OHD had no effect on the prevalence of keel deformity [22]. However, A recent study showed that dietary 25-OHD significantly increased the content of medullary ashes and decreased the concentration of cortical ashes in the femur and tibia [23]. There is a big difference between these findings. This may be related to the age of the laying hens, duration of the experiment, and the total vitamin D activity in the diet. Taken together, most of studies do not cover the pullet period and a complete cycle in laying hens. A recent study found that early and long-term supplementation of 25-OHD significantly improved the laying performance and egg quality during early laying period, when VD_3 was substituted for 25-OHD at 50%, in the birds' diets, at 5520 IU/kg of feed of the total vitamin D activity [8]. However, so far, there has been no report on the effect of 25-OHD on the performance, the quality of the egg, and bone traits of hens, when 25-OHD completely replaces dietary VD_3 for a complete cycle in laying hens.

Hence, the purpose of the this experiment was to study the effects of 25-OHD as a VD_3 substitute on hens' growth and bone development, laying performance, egg quality, and bone traits from 1 day to 72 weeks of age.

2. Material and Methods

2.1. Birds, Housing, and Treatments

All processes were permitted by the Animal Care and Use Committee of the Sichuan Agricultural University (SAUPN-19-02).

Initially, on the basis of the principle of no difference in body weight, a total of nine hundred one-day-old Roman Pink laying hens (three treatments $\times$ 15 replicates $\times$ 20 birds per replicate) were randomly distributed into three dietary treatments: VD_3 2800 IU/kg, 25-OHD 69 μg/kg and 25-OHD 125 μg/kg. Starting from 21 w, five replicate birds from each treatment were selected continued to feed on the same vitamin D diet as during the rearing period (1–20 w) and up to 72 w.

The experiment was conducted in a closed poultry house, and the laying hens were raised in cages with dimensions measuring 400 mm in length, 450 mm in width, and 450 mm in height. The chicken cage is 192 cm wide, 62.5 cm deep and 57 cm high. There was no restriction on chicken drinking and feeding throughout the experiment. Seven basic diets were assigned to seven stages (0–4, 5–8, 9–17, 18–19, 20–45, 46–65, 66–72 w) from 1 day to 72 weeks of age. the basic diet composition and nutrient levels at each stage are shown

in Table 1. The environmental conditions were controlled according to 'Roman Commercial Layer Management Guide (2018)'. The ambient temperature of the pullets at 1 day of age was controlled at 35–36 °C. Then the temperature gradually decreased to 20 °C at 25 days of age. The pullets were given 24 h of light at 1 day of age, then the light time gradually decreased, and the illumination time decreased to 8 h at 8 w. When hens were 18 weeks of age, light was supplemented to stimulate laying eggs until 14 h. At 24 w, light was then kept the same until the end of the experiment.

Table 1. Composition and nutrient level of the basal diet.

Ingredient (%)	0–4 w	5–8 w	9–17 w	18–19 w	20–45 w	46–65 w	66–72 w
Corn	59.92	63.98	68.34	62.42	57.00	58.02	60.58
Soybean meal	34.90	31.19	17.80	29.06	28.36	26.65	24.26
Wheat bran			9.22				
Soybean oil	0.44	0.52	0.75	1.50	3.24	3.24	2.93
DL-methionine	0.21	0.11	0.17	0.18	0.29	0.26	0.23
L-lysine HCL	0.17	0.03					
L-tryptophan			0.01	0.02			
L-threonine	0.04						
NaCl	0.18	0.17	0.16	0.16	0.16	0.16	0.16
Choline chloride, 60%	0.05	0.05	0.05	0.07	0.07	0.07	0.07
$NaHCO_3$					0.25	0.25	0.25
Calcium carbonate	1.27	1.26	1.36	3.90	8.67	9.42	9.75
Calcium hydrophosphate	2.08	1.95	1.40	1.95	1.57	1.54	1.38
Mineral premix [1]	0.5	0.5	0.5	0.5	0.15	0.15	0.15
Vitamin premix [2]	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Antioxidant (ethoxyquin)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Total	100	100	100	100	100	100	100
Calculated nutrient content, %							
Metabolizable Energy (kcal/kg)	2753	2789	2783	2775	2738	2729	2725
Crude protein	20.00	18.50	14.50	17.50	16.82	16.15	15.30
Calcium	1.05	1.00	0.90	2.00	3.73	4.00	4.09
Non-phytate P	0.48	0.45	0.37	0.45	0.38	0.37	0.35
Lysine	1.20	1.00	0.68	0.92	0.88	0.84	0.78
Methionine	0.51	0.40	0.39	0.45	0.55	0.51	0.47
Tryptophan	0.23	0.21	0.17	0.22	0.19	0.18	0.17
Threonine	0.80	0.71	0.54	0.67	0.65	0.62	0.59

[1] Provided per kilogram of diet (0–72 w): Cu ($CuSO_4 \cdot 5H_2O$) 5 mg, Fe ($FeSO_4 \cdot H_2O$) 25 mg, Mn ($MnSO_4 \cdot H_2O$) 100 mg, Zn ($ZnSO_4 \cdot H_2O$) 60 mg, I (KI) 0.5 mg, Se (Na_2SeO_3) 0.2 mg. [2] Provided per kilogram of diet (0–8, 9–17, 18–72 w): 10,000, 10,000, 10,000 IU vitamin A; 30, 30, 30 mg vitamin E; 3, 3, 3 mg vitamin K_3; 1, 1, 1 mg vitamin B_1; 6, 6, 4 mg vitamin B_2; 3, 3, 3 mg vitamin B_6; 20, 20, 25 μg vitamin B_{12}; 8, 8, 10 mg D-pantothenate; 30, 30, 30 mg niacin acid; 1, 1, 0.5 mg folic acid; 50, 50, 50 μg biotin. The three levels of vitamin D VD_3 2800 IU/kg, 25-OHD 69 μg/kg and 25-OHD 125 μg/kg were added to the seven diets used, respectively.

2.2. Data Collection and Sampling

Feed intake at each stage (1–8, 9–19, 1–19 w) and hen body weight at each time point (1 d, 8 w and 19 w) were recorded, and average daily feed intake and body weight gain were calculated. Body weight uniformity (BWU) of each replicate at 8 w and 19 w was calculated. BWU (%) = number of hens within the range of 10% add and subtract the birds average weight/ the number of all hens in the duplicate. At 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 16, and 19 w, the shank length of the hens (select 10 birds from each replicate) was measured and recorded, and the measurement of the shank length was measured with a vernier caliper. Starting from 21 w, egg production performance data were recorded daily. Then, the laying rate, average egg weight, feed conversion, feed intake, qualified egg number, total egg number, and total egg weight at the laying stage were calculated. At 10, 20, and 72 w, the birds were euthanized, and the full keel bones and left and right tibias and femurs were collected, and the keel bones were stored at 4 °C until measured, The femurs and tibias are stored at −20 °C. Chickens sampled at weeks 10 and 20 were selected in the first

five replicates of each treatment. Then, after 20 w, the middle five replicates were uniformly selected to continue the experiment.

2.3. Egg Quality

At 50, 60, and 70 w, three eggs were selected from each replicate to determine the egg quality. Egg quality parameters included the Haugh unit, albumen height, eggshell thickness, eggshell breaking strength, and proportion of eggshell. Haugh unit and albumen height were determined by a multifunctional egg analyzer (EMT-7, 300, Robotmation Co., Ltd., Tokyo, Japan). Eggshell strength was tested using an intensity measuring device (Robotmation Co., Ltd.). Using a vernier caliper to measure the big end, middle end and small end of the eggshell.

2.4. Bone Development and Bone Strength

At 10 and 20 w, after stripping off the left and right tibia and femur, the bone length and diameter (the middle of the bone) of tibias and femurs were tested with a vernier caliper, and bone plumpness was calculated. Bone plumpness (%) = bone diameter × 3.14/bone length × 100%. Then, bone strength was measured by the texture analyzer (TAXTPlus, Stable MicroSystems corp., Godalming, England). At 72 w, after stripping out the intact tibias and femurs, the bone strength was measured.

At 10 and 20 w, after separating the intact keel bones, the parameters (keel depth, keel length, and keel calcified rate) related to keel development and calcification were measured. The sites for the determination of the keel development and calcification parameters refer to the description of my previous article [24].

2.5. Statistical Analysis

All data were analyzed statistically by one-way ANOVA, using the general linear model procedure of SAS (version 9.1) with dietary treatment as the main effect. Duncan's method was used for multiple comparisons. When p was less than 0.05, the difference was significant. Otherwise, a quadratic regression was used to predict the bird shank length for each treatment using SPSS software (version 19) with the following model:

$$Y = AX2 + BX + C$$

where Y is the shank length, X is weeks of age, and C is the intercept of the equation, B is the linear term coefficient, and A is the quadratic term coefficient. The coefficient of determination (R^2) and p-value regression were used to define the equation with the best fit. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Growth Performance and Shank Length

The 25-OHD 125 μg/kg treatment group had the lowest BW (8 w), BWG (1–8 w), and ADFI (1–8 w and 1–19 w) among all treatments ($p < 0.05$; Table 2). No significant difference was found in BW (1 d), BWG (9–19 w), ADFI (9–19 w), BW (19 w), BWG (1–19 w), and BWU (8 w and 19 w).

The 25-OHD 125 μg/kg treatment group had the lowest shank length among all treatments at 4 w ($p = 0.027$; Figure 1). The 25-OHD 69 μg/kg treatment group showed a higher shank length, compared with the 25-OHD 125 μg/kg treatment group at 7 w ($p = 0.032$). However, no significant difference in the shank length was observed at 1, 2, 3, 5, 6, 8, 10, 13, 16, and 19 w ($p > 0.05$). By a regression analysis, we found the shank length of the birds in each treatment group reached the maximum (about 103 mm) at about 18 w (Table 3).

Table 2. Effect of 25-OHD as a VD3 substitute on hens' growth performance [1].

Items	VD_3 2800 IU/kg	25-OHD 69 μg/kg	25-OHD 125 μg/kg	SEM	*p*-Value
BW, g					
1 d	41.7	41.7	41.7	0.1	0.994
8 w	666.4 [a]	669.2 [a]	644.4 [b]	5.2	0.003
19 w	1624.5	1633.8	1611.2	10.6	0.326
BWG, g					
1–8 w	624.7 [a]	627.5 [a]	602.8 [b]	5.2	0.003
9–19 w	958.2	964.5	966.8	11	0.851
1–19 w	1582.9	1592	1569.5	10.6	0.326
BWU, %					
8 w	76.5	77.7	68.8	3.0	0.083
19 w	67.4	71.8	69.6	3.0	0.578
ADFI, g					
1–8 w	30.5 [a]	30.4 [a]	29.7 [b]	0.2	0.001
9–19 w	72.6	72.3	71.9	0.4	0.504
1–19 w	54.6 [a]	54.4 [a]	53.9 [b]	0.2	0.014

[a,b] Means with different superscripts within a row differ significantly ($p < 0.05$). Each mean repre–ents values from 15 replicates. [1] Abbreviations: BW, body weight; BWG, body weight gain; BWU, body weight uniformity; SEM, standard error of the mean.

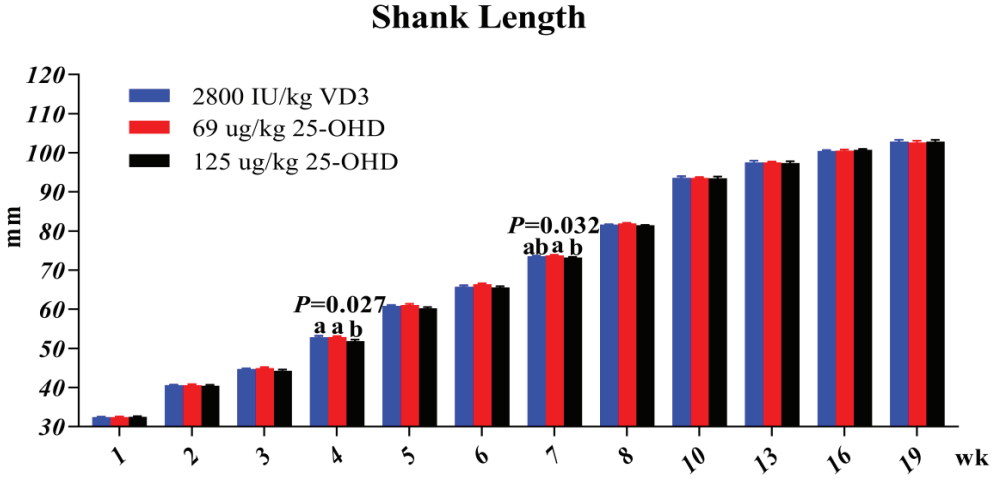

Figure 1. Effect of 25-OHD as a VD3 substitute on hens' shank length; SEM = standard error of the mean; each mean represents values from 15 replicates. a,b means significant difference ($p < 0.05$).

Table 3. Regression equations for the prediction of the shank length from the week age of laying hens [1].

Treatment	Prediction Equations	R^2	*p*-Value	Y (Max)	X
VD_3 2800 IU/kg	$Y = -0.278\ X^2 + 9.554\ X + 20.983$	0.989	<0.001	103.1	17.2
25-OHD 69 μg/kg	$Y = -0.281\ X^2 + 9.599\ X + 21.043$	0.991	<0.001	103.2	17.1
25-OHD 125 μg/kg	$Y = -0.275\ X^2 + 9.529\ X + 20.715$	0.988	<0.001	103.3	17.3

[1] R^2, the coefficient of determination; Y, shank length; X, week age of laying hens; max, maximum.

3.2. Bone Development and Bone Quality

Bone development results are presented in Table 4. The 25-OHD 125 μg/kg treatment group had the lowest femur bone diameter ($p < 0.001$; 20 w) and femur bone plumpness ($p = 0.002$; 20 w) among all treatments. The 25-OHD 125 μg/kg treatment group had a

lower keel length (p = 0.046; 10 w), compared with the 25-OHD 69 μg/kg treatment group. There were no significant differences in the tibia length, tibia diameter, tibia plumpness, femur length, and keel depth at 10 and 20 w among treatments ($p > 0.05$).

Table 4. Effect of 25-OHD as a VD3 substitute on bone development at 10 and 20 weeks.

Items	VD_3 2800 IU/kg	25-OHD 69 μg/kg	25-OHD 125 μg/kg	SEM	*p*-Value
10 w					
Tibia length, mm	98.9	101.5	97.2	1.3	0.112
Tibia diameter, mm	6.1	6.2	5.7	0.2	0.167
Tibia plumpness, %	19.2	19.2	18.5	0.5	0.587
Femur length, mm	69.7	70.1	69.8	0.8	0.945
Femur diameter, mm	6.7	6.6	6.6	0.1	0.701
Femur plumpness, %	30.3	29.6	29.8	0.5	0.681
Keel length, mm	76.0 ^{ab}	79.4 ^{a}	72.7 ^{b}	1.7	0.046
Keel depth, mm	30.1	30.3	30.2	0.8	0.982
20 w					
Tibia length, mm	116.9	116	116.1	0.8	0.684
Tibia diameter, mm	7.2	7.3	7.2	0.1	0.231
Tibia plumpness, %	19.3	19.9	19.4	0.2	0.136
Femur length, mm	80.9	77.8	78.8	1.1	0.159
Femur diameter, mm	7.7 ^{b}	7.9 ^{b}	7.0 ^{a}	0.1	<0.001
Femur plumpness, %	30.0 ^{b}	31.9 ^{c}	28.1 ^{a}	0.5	0.002
Keel length, mm	104.6	105.4	102.9	1.4	0.470
Keel depth, mm	39.5	40.6	39.5	0.7	0.379

$^{a–c}$ Means with different superscripts within a row are significantly different ($p < 0.05$). Each mean represents values from five replicates.

As shown in Table 5, the 25-OHD 125 μg/kg treatment group had a lower tibia strength at 10 w. compared with the 25-OHD 69 μg/kg treatment group. ($p > 0.05$), but both 25-OHD 69 and 125 μg/kg treatment groups had a higher femur strength at 72 w, compared with the VD_3 2800 IU/kg treatment group. No significant difference in the keel calcified rate was observed at 10 and 20 w ($p > 0.05$).

Table 5. Effect of long-term supplementation of 25-OHD as a vitamin D3 substitute on bone strength and keel calcification.

Item	VD_3 2800 IU/kg	25-OHD 69 μg/kg	25-OHD 125 μg/kg	SEM	*p*-Value
10 w					
Tibia strength, kgf	15.1 ^{ab}	17.4 ^{b}	12.8 ^{a}	0.9	0.023
Femur strength, kgf	21.0	21.9	21.7	1.3	0.872
Keel calcified rate, %	38.6	39.0	39.5	1.7	0.932
20 w					
Tibia strength, kgf	17.0	17.2	19.4	1.5	0.488
Femur strength, kgf	25.1	27.0	23.8	2.6	0.692
Keel calcified rate, %	85.3	86.6	86.9	1.7	0.789
72 w					
Tibia strength, kgf	16.0	18.3	18.2	1.1	0.262
Femur strength, kgf	23.9 ^{a}	34.0 ^{b}	31.5 ^{b}	1.7	0.006

^{a,b} Means with different superscripts within a row are significantly different ($p < 0.05$). Each mean represents values from five replicates.

3.3. Laying Performance and Egg Quality

As can be seen from Figure 2, the hen-day laying rate (HDLR) in each treatment reached peak production (more than 90%) at 23 w. Prior to 50 w, the HDLR was greater than 90% in each treatment group, then the HDLR gradually declined. At 72 w, the egg production rate of each treatment group was about 85%. As shown in Table 6, no significant difference was also found in the laying performance among all treatments except for the

feed intake. The ADFI in the 25-OHD 125 μg/kg treatment group was significantly lower than that in the 25-OHD 69 μg/kg treatment group (p = 0.030).

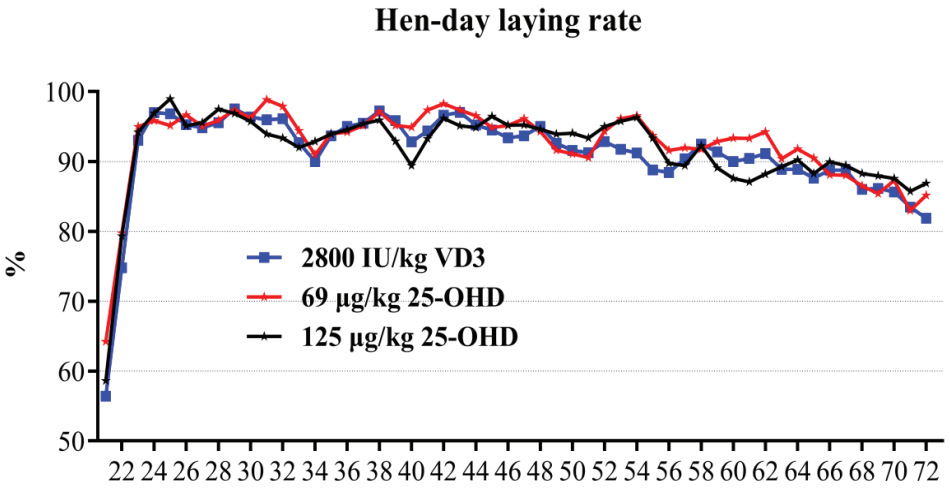

Figure 2. Effects of 25-OHD as a VD_3 substitute on the laying rate of the hens. Each mean represents values from five replicates.

Table 6. Effect of long-term supplementation of 25-OHD as a VD_3 substitute on the laying performance and mortality rate during the production stage [1].

Item	VD_3 2800 IU/kg	25-OHD 69 μg/kg	25-OHD 125 μg/kg	SEM	*p*-Value
Mortality rate, %	9.0	9.3	14.4	3.5	0.484
HDLR, %	91.6	93.3	92.3	0.5	0.147
HHLR, %	89.2	88.0	87.3	1.8	0.790
AEW, g	62.9	62.4	62.5	0.4	0.597
ENHD, No	330	336	332	2	0.184
ENHH, No	321	317	314	7	0.783
HDEW, kg	20.8	21.0	20.8	0.2	0.689
HHEW, kg	20.2	19.8	19.7	0.4	0.684
ADFI, g	115.5 [ab]	116.1 [a]	114.3 [b]	0.1	0.030
FCR	2.00	2.00	1.98	0.01	0.485

[a,b] Means with different superscripts within a row are significantly different (p < 0.05; n = 5). The number of hens-housed was calculated as the actual number of birds at 21 w. [1] Abbreviations: 25-OHD, 25-hydroxycholecalciferol; HDLR, hen-day laying rate; HHLR, hen-housed laying rate; AEW, The average egg weight; ENHD, egg number per hen-day; ENHH, egg number per hen-housed; HDEW, hen-day total egg weight; HHEW, hen-housed total egg weight; ADFI, average daily feed intake; FCR, feed conversion ratio.

As shown in Table 7, at 60 w, the 25-OHD 125 μg/kg treatment group had a lower eggshell thickness (p = 0.012) and proportion of eggshell (p = 0.022), compared with the 69 μg/kg 25-OHD treatment group. No significant differences in egg quality parameters were observed at 50 and 70 w.

Table 7. Effect of the long-term supplementation of 25-OHD as a VD3 substitute on egg quality at 50, 60, and 70 w.

Items	VD_3 2800 IU/kg	25-OHD 69 μg/kg	25-OHD 125 μg/kg	SEM	*p*-Value
50 w					
Eggshell strength, kg/cm^2	4.62	4.24	4.4	0.15	0.233
Eggshell thickness, mm	0.417	0.399	0.397	0.008	0.234
Proportion of eggshell, %	11.3	11	11	0.2	0.616
Albumen height, mm	7.2	6.8	6.6	0.3	0.321
Haugh unit	82	79.8	78.9	2	0.533
60 w					
Eggshell strength, kg/cm^2	4.38	4.5	3.99	0.17	0.113
Eggshell thickness, mm	0.386 ^{ab}	0.397 ^{a}	0.373 ^{b}	0.005	0.012
Proportion of eggshell, %	11.2 ^{ab}	11.5 ^{a}	10.9 ^{b}	0.1	0.022
Albumen height, mm	6.6	6.7	6.4	0.3	0.832
Haugh unit	78.7	79.3	77	2.3	0.763
70 w					
Eggshell strength, kg/cm^2	3.99	4.11	4.17	0.14	0.653
Eggshell thickness, mm	0.389	0.381	0.378	0.008	0.607
Proportion of eggshell, %	10.9	10.8	10.8	0.2	0.880
Albumen height, mm	6.7	6	6.4	0.3	0.300
Haugh unit	79.3	74	77.2	2.1	0.232

^{a,b} Means with different superscripts within a row are significantly different ($p < 0.05$; $n = 5$).

4. Discussion

In this experiment, the body weight of the birds in all treatments reached the standard of the 'Roman Commercial Layer Management Guide (2018)' at 8 and 19 w. However, in the starter period (1–8 w), the BWG and ADFI in the 25-OHD 125 μg/kg treatment group was the lowest among all treatments. Previous studies showed that adding 25-OHD to a VD_3-containing basal diet promoted bird growth and development up to 3 weeks of age, increasing the feed intake and body weight gain and reduced the FCR [6]. Otherwise, when laying hens were fed three vitamin D diets (VD_3 2760 IU/kg, VD_3 5520 IU/kg, and VD_3 2760 IU/kg + 25OHD 69 μg/kg) for a long period of time (0–95 w), there was no significant difference in the growth performance during the rearing period [8]. These results are inconsistent with ours, which may be caused by inconsistent experimental design. In our experiment, 25-OHD was used to completely replace VD_3 in the diet. In actual production, we hardly found VD_3 poisoning in laying hens. Earlier studies showed that dietary VD_3 15,000 IU/kg had no negative effect on the laying performance of Roman LSL White laying hens aged 20 to 68 weeks [25]. One study also showed that no poisoning was observed, when Hy-Line W36 laying hens (19–58 w) were fed VD_3 102,200 IU/kg [26]. However, a more recent study found that long-term (0–68 w) supplementation of VD_3 68,348 IU/kg had adverse impacts on the growth performance and egg production [27]. So far, there has been no report on the toxic dose of 25-OHD in laying hens. It has been reported that kidney production of 24,25 dihydroxycholecalciferol is 7–9 times higher than that of $1,25(OH)_2D_3$ in growing pullets [28], however, at the onset of laying, compared with 25-OHD 24-hydroxylase, the 25-OHD 1α-hydroxylase activity on the birds' kidneys, plasma levels of $1,25(OH)_2D_3$, and contents of intestinal $1,25(OH)_2D_3$ were improved significantly [29,30]. This suggested that immature pullets may have lower vitamin D requirements, compared with sexually mature laying hens. In China, the limit level for VD_3 used in poultry is 5000 IU/kg. In this experiment, compared with the VD_3 2800 IU/kg treatment, the growth performance of pullets in the 25-OHD 125 μg/kg treatment group was not improved. The optimal dose of 25-OHD for pullets at the rearing stage needs further research.

The bird's shank length is closely related to the skeletal development and frame size [31,32]. Previous studies have shown that hens fed a diet supplemented with 25-OHD have a longer shank length at 18 w, compared with the VD_3 3000 IU/kg treatment [6].

However, in our research, in addition to the growth performance, we also found that the shank length at 4 w in the 25-OHD 125 μg/kg group were the lowest among all treatments, which was associated with a lower feed intake at 1–8 w.

Similar to our results, a previous study found that supplementing with 25-OHD in the early (0–17 w) and long-term (0–90 w), had no effects on the cumulative egg production and egg weight of Hy-Line Brown hens from 18 to 87 w [6]. A recent report showed that early-term and the long addition of 25-OHD had no effect on egg production throughout the period (22–95 w) [8]. Furthermore, In addition, some studies on laying hens and broiler breeders also showed that the addition of 25-OHD to the diet had no beneficial effect on the laying performance [4,5,12,19,20,33]. In these reports, supplementation of 25-OHD was only in the laying stage. In our experiment, we found that the long-term (1–72 w) supplementation of 25-OHD as a VD_3 substitute did not affect the laying performance (HDLR, HHLR, AEW, ENHD, ENHH, HDEW, HHEW) of laying hens. This may be attributed to the fact that the total activity of vitamin D and the level of calcium in all treatment diets meet the requirements of laying hens, the addition of VD_3 and 25-OHD on top of this do not further improve production performance. One study also showed that the laying performance was significantly reduced when birds were fed a VD_3-free diet, however, there was no significant difference in egg production at VD_3 supplemental levels of 500, 1500, and 3000 IU/kg [34].

In this study, long-term supplementation of 25-OHD as a vitamin D_3 substitute had no beneficial effects on the eggshell quality at 50, 60, and 70 w. The 25-OHD 125 μg/kg treatment had a lower eggshell thickness and proportion of eggshell, compared with the 69 μg/kg 25-OHD treatment group. There has always been controversy about the effect of 25-OHD on eggshell quality. Some studies have shown that 25-OHD had no effect on eggshell quality [4,8,18–20]. However, other studies showed that dietary supplementation of 25-OHD or replacing VD_3 with 25-OHD improved the shell quality [12,17]. Silva (2017) also concluded that dietary supplementation of 25-OHD during the rearing and early laying period improved the eggshell thickness [6]. A recent study also found that the dietary supplementation of high levels of VD_3 or 25-OHD (125 μg/kg) improved the laying performance and eggshell quality of laying hens, compared to a control group (62.5 μg/kg VD_3) [35]. The mechanism of vitamin D in eggshell formation has not been well established. The $1,25(OH)_2D_3$, as an active metabolite of vitamin D, that may regulate the expression of Ca^2 + transport-related proteins, such as calcium-binding protein d28k and carbonic anhydrase in the eggshell gland [36]. The production of $1,25(OH)_2D_3$ in the kidneys is related to blood calcium levels. The decrease of blood calcium leads to the increase of parathyroid hormone secretion, which increases the production of $1,25(OH)_2D_3$ in the kidneys [1]. Therefore, the effect of vitamin D on the eggshell quality is inextricably related to dietary calcium levels. In our experiment, dietary calcium levels were adequate for the birds, this may be one of the reasons why 25-OHD as a VD_3 substitute did not further enhance the eggshell quality.

The bone quality of birds not only affects the laying performance and egg quality, but is also related to the welfare of laying hens [37,38]. The modern layers have a high incidence of osteoporosis at the later laying period [39,40]. Bone growth and development are concentrated in the early stages, so it is necessary to improve bone quality as much as possible during this stage. Studies on the effects of 25-OHD on bone development in pullets are limited. In our experiment, supplementation of 25-OHD as a VD_3 substitute had no beneficial effect on the bone development and bone strength (tibia and femur) at 10 and 20 w. The 25-OHD 125 IU/kg treatment was the worst among all treatments at 10 and 20 w, which may be associated with a lower feed intake and body weight. However, my previous research found increasing dietary VD_3 (2800 vs. 300 IU/kg) or the addition of 25-OHD 56 IU/kg during the pullet period improved the tibia quality during the early and later stages [24]. The difference in the results between the two studies was related to the level of VD3 in the control diets in the experiment. On the basis of my previous study, this study further demonstrated that VD_3 2800 IU/kg in hens' diet in the rearing period was sufficient

for the maintenance of bone quality. The results of this study were also inconsistent with the findings in broilers, one study showed that supplementing with 25-OHD via water, reduced the incidence of lameness [41]. One study found that supplementation of 25-OHD increased the bone mineralization and improved bone strength in broilers [42]. For laying hens in the laying stage, most of studies found that 25-OHD did not improve the bone quality [5,12,19,20]. In this study, the long-term addition of 25-OHD significantly improved the strength of the femur at 72 w. This indicates that the beneficial effect of 25-OHD on the bone quality in the late laying period is not caused by early supplementation, but due to continuous supply during the laying period. A recent study also found that the long-term supplementation with 25-OHD stimulated bone growth and had positive effects on laying hen's bone quality [43].

In the laying hen industry, keel bone damage has become a major concern. Keel bone damage has negative impacts on production in laying hens [2]. Studying the regularity of keel bone development and calcification may provide new ideas for reducing keel damage. In this study, supplementation of 25-OHD as a VD_3 substitute did not affect the development and calcification of the keel. There is no report on keel development and calcification in pullets. A recent study reported that dietary supplementation with 25-OHD significantly increased the sternal mineral accumulation in meat ducks [44]. A previous study found that the dietary addition of 25-OHD had no effect on the incidence of keel deformity [22]. Whether 25-OHD can be used to improve keel quality is worth further study.

5. Conclusions

The study confirmed that the dietary supplementation of VD_3 2800 IU/kg at the early stage (1–20 w) was sufficient to maintain the bone quality and growth of the pullet. The long-term supplementation of 25-OHD as a VD_3 (125 μg/kg) substitute improved the bone quality in the late laying period.

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Review

An Appropriate Genetic Approach to Endangered Podolian Grey Cattle in the Context of Preserving Biodiversity and Sustainable Conservation of Genetic Resources

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Abstract: In the context of the general phenomenon of diminishing genetic diversity, especially in cattle, the conservation of endangered species plays a primary role. The disappearance of some animal populations can irreparably affect the biodiversity of genetic resources. Among the most ancient European cattle are breeds that belong to the Podolian group, the history of which is still not well established. The common origin of these breeds is the wild ox (*Bos taurus primigenius*), which has been declared extinct since the 17th century. The purpose of this paper is to highlight and compare the latest studies on the origin, evolution, genetic diversity, and phylogenetic relationships of Podolian cattle, with special emphasis on the endangered Romanian Grey Steppe. The importance of studying these cattle derives from the special biological properties by which they have distinguished themselves over time (adaptability and resistance to diseases, severe climate and habitat conditions, hardiness, and longevity). The bibliographic references reviewed in this study confirm that these breeds are carriers of valuable genes that must be preserved for improvement of other cattle and protection of biodiversity. The information presented represents a valuable tool for efforts to conserve endangered cattle.

Keywords: biodiversity; *Bos taurus*; cattle production; genetic diversity; grey cattle; phylogeny; Podolian cattle

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1. Introduction

Throughout history, humans have been mainly focused on raising cattle. Initially, the emphasis was on meat and using them in the agricultural sector, transport, and construction. As time progressed, there was a shift towards prioritizing milk production. Cattle hold significant importance as a livestock species, playing a crucial role in shaping human history, culture, and civilization. The evolutionary history and genetic diversity of cattle are controversial and essential topics in this field.

Bos primigenius primigenius and *Bos primigenius namadicus*, two major wild aurochs subspecies that are common in Africa and Eurasia throughout the Middle Pleistocene, are the origin of the convoluted evolutionary history of cattle. Their domestication led to the current *Bos taurus taurus* (taurine) and *Bos taurus indicus* (zebuine) subspecies, which were separated approximately 250,000 years ago (YBP) [1–3]. Since domestication, which took place roughly 10,000 BC in the Fertile Crescent (*B. t. taurus*) and roughly 8000 BC in the Indus Valley (*B. t. indicus*) [1,4], cattle have followed colonization routes concurrently with Neolithic human expansion.

In terms of phylogenetic analysis and mitochondrial DNA evidence, the T haplogroup stands out as the predominant mitochondrial lineage in both contemporary and ancient

Neolithic European cattle. It is thought to have originated during the domestication event in the Fertile Crescent; nevertheless, other rare lineages are also scattered throughout Europe. Several studies have confirmed the presence of a P lineage, primarily identified in central and northern European aurochs. This lineage is also observed in certain contemporary taurine breeds and ancient domestic cattle. Additionally, the Q lineage is found in various European cattle and is recognized as part of the so-called Podolian trunk. An indicine gene pool component was observed in diverse breeds, such as the Turkish Grey and some Italian breeds that belong to the SO group (Chianina, Romagnola, Marchigiana, Romanian Grey Steppe, Maremmana, and Podolica Italiana). According to many studies, Podolian breeds are characterized by grey coats and upright and often long horns, with a common ancestral origin in Podolia (modern western Ukraine) [1,2,5–10]. The development of these breeds was significantly and favorably influenced by environmental conditions, which played a crucial role in shaping distinctive biological characteristics. These cattle experienced lower selective pressure, with limited use of artificial insemination and higher natural selection. Podolian breeds were typically raised in natural environments, where activities such as foraging for food, maternal care, and other natural behaviors were essential. This stands in contrast to other improved breeds that underwent different selection pressures and breeding practices. Regarding the origin and timing of the spread of the Podolian cattle, molecular evidence shows two alternative hypotheses: some authors suggest that Podolian cattle might have spread from the eastern steppe southward into Anatolia and westward into the Balkans and Italy in historical times (3rd–5th century AD) along with East European Barbarian people [1,2,9]; other authors suggest a more ancient migration (~3 kya BP) from the Near East to Central Italy through the Mediterranean Sea, together with a possible contribution from local wild aurochs through secondary local domestication/introgression events [1,2,7–10].

According to the Food and Agriculture Organization (F.A.O.), many Podolian breeds are seriously threatened by extinction in various European countries. Currently, this cattle group represents an important topic of discussion in scientific circles in European nations.

This study focused on the endangered Romanian Grey Steppe cattle, a valuable reservoir of genes with high importance for Romanian agriculture, belonging to the category of Podolian breeds, which have been threatened with extinction since 2000 according to the F.A.O. [11–13].

Archaeological and genetic evidence proves that the progenitor *Bos taurus primigenius (B. t. primigenius)*, which was declared extinct in the 17th century [7], is the ancestor of Podolian cattle and the wild ancestor of all the present-day breeds [14,15].

Owing to their valuable genetic pool for greater disease resistance and high adaptability to harsh environmental conditions, the Grey Steppe cattle are included in a genetic conservation program. Moreover, these indigenous cattle breeds form an integral part of our nation's history [16–20].

In the last decade, the population of the Grey Steppe breed has registered a significant numerical decrease [12]. Additionally, isolated individuals count to approximately 100 heads spread in the Danube Delta and the counties of Iasi, Neamt, and Pardina, northeastern Moldova [11,12,20]. A population of these cattle, which is part of a national genetic conservation effort, can currently be found at the Research and Development Station for Cattle Breeding in Dancu, Iasi, Romania.

Romanian Grey cows have a narrowed milk production, which is mainly used for calf feeding. They have a medium slaughter yield, and the bulls get to the slaughter weight at an older age compared to bulls of early maturing breeds (Angus, for instance). Their meat is darker and not particularly marbled as most of the suet forms subcutaneously, and their intestinal fat deposits have a lower arrangement of fat at the intramuscular level.

The majority of Grey Steppe varieties in Europe were not specifically bred for milk production. Instead, these were primarily raised for meat or as draft animals, leveraging their sturdy hooves, robust skeletal structure, and well-defined joints, which made them well-suited for tasks requiring strength and endurance. Cows raised in households typically

yield around 800–900 kg of milk per year, while those on farms produce higher quantities ranging from 1000 to 2500 kg. The milk from these cows generally has a fat content of 4–6%. In terms of slaughter yield, oxen exhibit a medium yield of approximately 50–51%, whereas cows have a slightly lower yield with a range of 47–49%. [12,16,20–23].

The preservation of local breeds is an important aspect of food security given the recent climatic changes as well as extreme phenomena that could bring new challenges in the future, with bovine species being the most exposed to these changes. Therefore, the Grey Steppe cattle could be an effective alternative to other improved breeds due to their special biological qualities. Owing to their valuable genetic resources, numerous studies in the specialized literature have demonstrated the importance of local cattle breeds in genetic conservation programs [1,24–27].

This paper reviews the most important research in the specialized literature regarding the origin, phylogeny, and genetic diversity of Podolian Grey Steppe in the context of its importance for preserving biodiversity and sustainable conservation of genetic resources.

2. The Importance of Cattle Biodiversity

Due to numerous advantages, including the rich nutritional content of raw animal products like milk and meat, cattle breeding plays a significant role in food and nutrition security. The conservation and sustainable utilization of cattle genetic resources are crucial due to the diverse benefits offered [28].

2.1. The Bovine Species: Origin, Evolution, and Domestication

Along with sheep and goats, according to the first archaeological evidence, cattle were domesticated about 10,000 BC (Figure 1A) [29].

The domestication of cattle represented a fundamental step in the development of humanity [30,31], leading to extensive changes in the diet, behavior, and socioeconomic structure of many populations. Genetically, the domestication process of animals can be reconstructed by employing phylogeographic analyses that rely on data from both the nuclear and mitochondrial genomes [32]. However, molecular studies in cattle have focused on the analysis of mitochondrial DNA (mtDNA) [33,34]. The wild ancestor of all domesticated cattle is *B. primigenius* [35], currently an extinct species, with the last recorded herd being found in 1627 A.D. in Poland [35–37]. It spread over much of Eurasia and North Africa during two major epochs, the Late Pleistocene and the Early Holocene [38], giving rise to two taxa (Figure 1C) that played a crucial role in the domestication of today's cattle.

The ancestors *B. taurus* and *B. indicus* are differentiated primarily by the presence of a hump (specific to *B. indicus*). The presence of a hump in *B. indicus* contributes to physiological adaptation and effectively dissipates heat; this adaptation is particularly important in warm tropical environments. The hump is primarily composed of a deposit of fatty tissue located on the back of the animal, just behind the neck and shoulders. This fatty tissue serves as an energy reserve, but its presence also plays a crucial role in the thermoregulation of body temperature [3,8,39].

The genus *B. taurus* encompasses the characteristic bulls found in Europe, Northeast Asia, and North Africa, specifically adapted to cold climates [40]. Various studies have consistently shown that modern European cattle breeds within the genus *B. taurus* fall under the mitochondrial haplotype T [9,41]. Regarding *B. indicus* (zebu), studies show India as the center of origin, later spreading to Africa and Southeast Asia in the period 2250–2000 BC, with zebu cattle later identified as being scattered all over India. As far back as the Harappan civilization in the Indus Valley, figures and paintings have been found depicting humped cattle resembling the ancestor *B. primigenius* (Figure 1B,C).

Allchin (2008, 2009) posits that the domestication of animals in India occurred through the efforts of two distinct groups. The first group consisted of hunters dispersed across the country during the Stone Age who engaged in the domestication of certain local animal species. The second group comprised nomadic shepherds originating from western

Asia [33,34]. The zebu bull, depicted on the Harappan seal, resembles the modern cattle of Gujarat, with a strong body and massive horns.

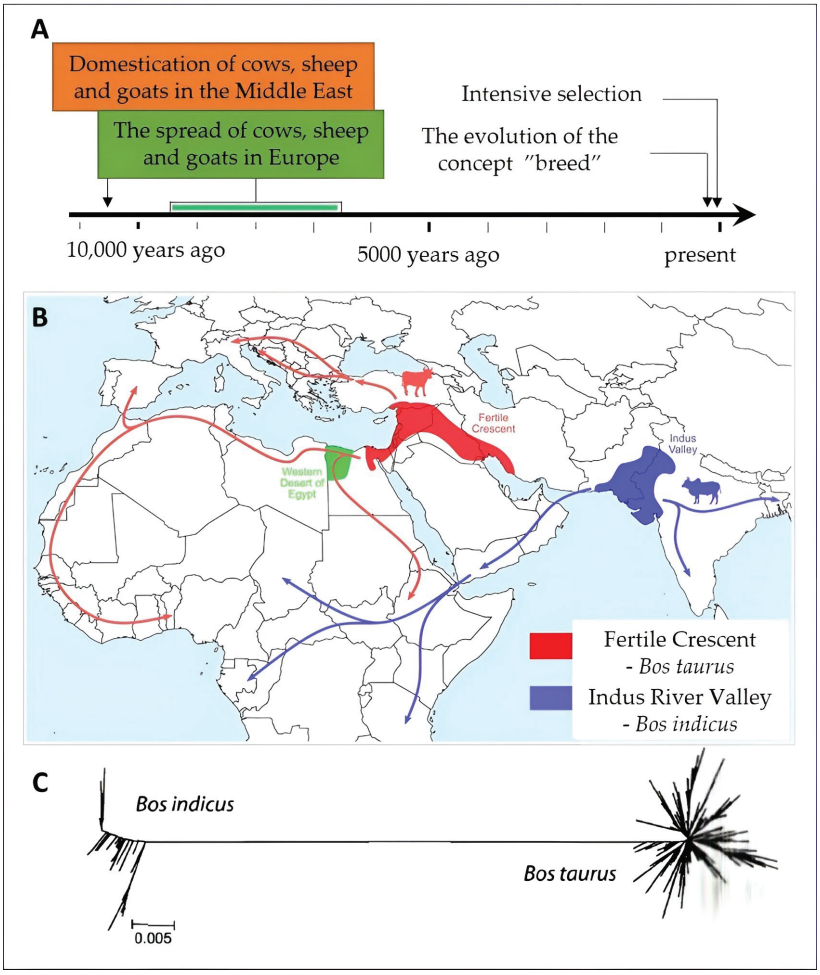

Figure 1. Archaeological data on the domestication of cattle, sheep, and goats. (**A**) Domestication centers. (**B**) Map of domestication centers for *B. indicus*—Indus River Valley and *B. taurus*—Fertile Crescent. (**C**) Unrooted neighbor-joining trees illustrating the mtDNA polymorphism of cattle, sheep, and goats. Graphic adapted: Copyright © 2008 S. Karger AG, Basel-Hiendleder et al. [8] and Copyright © 2011 Académie des sciences. Published by Elsevier SAS-Taberlet et al. [42].

Certain cave paintings found on the oldest Neolithic rocks portray zebu cattle characterized by a light build and prominent humps, showcasing distinct differences from northern cattle [34]. The spread of domesticated cattle across Europe coincided with the migration of first-generation farmers. As they settled in regions that harbored native European bulls, sporadic interbreeding between domesticated and native bulls occurred, which persisted in some regions until the Middle Ages.

Drawing on archaeological and genomic evidence, Taberlet et al., (2011) [43] showcased the existence of two mitochondrial haplogroups, suggesting two principal domestication events. The first event occurred in the Fertile Crescent during the Neolithic period, contributing to the domestication of *B. taurus.* The second event, transpiring 1500 years

later in the Indus Valley (present-day Pakistan) within the Indian subcontinent, led to the emergence of Indian cattle belonging to the *B. indicus* subspecies, commonly known as zebu [43]. Extensive hybridization has subsequently occurred in Africa [37,38,44].

Another study developed by Hiendleder et al., (2008) aimed to analyze complete mitochondrial genome sequences (16,338 bp for *B. taurus* and 16,339 bp for *B. indicus*), which indicated that the two bovine lineages separated 1.7–2.0 million years ago. Comparative phylogenetic analyses between 18 new sequences and 130 previously reported sequences in *B. taurus* and *B. indicus* with data from 32 specimens (*B. primigenius*) identified a maternal lineage across four genetic lineages. *B. primigenius* haplotypes were present in all but the *B. indicus* lineage, and one sequence from *B. taurus* clustered with *B. primigenius*, namely, the P haplotypes [8].

Achilli et al., (2009) conducted an extensive study on the origin of taurines based on mitochondrial genome analysis. By researching mtDNA control regions associated with taurines from numerous European breeds, a general grouping within haplogroups T1, T2, and T3, common to ancestors from the Near East, was confirmed, but eight mtDNA regions were also identified (1.3%) that did not fit into haplogroup T. The sequencing of the complete mitochondrial genome led to the hypothesis that four out of the eight mtDNA regions constituted a new haplogroup (haplogroup R). This haplogroup, following a bifurcation that resulted in two lineages, taurine and zebu, represented the oldest known split in the mitochondrial DNA phylogeny of the genus *B. primigenius*. The remaining four mtDNA regions formed the haplogroup Q, a discovery made more recently (Figure 2) [34].

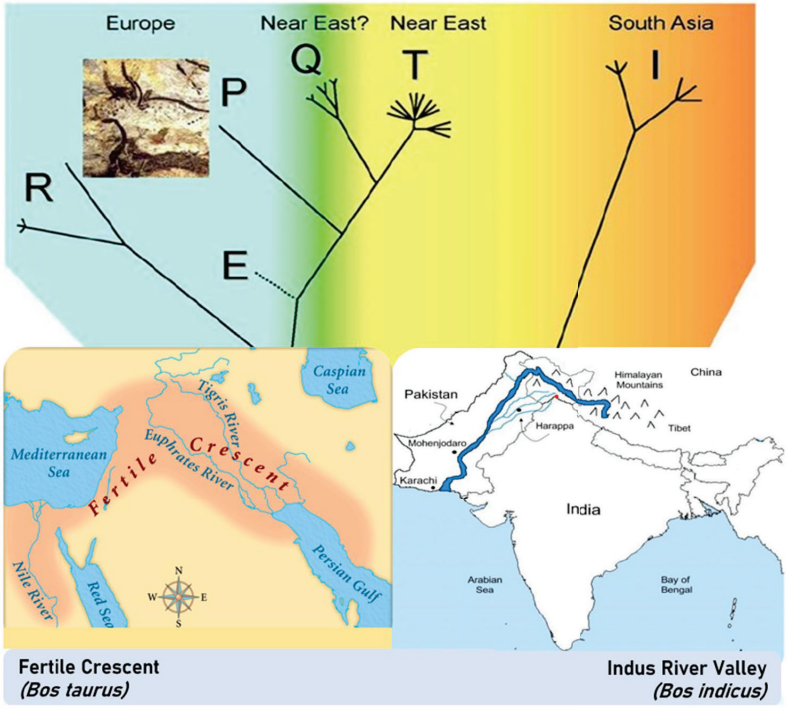

Figure 2. Phylogenetic analysis of cattle based on mtDNA haplogroups—R, E, P, Q, T, I. Graphic adapted: Copyright: © 2009 Achilli et al. [34].

The available data suggest that haplogroups Q and T were involved in the same Neolithic Near Eastern domestication event, while the existence of new (and rare) haplogroups points to genetic inheritance from distinct populations of *B. primigenius* [8,34,42].

Another analysis of the phylogenetic relationships between the aurochs and *B. taurus* (taurines) and *B. indicus* (zebu) was carried out by Sinding and Gilbert (2016) based on mitochondrial studies of seven lineages, C, I, P, Q, R, and T (Figure 3) [45]. This suggests that the domestication of the aurochs occurred through at least two distinct events: one leading to taurines *(B. taurus)* originating in the Near East, and a second event resulting in zebu *(B. indicus)* originating in South Asia [2,3].

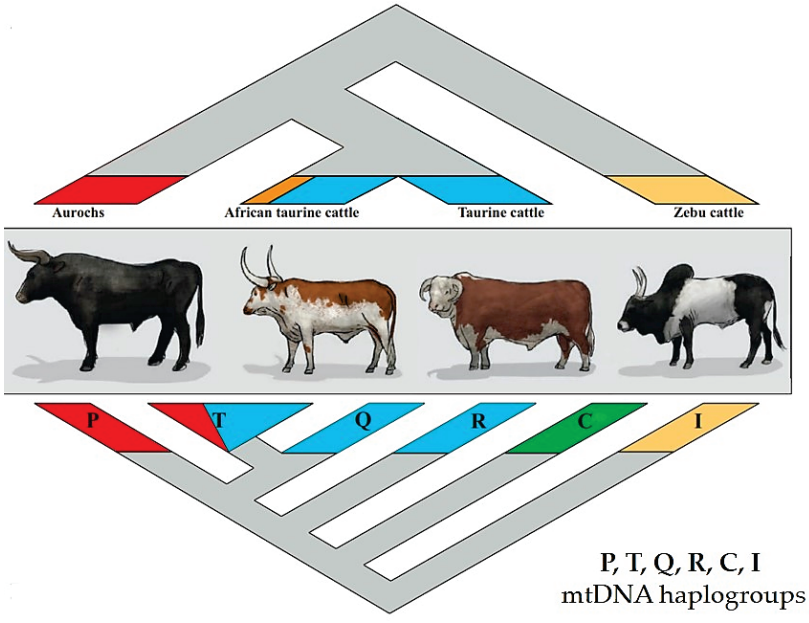

Figure 3. *Boviadae* family cluster based on phylogenetic analysis between *B. primigenius*, *B. taurus*, *B. indicus*, and *African taurine* (neighbor-joining algorithm). Graphic adapted: Copyright: © 2016 Sinding et al. [45].

By interpreting mitochondrial data in detail, three important aspects can be observed. First, most modern taurines belong to the T lineage, which is believed to have been derived from the first domesticated taurines in the Near East. Second, all zebu cattle carry lineage I. The distinct sister relationship of this clade with all other lineages was as expected given zebu's independent domestication. For this lineage, the highest genetic diversity was observed within the Indus Valley, which is one of the hypothesized domestication centers alongside South India [3,45]. Third, all remaining northern, central, and eastern European aurochs studied to date carried lineage P. The P lineage has also been identified in some modern taurine breeds and ancient European domestic cattle, suggesting the introgression of northern, central, and/or eastern European aurochs [34,45–47].

Approximately 200 years ago, with the emergence of the concept of breed, the situation changed dramatically because of the selection process [43,48], which led to the fragmentation of the initial genetic background. The enhancement of production performance in industrial breeds has coincided with a decline in the genetic resources of traditional native breeds, leading to the extinction of many cattle breeds. The genetic diversity of both *B. taurus* and *B. indicus* cattle has been diminished, primarily concentrating on breeds with high production potential. Most wild varieties of cattle have already disappeared or are in danger of extinction, as is the case for the Podolian cattle group that is the subject of this study.

2.2. Systematics and Phylogeny of Cattle

The *Bovidae* family presents a great variety, with more than 300 extinct or endangered species described and approximately 140 existing species [49], which is extremely difficult to achieve, representing one of the largest groups of mammals. According to taxonomic data, cattle are classified as follows: animal kingdom—*nimalia*; subkingdom—*Metazoa*; phylum—*Chordata*; subphylum—*Vertebrata*; class—*Mammalia*; subclass—*Eutheria*; order—*Artiodactyla/Paricopitatae*; family—*Bovidae*; subfamily—*Bovinae*; tribe—*Bovini* [49].

Due to its importance as a primary source of food (milk and meat), cows were the first mammal whose genome were completely sequenced [50,51]. Advances in genome sequencing analysis have simplified the molecular systematics of the *Bovidae* family.

The analysis of both the complete genome and mitochondrial regions, as represented by the cytochrome b gene (Cyt-b) and the mitochondrial control region (D-loop), has attracted considerable attention in the phylogenetic analysis of various taxa [52].

Despite ongoing research efforts, the phylogenetic relationships and taxonomy within the *Bovidae* family remain contentious. There have been numerous controversies over time concerning the taxonomy of the *Bovidae* family. According to some authors [52,53], it comprises two major subfamilies, *Bovinae* and *Antilopinae*, with origins in both Eurasia and Africa [54]. According to studies conducted by Feldhamer et al., (2007) and Gentry (2011), based on phylogenetic analyses, the *Bovidae* family consists of eight distinct subfamilies belonging to the *Artiodactyla* order (Figure 4A,B) [55,56]. The same hypothesis is also supported by Hernandez Fernandez M. and Vrba Elisabeth (2005), who, as part of research on the systematics of the *Bovidae* family, developed a phylogenetic tree that includes the eight subfamilies (Figure 4B) [57].

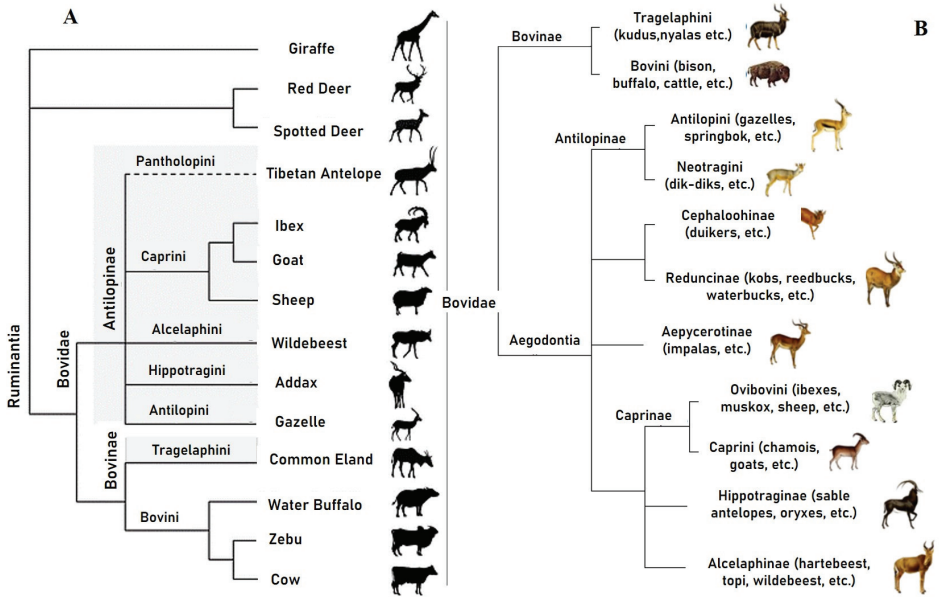

Figure 4. (**A**) Phylogenetic tree representing 11 species: two subfamilies and seven tribes belonging to the *Bovidae* family and two ruminant families used as out groups (giraffe and deer). (**B**) Systematics of the *Bovidae* family. Graphic adapted: Copyright: © 2015 Dekel et al. [51] and Hernandez-Fernandez et al. [57].

Dekel et al., (2015) analyzed the TP53 P1 promoter (a key tumor suppressor gene that is indispensable for diverse developmental processes) of three species of the subfamily *Bovinae:* one belonging to the tribe *Tragelaphini* (the spiral-horned eland—*Taurotragus oryx*) and two belonging to the tribe *Bovini* (water buffalo—*Bubalus bubalis* and zebu—*B. indicus*).

In the case of the zebu and buffalo species, similar to the *Bovinae* species, an additional long segment of 272 bp (base pair) was not found, whereas in the common antelope, this segment was identified with a length of 522 bp. Examination of the same promoter region in two families closely related to *Bovidae*, *Cervidae* (represented by the axis axis—Indian Spotted deer/chital) and *Girraffidae* (represented by the giraffe, *Giraffa camelopardalis*), did not reveal any insertion, similar to the *Bovinae* species (Figure 4A) [51].

Bovines, belonging to the subfamily *Bovinae*, form a diverse group encompassing 10 genera of medium- to large-sized ungulates. This group includes various species such as cattle, bison, African buffalo, water buffalo, and both four-horned and spiral-horned antelopes. The evolutionary relationships among members of this group remain a subject of debate, and their classification into loose tribes rather than formal subgroups reflects the ongoing uncertainty in this field [55,56].

Antilopinae belonging to the *Bovidae* family include antelopes, gazelles, and other related species. These are even-toed ungulates and occur in much of Africa and Asia, with the highest concentration of species occurring in East Africa in Sudan, Eritrea, Ethiopia, Somalia, Kenya, and Tanzania [58]. Another subfamily of the *Bovidae* family is *Cephalophinae*, which includes forest antelopes, which are widespread in the area of sub-Saharan Africa and are classified into three genera (*Cephalophus*, *Philantomba*, and *Sylvicapra*) and 18 species [59].

The *Bovidae* family is widely spread, including the subfamilies *Reduncinae* [59,60], composed of nine species of antelope, all of which dwell in marshes, floodplains, or other well-watered areas, including waterbucks and reedbucks; *Aepycerotinae*, an African antelope that contains a single living species, the Impala [59]; and *Alcelaphinae*, a subfamily with under 10 species, grouped in turn into four genera [56].

The subfamily *Caprinae*, also sometimes referred to as the tribe *Caprini*, is part of the ruminant family *Bovidae*, and consists mostly of medium-sized animals, including sheep (*Ovis*) and goats (*Capra*) and mountain-adapted mammals with either short and sharp horns or large and ornate horns. The geographical distribution is represented by Europe, America, Africa, and Asia [59].

The last subfamily, according to the above tree, is *Hippotraginae*, which is represented by large antelopes. This subfamily includes three genera, *Hippotragus*, *Oryx*, and *Addax*, and eight species. Their habitats are represented by areas in Africa and the Middle East [53,61–63].

3. The Origin and Phylogeny of the Podolian Cattle Breeds

Podolian cattle constitute a group of breeds distinguished by grey coats and upright and often long horns that are believed to have originated in the Podolian steppe. These are classified by geographical area as follows: Podolian Grey Steppe breeds from Eastern Europe, Podolian–Istrian breeds from Central Italy, and Podolian–Illyrian breeds from the Balkans and Anatolia, as shown in Table 1.

Table 1. Classification of Podolian cattle breeds according to their geographical distribution.

Podolian Cattle Breeds/Geographical Distribution		
1. Eastern Europe	2. Italy and Istria	3. Balkans and Anatolia
Ukrainian Grey (*Seraya Ukrainskaya*)	Istrian cattle (*Boškarin*)	Bulgarian Grey (*Iskar*)
Romanian Grey *(Sură Stepă)*	Mursi	Katerini cattle
Hungarian Grey (*Magyar Szűrke*)	Istarsko (*Istarsko govedo*)	Sykia cattle

Table 1. *Cont.*

Podolian Cattle Breeds/Geographical Distribution		
Slavonian–Syrmian Podolian (*Slavonsky Podolac*)	Maremmana	Anatolian Grey (*Boz Irk*)
Serbian (Srem) Podolian (*Podolsko Serbia*)	Podolica	

Data processed from Rieznykova et al. [5], D'Andrea et al. [7], and Pariset et al. [15].

Podolian cattle exhibit morphological variations primarily attributed to their adaptation to the specific climatic conditions of their geographic region. The distinctive characteristics of these cattle result from the influence of the natural environment (Table 2).

Table 2. Morphological characteristics of Podolian grey cattle.

Podolian Cattle Breeds and Their Photo		Average Adult Weight (kg)		Average Wither Height (cm)		References
		Males	Females	Males	Females	
	Romanian Grey Steppe (*Sura de Stepa*)	780	480	137	129	[64–66]
	Iskar Grey (*Bulgarian Grey*)	750	350	140	118	[67,68]
	Istrian Grey (*Boskarin*)	900	625	148	138	[69,70]
	Slavonian Podolian (*Slavonian–Syrmian*)	600–800 (1000 for oxen)	470	135–145	128	[71,72]
	Katerini cattle	400	285	123	113	[73,74]
	Hungarian Grey Steppe (*Hungarian Grey*)	900	600	150	140	[75,76]

Table 2. *Cont.*

Podolian Cattle Breeds and Their Photo		Average Adult Weight (kg)		Average Wither Height (cm)		References
		Males	Females	Males	Females	
	Turkish Grey Steppe (*Boz Irk/Boz Step*)	470	375	126	117	[77,78]
	Ukrainian Grey	780	480	137	129	[79,80]

Notable differences in body development are evident in breeds such as Istrian Grey, with an average wither height of 148 cm for males and 138 cm for females, as well as in the Hungarian Grey Steppe breed, with an average wither height of 150 cm for males and 140 cm for females.

Podolian cattle breeds are known for their adaptability to extreme conditions and hardiness against diseases. "Podolica/Podolian" indicates a possible origin of these breeds in Podolia (a region of Ukraine) [81] and their spread southward to Anatolia and westward to the Balkan and Italian peninsulas [82].

The results of Senczuk et al., (2021) indicated that Podolian cattle show higher values of genetic diversity indices than African or Asian cattle breeds. Analysis of mitochondrial DNA revealed close phylogenetic relationships among Podolian breeds, indicating a shared genetic ancestry (Figure 5) and a common evolutionary history [69].

Di Lorenzo et al., (2018) conducted a study on the origin of Podolian cattle through mitochondrial DNA analysis. The biological material for the study comprised 18 breeds within the Podolian group (Piedmontese—PI, Romagnola—RO, Marchigiana—MR, Chianina—CH, Maremmana—MA, Podolica Italiana/Italian Podolian—IP, Mucco Pisano—MP, Calvana—CA, Bianca di Val Padana—BP, Hungarian Grey—HG, Bulgarian Grey—BG, Istrian cattle—IC, Katerini—KA, Romanian Grey—RG, Slavonian–Syrmian Podolian—SS, Turkish Grey—TK, Ukrainian Grey—UK, and Podolsko—PO) and 9 non-Podolian breeds, representing the control group (Valdostana—VA, Bruna Italiana—IB, Grigio Alpina—GA, Pezzata Rossa Italiana—RP, Modicana—MO, Reggiana—RE, Agerolese—AG, Cinisara—CI, and Cabannina—CB). Following this study, genetic closeness was found between five cattle breeds (Chianina, Marchigiana, Maremmana, Podolica Italiana, and Romagnola) (Table 3 and Figure 6) [83].

A plausible hypothesis posits a dual ancestral contribution to the present genetic background of Podolian breeds stemming from both Eastern European and Middle Eastern cattle.

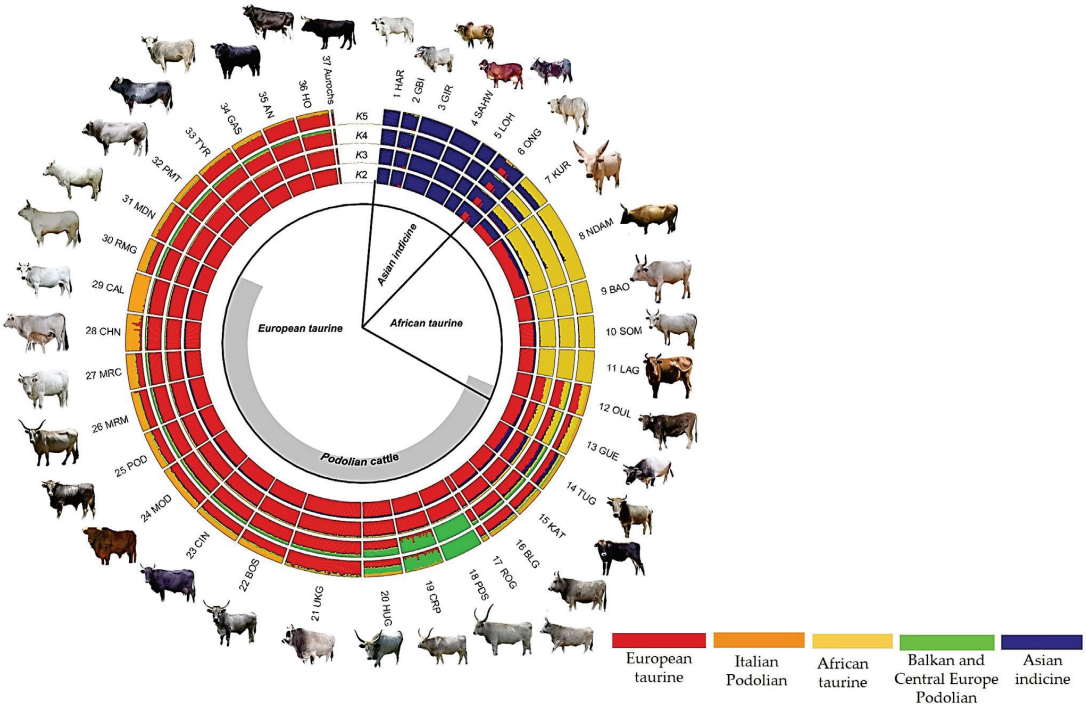

Figure 5. Analysis of the phylogenetic relationships between Podolian cattle breeds and African/Asian cattle breeds. Graphic adapted: Copyright: © 2021 Senczuk et al. [69].

Table 3. Cattle breeds from the northern (N), central (C), and southern (S) areas of Italy; Podolian breeds (P-underlined) non-Podolian breeds (non-P) [83].

Northern Italy	Central Italy	Southern Italy
		
Piedmontese—PI	Romagnola—RO	Italian Podolian—IP
Bianca di Val Padana—BP	Mucco Pisano—MP	Agerolese—AG
Valdostana—VA	Calvana—CA	Cinisara—CI
Grey Alpine—GA	Chianina—CH	Modicana—MO
Italian Brown—IB	Maremmana—MA	
Italian Red Pied—RP	Marchigiana—MR	
Cabannina—CB		
Reggiana—RE		

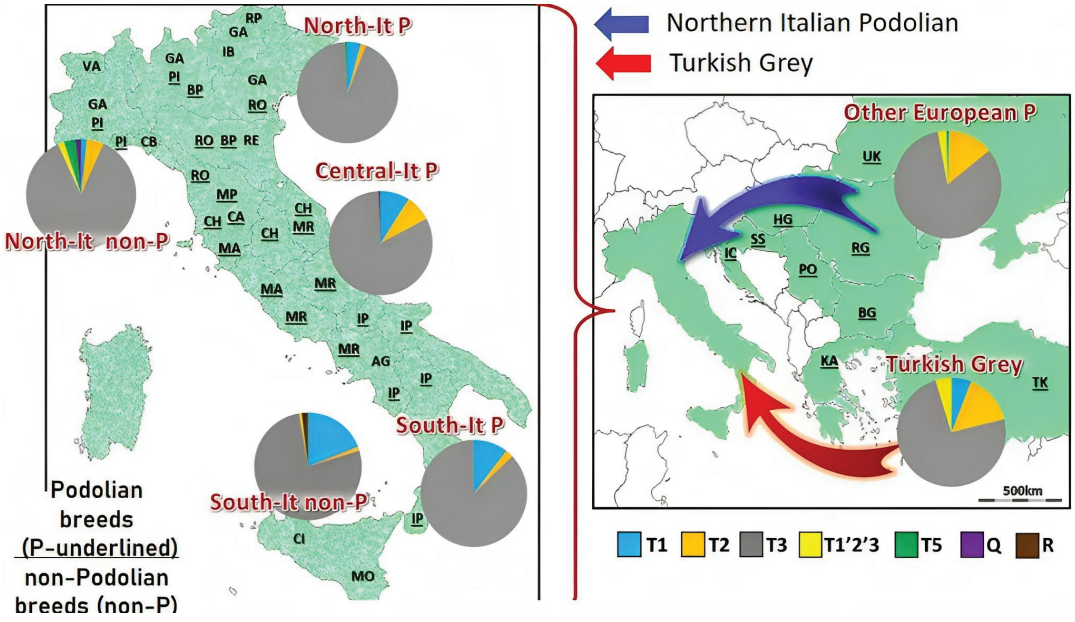

Figure 6. Analysis of frequency distributions of mitochondrial haplogroups for 18 Podolian breeds (P-underlined) and 9 non-Podolian breeds (non-P). Graphic adapted: Copyright: © 2018 Piera Di Lorenzo et al. [83].

3.1. Romanian Grey Steppe: Origin and Phylogeny

In Romania, Grey Steppe cattle represent one of the oldest autochthonous breeds, formed in the pedoclimatic conditions of the country, with its origin in *B. t. primigenius* (wild ox).

According to the research by Carsai (2008), until around 1850, the total taurine in Romania was predominantly represented by the Grey Steppe breed. Since 1892, with the first imports of non-native breeds (Simmental, Schwyz, and Pinzgauer), the local breeds have faced a sharp decline. Thus, the numerical total had decreased to approximately 57.3% of the total cattle in 1935 and to only 0.6% in 1977 [84–86].

This breed is included alongside other breeds in the Podolian group found in various European countries (*Podolica Italiana/Italian Podolian*, *Hungarian Grey*, *Bulgarian Grey*, *Istrian cattle*, *Katerini*, *Turkish Grey*, *Ukrainian Grey*, etc.) (Figure 7) [20,23,83,86].

A crucial aspect is that the natural environment played a nearly exclusive role in shaping and evolving the Romanian Grey Steppe breed. This environmental influence endowed the breed with distinctive qualities such as hardiness, resistance, adaptability to various maintenance conditions, and specific feeding methods [16].

The external peculiarities of this breed distinguish it from others by the craniological type and characteristic shape of the horns, which are bicolor, white at the base, and have a characteristic black point. At the moment of birth, the calves have the robe color of yellow-reddish shade, and after a period of approximately 2–3 months, it changes to grey [20].

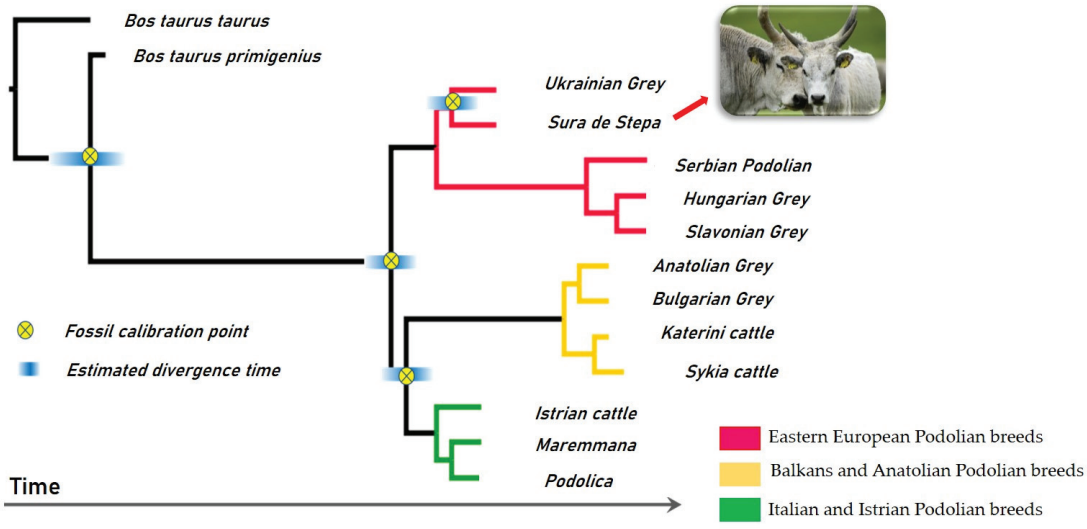

Figure 7. Phylogeny relationships between Romanian Grey Steppe and Podolian cattle breeds. Graphic adapted: Copyright: © 2018 Piera Di Lorenzo et al. [83].

A number of studies on the Grey Steppe breed have focused on the study of genetic markers associated with production traits (milk or meat) [21,22,87,88]. These studies have helped to assess the conservation value of genetic resources and establish the degree of uniformity of the breed.

In Romania, the study of the polymorphisms of the major milk proteins (casein, lactalbumin, and lactoglobulin) was conducted by researchers Bâlteanu and Ilie Daniela (2007, 2008, and 2010) through isoelectric focusing (IEF) and PCR techniques. These results led to the identification of the αS1-casein ISM allele in the Grey Steppe breed. This allele has not been identified in any other European cattle breeds. It represents an ancestral allele directly inherited from wild ancestors, offering the initial molecular evidence for the phylogenetic position of this Romanian Grey cattle [21,22,88,89].

Davidescu et al., (2022) studied the genetic diversity of a population of 32 cattle from northeast Moldova–Romania through the sequencing and analysis of two mitochondrial markers, cytochrome b and the d-loop, which have been proven to be relevant to studies of genetic diversity and phylogeny. The results obtained in this study, based on the statistical analysis of the data using nucleotide sequence analysis software (DnaSP, SeaView, MegaX, PopArt, etc.), demonstrated that the breed belonged to the ancestral P′QT haplogroup with direct descent from *B. t. primigenius*. Within this haplogroup, five cattle were identified that could be used in the selection of crosses with the aim of preserving valuable genetic resources for improving other cattle breeds and the protection of biodiversity [66].

3.2. Genetic Relationships among Romanian Grey Cattle and Podolian Cattle Breeds

Among the most ancient European cattle with direct descent from *B. t. primigenius* are wild ox are breeds belonging to the so-called Podolian trunk [90]. They have spread over time in several areas, such as Eastern Europe, Italy, Istria, the Balkans, and Anatolia. In this study, Podolian cattle breeds from the three geographical areas will be presented.

3.2.1. Genetic Diversity of Podolian Cattle Breeds

According to recent studies [14,32,77,78,86,89], Podolian breeds have a high level of genetic diversity.

Ilie et al., (2015) investigated the genetic diversity of Romanian Grey cattle (n = 29), based on 11 microsatellite loci. A total of 100 alleles were found, with an average number of

alleles per locus of 9.091, which is higher than that reported in other Podolian cattle, such as Italian Podolica (8.5) and Bulgarian Grey (7.6), and lower than that reported in Istrian cattle (12.55). The highest number of alleles was found at the TGLA122 locus (20). The Romanian Grey breed showed a high mean expected (0.794 ± 0.083) and observed heterozygosity (0.940 ± 0.127). The observed heterozygosity at the TGLA122 locus was 0.740 in Italian Podolian cattle, 0.570 in Slavonian–Syrmian Podolian cattle, and 0.644 in Austrian and Hungarian cattle [59,60,65]. The observed heterozygosity was also higher (0.940) than that reported for the Italian Podolian breed (0.73), Slavonian–Syrmian Podolian cattle (0.70), Bulgarian Grey (0.78), and Hungarian Grey (0.67). In addition, Wright's fixation index (FIS) was negative (−0.189), indicating that there was no inbreeding or selection pressure [23,89]. These results confirmed that the breed's genetic diversity is correctly preserved; however, the number of Romanian Grey individuals is extremely low and needs to be urgently increased.

Polymorphism information content (PIC) was also analyzed in the Romanian Grey breed. None of the loci showed PIC values <0.5. The PIC of all 11 loci in the Romanian Grey breed was quite high, with an average of 0.752. Analysis of the individual loci revealed that the highest value for this parameter was observed among the TGLA122 (0.863), TGLA53 (0.842), and INRA23 (0.810) loci [89].

Demir et al., (2019) investigated the genetic diversity of three Podolian cattle breeds in Turkey, including Turkish Grey Steppe, Eastern Anatolian Red, and Anatolian Black, based on 20 microsatellite markers [78]. A total of 204 different alleles, of which 31 were private alleles, were detected at 20 microsatellite loci in all populations. All private alleles had frequencies lower than 3%. The number of alleles per locus ranged from 5 (TGLA227) to 17 (ETH185) with a mean of 10.2, whereas the number of effective alleles per locus ranged from 2.39 (DRBP1) to 7.78 (SPS113) with a mean of 4.44. The observed heterozygosity ranged from 0.30 (DRBP1) to 0.98 (ILSTS011) with a mean of 0.63, whereas the expected heterozygosity ranged from 0.51 (INRABERN172) to 0.88 (SPS113) with a mean of 0.74. This study showed that Turkish native cattle breeds had a higher level of inbreeding, ranging from 0.128 (Anatolian Black) to 0.216 (Turkish Grey Steppe), which indicated lower effective population sizes. To decrease the level of inbreeding and increase the effective population size of native cattle breeds, comprehensive conservation programs are needed [78].

Another study, conducted by Maretto et al., (2012), regarding the genetic diversity and population structure of five Italian Podolian breeds (Romagnola, Marchigiana, Chianina, Maremmana, and Podolica) and their genetic relationships with the Istrian Cattle (IST) of Croatia using 20 microsatellite markers showed that the Maremmana breed presented the highest genetic diversity over all loci (0.726), followed by the Podolica (0.719) and Istrian Cattle (0.659). To characterize genetic differentiation among Podolian cattle breeds, the Genepop 4.0 version was used. This software estimated overall and pairwise F_{ST} values; for clarity of presentation, only F_{ST} values lower than or equal to 0.090 were reported. Genetic distances between breeds were estimated following Nei's genetic distance and plotted as a neighbor network using SplitsTree4 software. The mean observed heterozygosity was 0.580, which was lower than that expected for all breeds. The Istrian cattle breed appeared genetically very close to the Italian breeds, more similar to Podolica and Maremmana, with genetic distances of 0.058 and 0.080. These results emphasize the importance of monitoring genetic variability in native populations for conservation and maintaining breed identity and genetic diversity [6].

In addition, there are many other genes associated with milk production, such as the Pituitary Transcription Factor and the Growth Hormone genes. Polymorphisms in Pituitary Factor 1 (POU1F1 or PIT1) and Growth Hormone Receptor (GHR) genes were investigated using the Romanian Grey Steppe. The investigation of 60 blood samples showed two alleles at the PIT1 locus, the B allele being prevalent to A variant also in the Podolian breed, although the A allele was found to be desirable for milk production and body conformation, for example in Holstein Friesian, Polish Black and White, and Simmental [87].

3.2.2. Podolian Cattle Breeds from Eastern Europe

In eastern Europe, five Podolian cattle breeds have been identified over time, named after the area of formation: Ukrainian Grey, Romanian Grey (Sura Stepa in Romanian), Hungarian Grey, Slavonian–Syrmian Podolian, and Serbian (Srem) Podolian (Figure 8).

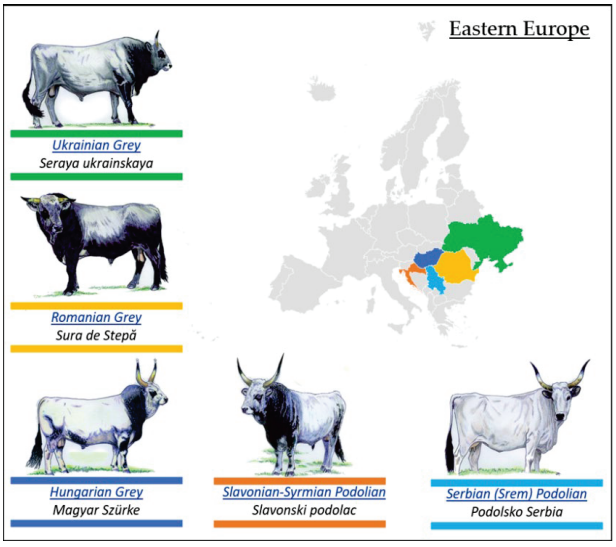

Figure 8. Graphical representation of Podolian cattle breeds from Eastern Europe (created with Bing ©GeoNames, Microsoft, TomTom, produced by Microsoft Corporation—Redmond, WA, USA).

- Romanian Grey Steppe Breed

In Romania, the last local cattle breed is Romanian Grey Steppe, which has the wild ox, *B. t. primigenius*, as its ancestor and is included in the group of Podolian cattle breeds together with other breeds from various European countries [86]. Developed over many centuries, this breed has evolved exclusively under the influence of natural environmental conditions. The number of individuals in Romania was predominant until the middle of the 19th century; however, starting from the 20th century, an increasingly pronounced decline was recorded. It was spread across four geographical areas of the country in the form of four varieties named after the area of formation: Moldavian, Ialomitean, Transylvanian, and Dobrogean varieties [16,19]. According to F.A.O. reports regarding the risk status of the breed, the Grey Steppe is endangered.

- Ukrainian Grey Breed

The Ukrainian Grey breed, with its origins in *B. t. primigenius*, is one of the oldest native breeds that is currently endangered. The evolution of this breed has been characterized by a lack of interbreeding for several centuries. While it exhibits an exclusive capacity for resistance and fattening, it demonstrates lower performance in milk production. Simultaneously, this breed serves as an intriguing research tool. These cattle proved to be indispensable to peasants and represented the most numerous breeds in Ukraine until the 20th century, being widely distributed in the southern part of Europe and the steppe zone of the Mediterranean coast and Black Sea [91].

- Hungarian Grey Breed

The Hungarian Grey is an indigenous breed, representing a national symbol of Hungary. There are several theories regarding the origin of this breed, one of which is that it arrived in the Pannonian Basin, where domestication also took place, together with Hungarian immigration in the 9th century, during the reign of King Árpád, having descended

from wild boar (*B. t. primigenius*) [92]. The first written document that refers to Hungarian Grey cattle is entitled "Magnus cornuotes boves Hungaricos" and dates back to the 16th century. The breed was bred specifically for its exceptional meat quality. By 1925, after the First World War when Hungary lost about 72% of its territory, there were approximately 321,000 specimens of the Hungarian Grey breed [93]. Currently, the herd of cattle of this breed is facing a sharp decline.

- Slavonian–Syrmian Podolian Breed

The Slavonian–Syrmian Podolian is a native cattle species found in Slavonia, a region in the northeastern part of Croatia. These cattle are irreplaceable for agricultural activities because of their physical strength and endurance. In Podravina, Slavonia, and Syrmia, this breed was predominant until the first half of the 20th century, representing 90% of all cattle [94,95]. Since 1998, this breed has been included in a national genetic conservation program, and since 2000, according to the F.A.O., it is endangered, along with other breeds of Podolian cattle.

- Serbian (Srem) Podolian Breed

The Serbian (Srem) Podolian breed in Serbia faced gradual replacement due to the extensive importation of specialized cattle breeds for meat and milk production. In the early 20th century, native cattle accounted for 83% of the total population. Among the autochthonous breeds in Serbia, two varieties, Busha and Podolian, have been identified, and they are currently classified as endangered [96].

3.2.3. Podolian Cattle Breeds from Italy and Istria

In Italy and Istria, five breeds of the Podolian group have been distinguished over time: Istrian cattle (Boškarin), Mursi, Istarsko (Istarsko govedo), Maremmana, and Podolica (Figure 9).

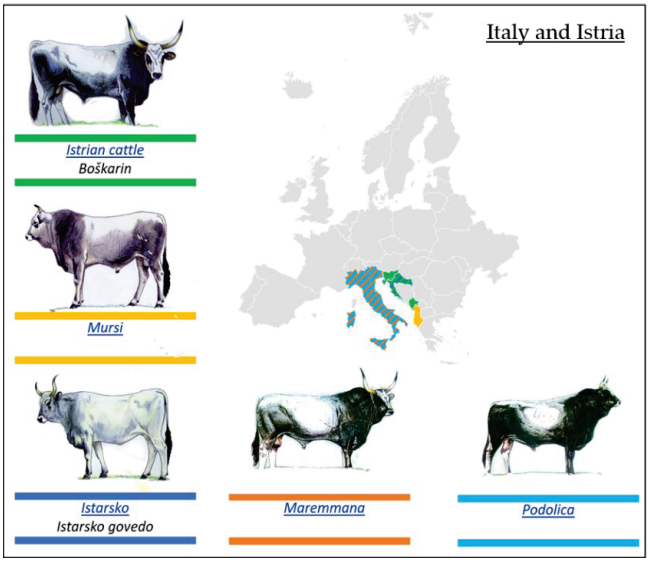

Figure 9. Graphical representation of Podolian cattle breeds from Italy and Istria (created with Bing ©GeoNames, Microsoft, TomTom, produced by Microsoft Corporation—Redmond, WA, USA).

- Boškarin or Istrian Breed

Indigenous cattle, also called Boškarin or Istrian cattle, are widespread on the Istrian Peninsula. The breed belongs to the group of Podolian cattle found in the Balkans and

neighboring countries (Croatia, Bulgaria, Greece, Hungary, Italy, Romania, Serbia, Turkey, and Ukraine), with direct descent from the aurochs (*B. t. primigenius*) [70]. Over the past 50 years, the cattle population in Istria has experienced a significant decline. Similar to the situation with the Romanian Grey Steppe, the Boškarin breed is also endangered.

- Mursi Breed

Mursi cattle are widespread in the South Omo area of southwestern Ethiopia [97]. They typically have inward-curving horns, prominent humps, and well-developed bodies. They have a combination of colors: grey, white, and black, with spots or stripes. The Mursi breed is raised mainly for high milk production, but it also lends itself well to meat production.

- Istarsko Govedo Breed

Istarsko govedo is a domestic cattle breed recognized for producing high-quality meat. It is currently on the verge of extinction, and the size of the population has decreased sharply since the second half of the 20th century. The conservation program for this breed was launched in the early 1990s [65].

- Maremana and Podolica Breeds

The cattle breeds of Italy, namely, the Maremana and Podolica, were very popular and widespread in this area before the Second World War but saw a consistent reduction in their numbers due to three major factors: mechanization in agriculture, urbanization, and breed competition with high production efficiency. Their numbers of 288,000 and 630,000 heads [98] were reduced by 90% and 80%, respectively [13]. The two breeds belong to the group of grey cattle whose ancestral origin is *B. primigenius* [99].

3.2.4. Podolian Cattle Breeds from Balkans and Anatolia

In the Balkans and Anatolia regions, four breeds of Podolian cattle have spread over time: Bulgarian Grey (Iskar), Katerini cattle, Sykia cattle, and Anatolian Grey (Figure 10).

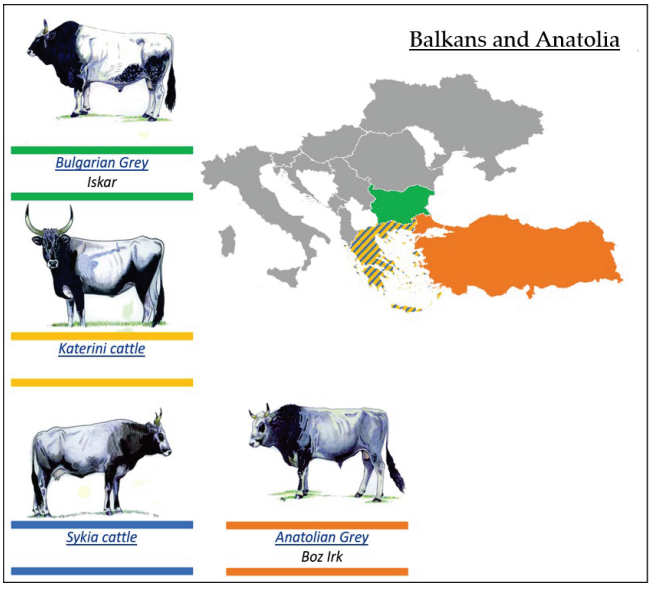

Figure 10. Graphical representation of Podolian cattle breeds from Balkans and Anatolia (created with Bing ©GeoNames, Microsoft, TomTom, produced by Microsoft Corporation—Redmond, WA, USA).

- Bulgarian Grey (Iskar) Breed

According to studies in the field of cattle phylogeny, based on craniological analyses [3,14,69,83], the Bulgarian Grey (Iskar) breed is the result of the cross between *B. taurus brachiceros* and *B. t. primigenius*. The breed is characterized by high vitality and fertility, ease of calving, resistance to parasitic and infectious diseases, and low feed requirements. Currently, there are fewer than 1000 heads. Cattle are mainly distributed in the regions of Plovdiv, Haskovo, and Shumen and in the mountainous regions of Strandzha and Sakar. Bulgarian Grey is the first native cattle included in the National Program for the Conservation of Genetic Resources of Animal Origin [100,101], representing a valuable genetic reserve for Bulgaria.

- Katerini Breed

Katerini cattle is a breed with a special quality of meat production. Similar to other Podolian cattle breeds, their ancestral origin is *B. t. primigenius* [73]. From the reports issued by the F.A.O. regarding the risk status of this breed, it appears that it is at critical risk, with the effective population size gradually decreasing.

- Sykia Cattle Breed

Sykia cattle is another ancient breed found in Greece that is currently on the verge of extinction, mainly due to random crossbreeding [65]. Current data suggest that there are less than 200 purebred females, with the F.A.O., placing the breed in the critical risk class in terms of herd size [13].

- Anatolian Grey Breed

Anatolian Grey is a cattle population widespread in Turkey. Currently, the numerical status of this Anatolian breed is critical [102]. To protect these genetic resources, a national project was initiated in Turkey with the main objective of in vitro conservation and identification of valuable genetic resources of native cattle [78,103], resulting in a numerical increase in the population of the Anatolian Grey breed through different breeding biotechnologies.

According to certain researchers, there are distinct phenotypic features observed in Podolian cattle. Long horns, characteristic of breeds like Hungarian Grey, Katerini, or Slavonian–Syrmian, are considered breed-specific traits. However, some breeds, including Podolica Italiana, Romanian Grey Steppe, Ukrainian Grey, Turkish Grey, and other Balkan breeds, may not necessarily exhibit long horns but retain other characteristics, such as the light grey color in adult cows [83].

4. Trends and Management Recommendations in Genetic Conservation Programs, including the Endangered Grey Steppe Cattle

The production systems of Podolian cattle are rooted in a combination of traditional breeding practices, extensive grazing management, and recognition of the breed's economic and cultural importance. As agriculture progresses, maintaining a balance between modern techniques and the preservation of indigenous breeds, like Podolian cattle, becomes essential for sustainable and resilient food production systems. Typically, Podolian breeds are raised in pastures, offering an environment that allows animals to exhibit their natural behaviors.

Natural pastures allow selection among a diverse array of herbaceous and arboreal plants based on nutrient requirements and individual preferences and influenced by physical characteristics, accessibility, and palatability. Thus, akin to wild herbivores, Podolian cattle have the opportunity to exhibit their natural behavior and choose a balanced diet based on their evolving nutritional requirements and physiological conditions. This capacity is deemed crucial for ensuring the welfare of the animals [104,105]. Another important aspect is that the reproductive characteristics of Podolian cattle are influenced by environmental factors. Podolian cattle are recognized for a significant increase in births during the spring season, when natural pastures and meadows are at maximum productivity, with direct effects on the physiological state of cows and milk production. Despite the cost-

effectiveness of rearing, the trade in calves is contingent on age and live weight, reaching its peak between August and December (age 15–18 months) [104–106].

The preservation of the gene pool of the Grey Steppe cattle breed requires the application of additional measures and the allocation of considerable financial resources. To design appropriate and effective conservation strategies, accurate knowledge of all existing individuals within a population must be considered. The present tools for genomic evaluation have made it possible to use mitochondrial DNA as a tool for phylogenetic and biodiversity studies [107]. The methods used to analyze the phylogeny of cattle have progressed from traditional morphometric analyses to contemporary molecular genetics techniques.

Further research considering the analysis of the complete mitochondrial genome, Y chromosome, and microsatellite analysis, as well as the study of DNA extracted from fossils or bone remains belonging to taurine with direct descent in *B. taurus*, could provide a better understanding of the genetic structure and evolutionary history of this breed [11,66].

Animal husbandry and cattle breeding technologies are currently facing several problems, such as the conservation of breeds at risk of abandonment, sustainable management of genetic resources, and appropriate management. To ensure sustainability, modern animal husbandry, classic breeding, and selection programs are insufficient. In addition to being expensive in terms of time and money, they are characterized by limited accuracy. The limitations imposed by these classic techniques can be eliminated by applying modern reproduction techniques (in vitro fertilization and embryo transfer), to which genetic screening methods can be added. An important aspect of ensuring an effective conservation program is the preservation of genetic material by cryogenically freezing samples (tissue and genomic DNA) and creating a database of archived samples, phenotypic parameters, and genotypic profiles of each individual to ensure propagation (Figure 11) [11,66].

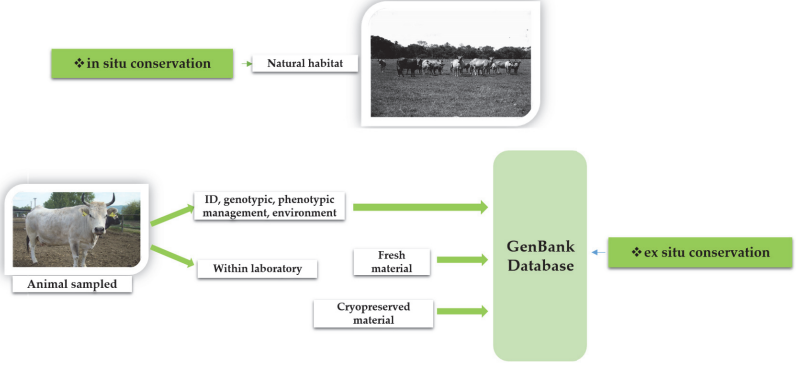

Figure 11. In situ and ex situ genetic conservation plan for Podolian cattle breeds.

The European representatives of the (F.A.O.) have classified Podolian Grey Steppe cattle as endangered and in urgent need of conservation to preserve genetic diversity. Currently, several European countries, including Romania, are attempting to implement in situ conservation programs for Podolian cattle breeds. These projects aim to ensure a natural habitat similar to that in the wild, in which cattle can manifest their innate behavior and benefit from environmental conditions favorable to their growth and development. In addition, a series of methods for conserving the genetic resources of these cattle breeds ex situ (e.g., cryopreservation, such as processing and freezing of genetic material, and DNA banks) have been implemented, all of which aim to preserve the biodiversity of Podolian cattle breeds threatened with extinction [108–110].

The issue of maintaining and conserving local breeds is important for ensuring the food security of the population, not only considering the recent climate changes as well as extreme phenomena, which could bring new challenges in the future. Bovine species are

the most exposed to these changes; therefore, the Grey Steppe breed could be an effective alternative to improved breeds because of its special qualities of resistance and adaptability to different stress factors and diseases.

5. Conclusions

The information presented in this study regarding the genetic diversity, origin, and phylogeny of Podolian cattle breeds demonstrates their ancestral origin, with direct descent from *B. t. primigenius* (the aurochs). Their biologically valuable characteristics, such as rusticity, high longevity, adaptability, hardiness, and resistance to diseases, are important aspects of breeding and conservation programs.

According to the cited studies, it has been concluded that many populations of cattle from Eastern Europe, including the Romanian Grey Steppe, have a high level of genetic diversity and close phylogenetic relationships with wild ox.

The information presented in this study can help to improve the present conservation program to preserve biodiversity and protect Podolian Grey breeds from genetic loss, contributing to the general knowledge of the genetic diversity of European cattle breeds and could prove a valuable tool for conservation efforts of animal genetic resources.

Therefore, we strongly recommend taking immediate action and allocating appropriate financial resources to conserve the valuable genetic diversity of the Romanian Grey cattle breed, which still represents a valuable gene pool. Preserving Podolian Grey cattle from extinction will yield benefits for both the agricultural sector and the international genetic heritage.

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Review

Use of *Spirulina platensis* and *Curcuma longa* as Nutraceuticals in Poultry

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Abstract: Since the banning of antibiotics in animal feeds (2006), there has been an increase in the number of studies looking for alternatives to stimulate the gut immune system. The main objective of our review article is to underline the nutraceutical properties of *Curcuma longa* and *Spirulina platensis* in the broiler chicken industry, and the experimental data were obtained by analyzing literature sources. *Spirulina platensis* is widely recognized as a valuable protein source, containing approximately 55–70% protein, 25% carbohydrates, essential amino acids, and 18% fatty acids. It is also rich in various vitamins like thiamin, riboflavin, pyridoxine, vitamin B12, vitamin C, gamma-linolenic acid, phycocyanins, tocopherols, chlorophyll, beta-carotenes, carotenoids, exhibiting positive effects on growth performance, gut integrity, and immunity. The anti-inflammatory effect of spirulina supplementation at different levels showed a decrease in caspase-3 and the TNF-α immunolabeling index; a reduction in IL-1β, IL-2 and IFN-γ; and an increase in the expression of the anti-inflammatory cytokine IL-4. Spirulina inhibits the synthesis of cytokines IL-1, IL-6, and TNF-gamma in addition to the activities of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) enzymes. Turmeric also positively influences the growth, egg production, and overall health of chickens. Curcumin, the most potent component of turmeric, possesses additional pharmacological activities, including hepatoprotective, immunostimulant, and anticancer effects. Its immunomodulatory properties greatly enhance the immune system response, acting as a natural antibiotic against pathogens and decreasing levels of proinflammatory interleukins IL-1β, IL-6, IL-2, IL-18, and TNF-α.

Keywords: chickens; spirulina; turmeric; growth promoters; immunomodulators

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1. Introduction

Poultry production, particularly in developing regions, is experiencing rapid growth within the agricultural sector, making a valuable contribution to ensuring food security [1]. The rising demand for alternative feed resources is driven by factors such as intensified competition with other agricultural sectors over land, the impacts of climate change, and the ongoing need to enhance productivity and meat quality. Furthermore, there has been a growing focus on producing innovative functional foods using environmentally friendly sources [2].

Nutraceuticals refer to the essential nutrients or dietary components derived from animals that hold significant nutritional and pharmaceutical value. They exhibit immunomodulatory properties and play a crucial role in preventing diseases, promoting overall health, and subsequently enhancing productivity [3–6]. In addition, these nutraceuticals aid in safeguarding the host from infectious diseases [7], as well as influencing and sustaining essential physiological functions that contribute to the well-being of the host [8].

The term "nutraceutical" was coined in 1989 by Stephen DeFelice, combining the words "nutrition" and "pharmaceutical". He clarified that a nutraceutical could encompass either a food or a component of food with the potential to prevent and/or treat diseases [9,10]. Since then, the term has been the subject of ongoing global discussions regarding its precise definition and scope.

Nutraceuticals encompass a wide range of substances, including isolated nutrients, such as vitamins, minerals, amino acids, and fatty acids. They also include herbal products, such as polyphenols, herbs, and spices, as well as dietary supplements, such as probiotics, prebiotics, synbiotics, organic acids, antioxidants, and enzymes. Additionally, nutraceuticals can extend to genetically modified foods [8,11,12].

Amino acids [13], minerals [14], and vitamins [15] play a significant role as common components in poultry diets, either individually or in combination [16,17], and can be considered as nutraceuticals with particular importance in poultry feeding. While natural feedstuffs generally provide essential nutrients for poultry, certain crucial amino acids (lysine, methionine, threonine, and tryptophan), vitamins, and minerals are often supplemented synthetically [18]. The refined form of these nutraceutical constituents in the diet can lead to improved digestion, absorption, utilization, and metabolism, ultimately resulting in beneficial health effects compared to conventional forms.

The nutraceutical value and conversion efficiency in poultry are subject to the influence of multiple factors, such as the genetic potential of the birds, environmental conditions, dietary quality, and gut health. Consideration of these factors is crucial for maximizing the productive efficiency of the birds [19–21].

In recent times, nutraceuticals have garnered significant attention in poultry science. This is attributed to the recognition of the nutritional and healthier attributes of feed ingredients, as well as growing concerns regarding the adverse effects associated with chemical pharmaceuticals, such as antibiotic resistance and the presence of drug residues [22,23].

The use of antibiotics in poultry has been known to reduce the microbial load in the gut, thereby increasing nutrient availability for the host [24]. However, concerns regarding the development of antimicrobial resistance and the transfer of antibiotic-resistance genes from animals to human microbiota prompted the European Union to ban the application of antibiotics as growth promoters from 1 January 2006 [25]. The removal of antibiotic growth promoters (AGP) from poultry diets has resulted in challenges related to animal performance and an increase in the occurrence of certain poultry diseases, such as subclinical necrotic enteritis and dysbacteriosis [26]. Consequently, there is a pressing need to find alternatives to AGP.

Ideally, these alternatives should provide similar beneficial effects as AGP. In recent years, there has been a significant increase in nutrition-based research focused on finding alternatives to AGP in various farm animals, including poultry [26].

In this context, nutraceuticals have been discovered to possess numerous beneficial health applications and potential roles in improving production performances in poultry. They serve as antioxidants in promoting health, modulating the composition of gut microbiota, and enhancing poultry immunity [3,11,27–29].

1.1. Spirulina spp.

The utilization of microalgae as a feed ingredient offers several advantages in terms of environmental protection and conservation of natural resources, including the prevention of land degradation and water scarcity issues [30]. In the field of poultry nutrition, there is a growing trend toward the incorporation of natural ingredients as alternatives to antibiotics, growth factors, and other chemical substances.

Spirulina platensis (SP), a blue-green filamentous photosynthetic alga, is widely recognized as a valuable protein source, containing approximately 55–70% protein, 25% carbohydrates, essential amino acids, and 18% fatty acids. It is also rich in various vitamins and minerals. Notably, spirulina is known for its high content of thiamin, riboflavin, pyridoxine, vitamin B12, vitamin C, gamma-linolenic acid, phycocyanins, tocopherols, chlorophyll,

beta-carotenes, and carotenoids [31]. Recent studies have demonstrated that spirulina exhibits positive effects on growth performance, gut integrity, and immunity. Additionally, it has shown modulating activity and has been associated with various pharmacological properties.

Several studies have been conducted to investigate the potential benefits of SP in broilers. These studies have explored its use as a growth promoter [32,33], regulator of gut health [34], immune stimulator [35], and enhancer of meat yield and quality [2]. These studies have reported various findings highlighting the positive effects of SP in these different aspects of broiler production.

A significant and expanding body of research has provided evidence for the immunostimulatory, hepatoprotective, anti-inflammatory, antimicrobial, antiviral, and antioxidative activities of SP [36,37]. These activities are attributed to its ability to enhance disease resistance, stimulate the production of antibodies and cytokines, effectively scavenge free radicals, and inhibit lipid peroxidation. Consequently, the inclusion of SP in poultry production has shown promising results in improving overall production outcomes and attaining greater profitability [38–41].

Spirulina has gained recognition for its diverse biological effects, including the prevention of anemia due to its high iron and vitamin content [42]. It has also shown a potential to inhibit herpes simplex infection [43]. Studies have revealed that the ethanolic extract of SP contains various bioactive compounds, such as alkaloids, flavonoids, glycosides, tannins, phenolic compounds, steroids, and saponins [44].

Numerous studies have indicated the therapeutic effects of spirulina. It has been shown to reduce cholesterol levels and potentially have anticancer properties by enhancing the immune system. Additionally, spirulina has been found to increase the population of beneficial intestinal lactobacilli, reduce nephrotoxicity caused by heavy metals and drugs, and offer radiation protection [45]. Furthermore, spirulina is widely recognized for its antioxidant properties, which can be attributed to molecules such as phycocyanin, beta-carotene, and tocopherol. These antioxidant properties contribute to spirulina's ability to inhibit carcinogenesis and mitigate organ-specific toxicity [46].

Phycocyanin (PC) is a blue pigment found in cyanobacteria and certain red algae of the phycobiliprotein family. It is soluble in water and primarily located in the cytoplasmic membrane. However, it can be released outside the cells when the thylakoid membrane is disrupted by lysozyme enzymes and EDTA-chelated cations [47,48]. PC exhibits a range of beneficial properties, including antioxidant, radical scavenging, anti-inflammatory, anti-arthritic, hepatoprotective, antitumor, and immune-enhancing activities [49,50]. In broiler production, natural antioxidants are preferred due to their health-promoting characteristics [51]. These antioxidants help reduce the production of reactive oxygen species (ROS) and subsequent oxidative stress [52].

In the current global market, there is significant competition between microalgae carotenoids and artificial, synthetic, and unnatural pigments [53]. Despite their relatively high cost, natural algae are used in smaller quantities and have no known side effects [54].

Similar to other animals, the digestive tract of poultry plays a critical role in the utilization and intake of feed while also being important in terms of exposure to environmental pathogens [25]. Any functional disturbances in the digestive tract can pose risks to the health and performance of poultry, as it can disrupt the absorption and digestion of nutrients. The small intestine is particularly crucial for nutrient absorption [55]. The intestinal mucosa plays a vital role in enhancing nutrient absorption and acts as a barrier between the internal tissues of the host and the intestinal contents, thereby serving as an immune defense mechanism [19]. The proper functioning of the mucosal layer is the result of a delicate balance between the mucosal layer, epithelial cells, immune cells, and the microbiota [56].

The intestinal microbiota plays a crucial role in maintaining immune homeostasis and regulating inflammatory responses [57]. Commensal bacteria in the intestine produce short-chain fatty acids (SCFAs), which have anti-inflammatory properties and help protect

against intestinal injury [58]. Modulation of the intestinal microbiota can be achieved by incorporating nutraceuticals into the poultry diet. Nutraceuticals have the potential to promote the growth of beneficial bacteria while suppressing the growth of harmful bacteria [59]. This modulation of the intestinal microbiota can have significant implications for the overall health and well-being of poultry.

The gut microbiota plays crucial roles in the regulation of epithelial cell proliferation in the gut, synthesis of vitamins, and host energy metabolism. In poultry, the gastrointestinal tract (GIT) harbors a complex and dynamic microbiome primarily composed of bacteria, with a lesser presence of fungi, protozoa, bacteriophages, yeast, and viruses. This diverse microbial community has significant impacts on various aspects of poultry health and physiology.

Microbes within the gastrointestinal tract (GIT) interact extensively with the host and the consumed feed. Different regions of the GIT harbor distinct populations of microbes, creating specific niches [21]. In chickens, imbalances in the gut microbiota have been associated with adverse effects on intestinal health [60,61]. It is widely acknowledged that maintaining an appropriate microbial balance, with favorable bacteria comprising about 85% of the total bacterial population, is crucial for the host's well-being [62]. The elimination of antibiotics from feed further compounds bacterial imbalances. Correspondingly, Kabir demonstrated that a balanced gut microbiota is vital for maximizing chicken growth performance and promoting a healthy gut [63].

Fortunately, dietary interventions, in conjunction with promoting the growth of beneficial bacteria in the chicken intestine, can be used to modulate the gut microbial population [59]. Studies by Humphrey and Klasing have shown that changes in the microbiota can impact gut wall morphology, elicit immune responses, and ultimately influence energy expenditure and chicken development [64]. These findings highlight the importance of maintaining a healthy and balanced gut microbiota for optimal poultry growth and overall well-being.

1.2. Curcuma spp.

Turmeric (*Curcuma* spp.) is a perennial herb with rhizomatous growth, belonging to the Zingiberaceae family. It is extensively cultivated and utilized in tropical and sub-tropical regions worldwide [65]. Turmeric is highly regarded for its medicinal properties, exhibiting a wide array of pharmacological effects, such as antioxidant, anti-protozoal, antimicrobial, anti-inflammatory, and antitumor activities [66]. The primary active compound in turmeric is curcumin, which demonstrates potent antioxidant capabilities [67,68]. Turmeric belongs to a category of medicinal plants that offer an alternative natural antibiotic approach for poultry farming. Supplementation of turmeric effectively influences the growth, egg production, and overall health of chickens [69]. This medicinal plant possesses rhizomes and subterranean stems resembling roots [70], originally used as a food additive in curries to enhance food storage, appearance, flavor, palatability, and preservation. The addition of turmeric powder and extracts has beneficial effects on the performance of birds and animals [71].

Turmeric contains several active ingredients, namely curcumin, demethoxycurcumin, bisdemethoxycurcumin, and tetrahydro curcuminoids [72]. Among these, curcumin, extracted from turmeric rhizomes, is the primary bioactive compound of *C. longa*, exhibiting antioxidant, antiviral, and antibacterial properties [73]. Curcumin, the most potent component of turmeric, represents 3–5% of the curcuminoids found in the rhizomes and acts as a robust phenolic antioxidant [74]. Turmeric also possesses additional pharmacological activities, including hepatoprotective, immunostimulant, and anticancer effects [75]. Its immunomodulatory properties greatly enhance the immune system's response, acting as a natural antibiotic against pathogens. Turmeric effectively regulates inflammation, playing a crucial role in preventing inflammation-related disorders in poultry [76]. The World Health Organization (WHO) has declared turmeric safe for dietary consumption in humans and animal feed [77].

Curcumin, a polyphenolic compound, possesses various bioactive properties, including antibacterial, anti-inflammatory, anti-carcinogenic, anti-proliferative, and antioxidant effects [78–81]. It has been observed that curcumin affects bile production and lipid metabolism in the liver, leading to the belief that the active constituents in turmeric can enhance liver function by reducing cholesterol levels in the liver and serum. Consequently, they have the potential to regulate cholesterol levels and lipid profiles [82–86].

Turmeric (*Curcuma longa*) is a widely utilized natural remedy with a plethora of pharmacological properties. In terms of its biochemical composition, turmeric consists of approximately 69.4% carbohydrates, 6.3% protein, 5.1% fat, 3.5% minerals, and 13.1% moisture [78]. It also contains approximately 5% essential oils and 5% curcumin, a bioactive polyphenol [87]. Curcumin, the principal component of turmeric, is insoluble in water and ether but can disperse in ethanol and other organic solvents [88]. Among the noteworthy bioactive constituents in turmeric are curcumin, dimethoxy curcumin, tetrahydro curcuminoids, and bis-methoxy curcumin [87]. The diverse biological activities of turmeric stem from the presence of these various bioactive molecules. Curcumin exhibits specific antimicrobial, anti-inflammatory, antioxidant, and anticancer properties [89]. Moreover, curcumin enhances insulin release, facilitates fatty acid uptake, reduces lipogenesis, and elevates nitric oxide (NO) levels [90]. Turmeric has so far been reported to possess 326 biological activities [64,84,86].

Curcumin possesses a distinctive chemical structure that is primarily responsible for its antioxidant properties. It contains carbon–carbon double bonds, a β-diketo group, and phenyl rings with hydroxyl and methoxy substituents [91]. This unique structure enables curcumin to donate hydrogen atoms (from its phenolic groups) to effectively neutralize free radicals. Aside from curcumin, which is the main polyphenol, turmeric powder also contains dimethoxycurcumin, bis-dimethoxycurcumin, 2,5-xylenol [92], flavonoids, tannins, and ascorbic acid [93]. These compounds can also exhibit neutralizing activity toward free radicals (Figure 1).

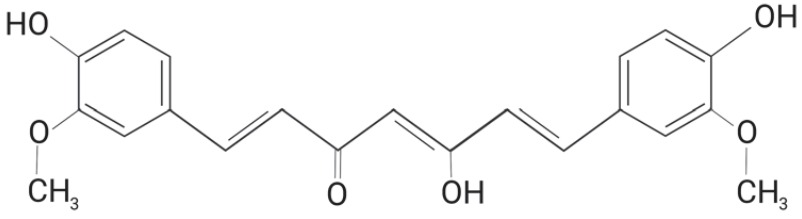

Figure 1. Curcumin chemical structure. The figure was created using BioRender.com accessed on 19 June 2023.

Turmeric is widely used as a treatment for inflammatory conditions and has various applications in traditional Chinese medicine, including its use as a stimulant, aspirant, carminative, emmenagogue, astringent, detergent, and diuretic [94]. Over the past decade, the use of turmeric in poultry feed has become widespread due to its medicinal properties [69]. Curcuminoids, including bisdemethoxycurcumin (BDMC) and demethoxycurcumin (DMC), along with the colorless metabolite tetrahydrocurcumin (THC), act as potent antioxidants [95]. Turmeric powder, which contains approximately 3 to 5 percent curcuminoids, exhibits a broad spectrum of biological activities, including antioxidant, antibacterial, antifungal, antiprotozoal, antiviral, anticoccidial, and anti-inflammatory properties [96]. Turmeric possesses favorable pharmacological properties and can serve as a beneficial natural growth promoter and a safe alternative to antibiotics. However, the dietary supplementation of curcumin is limited due to its low solubility in alkaline pH and its susceptibility to hydrolysis when exposed to light, resulting in poor absorption in animals [97]. Studies conducted on broiler chickens have demonstrated increased weight gain and improved feed conversion ratio (FCR) with turmeric supplementation. Conversely, in

studies by [98–100], no significant effects of turmeric on FCR were observed. The research findings regarding turmeric supplementation in poultry diets are not consistently aligned.

Curcumin, the primary phenolic compound in *Curcuma longa* powder, has been found to possess antioxidant effects [101–103]. Alongside its antioxidant properties, *Curcuma longa* has also been reported to exhibit free radical scavenging abilities [104], hypolipidemic effects [105], protection of biological membranes from peroxidative damage [106], enhancement of immune function [107], and antiviral and antibacterial properties [108]. As a result of its antioxidant properties, *Curcuma longa* suppresses lipid peroxidation [109] while promoting the actions of detoxifying enzymes [110].

Previous studies have additionally highlighted the biological activities of *Curcuma longa*, such as its anticoagulant effects [111] and in improving nutrient digestibility and metabolism [35], enhancing hepatic functions [112], and reducing serum LDL, cholesterol, triglycerides, and blood glucose levels [103]. Recent research has demonstrated that curcumin from *Curcuma longa* can improve the performance of broiler chickens, particularly under thermal stress conditions [101,113].

The supplementation of turmeric in chicken feed as a feed additive has led to significant advancements in chicken performance and economic efficiency [114,115]. As an herbal feed additive, turmeric has been found to enhance the average daily weight gain (DWG) and reduce feed costs in poultry production [114]. It also exhibits positive effects on the DWG, feed conversion ratio (FCR), and carcass characteristics of broiler chickens [115]. Turmeric, a commonly used culinary ingredient in Vietnam, is known for providing artificial color and a distinct aroma to human food. It is readily available in household backyards and local markets. The key active component, curcumin, contributes to its various therapeutic properties, including antibacterial, anticoccidial, antioxidant, hypercholesteremic, and hypolipidemic effects [114–118]. Additionally, turmeric has been found to have substantial levels of crude protein (10.07%), ether extract (6.64%), crude fiber (4.87%), nitrogen-free extract (66.76%), and ash (2.76%) [119,120]. Moreover, turmeric is rich in essential minerals and nutrients crucial for bone and muscle development [120].

The present review focuses on the potential role of nutraceuticals in enhancing the growth performance, immune system, gut microbiota, and health of poultry. In particular, it explores the utilization of *Spirulina* spp. and *Curcuma* spp. as an alternative strategy to reduce the reliance on antibiotic growth promoters (AGP) in poultry diets. To gather relevant information, we conducted a comprehensive search using databases such as Science Direct, Google Scholar, and Web of Science. The keywords "nutraceuticals", "Spirulina", "Curcuma", and "poultry" were employed to identify relevant articles. The search encompassed papers published between 2000 and 2023, and the selection was based on the content and relevance of the studies. Specifically, articles written in English that addressed the aforementioned topics were included in the review.

2. Growth-Promoting Effects on Broiler Chicken

2.1. Growth-Promoting Effects of Spirulina

The primary objectives of poultry farming are to achieve rapid growth and maximize feed efficiency. To attain optimal performance in livestock, several factors need to be taken into account, including the genetic potential of the animals, feed quality, environmental conditions, and disease management [19]. These factors play crucial roles in determining the overall productivity and health of poultry. By considering these aspects and implementing appropriate strategies, farmers can enhance growth rates and improve feed utilization, leading to better outcomes in poultry production.

The supplementation of algae has shown beneficial effects on broiler performance. A study conducted by [121] found that the inclusion of supplemental algae in broiler diets improved feed intake and body weight gain. This indicates that algae can contribute to enhancing growth performance in broilers (Figure 2).

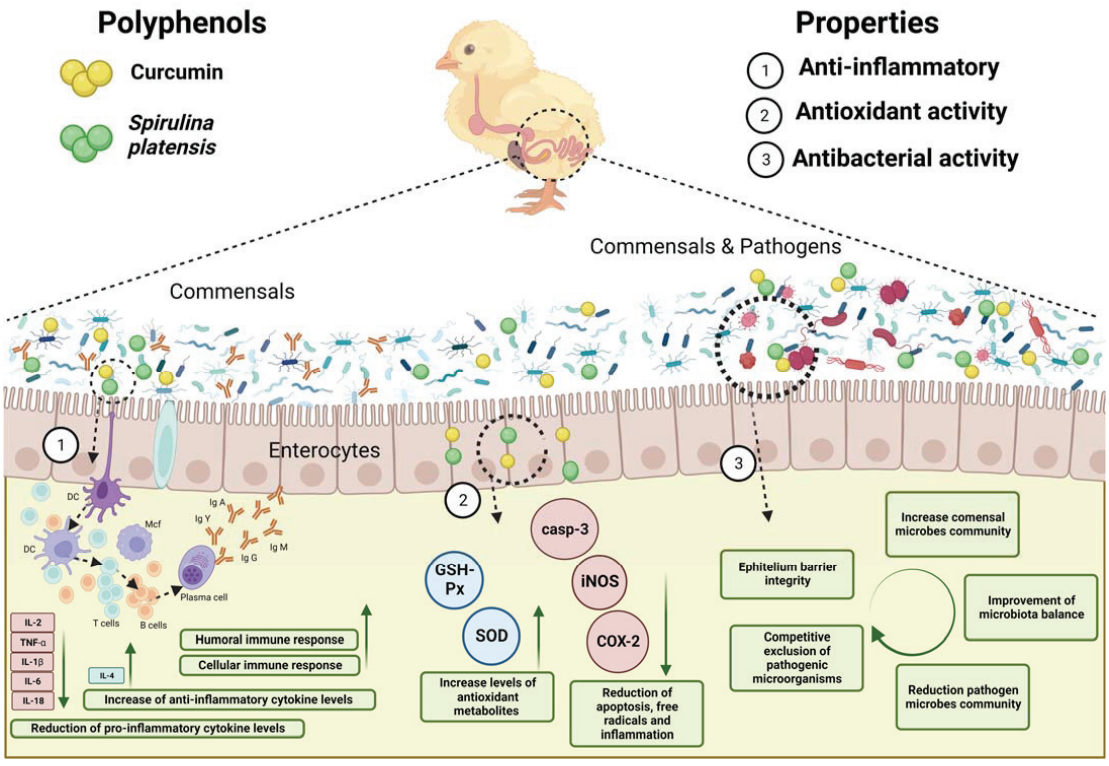

Figure 2. Anti-inflammatory properties and antioxidant and antibacterial activity of curcumin and *Spirulina platensis* on GALT in chickens. IL-2 (interleukin-2), TNF-α (tumor necrosis factor-alpha), IL-1β (interleukin-1 beta), IL-6 (interleukin-6), IL-18 (interleukin-18), IL-4 (interleukin-4), GSH-Px (glutathione peroxidase), SOD (superoxide dismutase), casp-3 (caspase-3), iNOS (nitric oxide synthases), and COX-2 (cytochrome c oxidase subunit 2). The figure was created using BioRender.com accessed on 19 June 2023.

Additionally, research [122] suggests that the nutrient composition and physiological functions of spirulina may have positive effects on the metabolic systems associated with broiler growth performance. This indicates that spirulina, with its specific composition and properties, can potentially support the growth and development of broilers.

These findings highlight the potential of incorporating algae, such as spirulina, into broiler diets as a means to optimize growth performance and maximize productivity in the poultry industry. The studies by [123–126] provide evidence of the positive impacts of spirulina supplementation on the productive performance of broilers, indicating that the inclusion of SP in broiler diets improves body weight gain (BWG) and feed conversion (FC). Spirulina supplementation at different levels, such as 0.9 g/kg, has been shown to promote BWG in broilers, which may be attributed to the absorption of minerals and vitamins present in SP. Furthermore, the studies highlight that SP supplementation enhances feed utilization efficiency, leading to improved feed conversion. This improvement in feed conversion is associated with the balanced microbial population in the gastrointestinal tract, which enhances the absorbability of dietary vitamins and minerals. This, in turn, contributes to the overall performance and health of broilers. Overall, the findings suggest that the inclusion of SP in broiler diets can have positive effects on body weight gain, feed conversion, and overall productivity. The study by [123] found that the inclusion of feed containing 1% SP led to a decrease in abdominal fat in broilers compared to the control

group and other supplemented groups. This suggests that spirulina supplementation may have a positive impact on reducing abdominal fat deposition in broilers.

Additionally, ref. [127] reported that dietary supplementation with spirulina significantly improved the carcass parameters of broilers in terms of factors such as meat yield and composition. Furthermore, ref. [128] observed that feeding birds with spirulina at levels of 40 and 80 g/kg in broiler diets resulted in significant differences ($p < 0.01$) in the meat color of chick muscles. This suggests that spirulina supplementation may influence the color of meat in broilers, potentially enhancing its visual appeal and quality.

It is important to note that not all studies investigating the addition of SP or other microalgae to broiler diets have reported positive effects on growth performance. Some studies have found no significant effect or even adverse effects on growth parameters [2,129]. El-Bahr et al. [130] reported that the inclusion of a 1 g SP/kg diet did not influence feed intake (FI), feed conversion ratio (FCR), or BWG in broilers. Similarly, Altmann et al. [129] found no noticeable effect on live weight or carcass weight when SP replaced half of the soy protein in broiler diets.

These contrasting results may be attributed to various factors, including the dosage and duration of microalgae supplementation, variations in experimental conditions, differences in bird strains, and variations in the composition and quality of the microalgae used. Additionally, the presence of anti-nutritional factors or interactions with other dietary components may also contribute to the observed effects.

Overall, the effects of microalgae supplementation on broiler growth performance can vary depending on the specific conditions and experimental setups. Further research is needed to better understand the optimal dosage, duration, and potential interactions of microalgae supplementation in broiler diets to achieve consistent and positive growth outcomes.

In this regard, ref. [32] highlights a potential negative effect of spirulina supplementation on broiler performance. The addition of spirulina to broiler diets led to a 15% lower performance compared to non-supplemented birds during a specific two-week period (21–35 days old). The researchers attributed this negative effect to the high digesta viscosity caused by the gelation of indigestible proteins present in spirulina.

Interestingly, even the addition of exogenous enzymes, such as lysozyme or Rovabio Excel AP, did not improve upon the negative findings. Although lysozyme was able to break down the cell walls of spirulina, it did not prevent the harmful gelation of the microalga proteins. The researchers suggested that combining lysozyme with a specialized exogenous peptidase could potentially enhance the digestion of spirulina proteins and prevent gelation.

This study emphasizes the importance of considering factors such as digestibility and interactions with other dietary components when evaluating the effects of microalgae supplementation in broiler diets. The gelation of proteins and subsequent increase in digesta viscosity can negatively impact nutrient utilization and performance in broiler chickens. Further research is needed to explore potential strategies to overcome these challenges and optimize the use of spirulina or other microalgae as feed additives in broiler production.

Indeed, the contradictory findings among studies regarding the effects of spirulina supplementation in broiler diets can be attributed to various factors. Here are some possible factors that may contribute to the inconsistencies.

1. Levels of spirulina: The concentration or inclusion level of spirulina in diets can vary among studies. Different levels of supplementation may have different effects on broiler performance.
2. Broiler hybrids: Different broiler strains or hybrids may respond differently to spirulina supplementation. Genetic variations among broiler breeds can influence their ability to utilize nutrients and respond to dietary interventions.

3. Broiler age: The age of broilers at the time of spirulina supplementation can influence the outcomes. The physiological and metabolic status of broilers change as they grow, which can affect their response to dietary interventions.
4. Housing conditions: Variances in housing conditions, such as temperature, humidity, and ventilation, can impact broiler performance. Environmental stressors may interact with spirulina supplementation and influence the results.
5. Feed preparation: The processing and formulation of broiler diets, including the method of spirulina incorporation, can affect the availability and digestibility of nutrients. Differences in feed preparation techniques among studies may contribute to variations in the results.
6. Administration method: The way spirulina is administered to broilers can vary. It could be mixed directly into the diet, offered as a separate supplement, or administered via drinking water. The mode of administration may influence the interaction between spirulina and the birds' digestive system.

Considering these factors, it is important to interpret the findings of individual studies within the context of the specific experimental conditions and methodology used. Further research with standardized protocols and larger sample sizes may help to establish more consistent conclusions regarding the effects of spirulina supplementation on broiler performance.

2.2. Growth-Promoting Effects of Curcuma

Nouzarian et al. [131] also found no significant effect on daily feed intake and body weight gain in chickens. Kumari et al. [99] observed that supplementation with 7.5 g/kg turmeric powder in feed resulted in the highest weight gain in birds. The variations in body weight values could be attributed to differences in agroclimatic conditions [100]. Ahlawat et al. [132] reported that supplementation of 3.3, 6.6, and 10 g/kg turmeric powder in broiler chicken improved feed efficiency, which is consistent with the findings supported by Kafi et al. [72].

Arslan et al. [132] found that turmeric supplementation at different rates improved feed conversion efficiency, with the best result observed at a supplementation rate of 1.5 percent. Shohe et al. [133] also observed that feed conversion efficiency was lowest in the group supplemented with 7.5 g turmeric powder/kg feed, followed by groups supplemented with 5, 2.5, and 1.5 g/kg turmeric powder.

It is important to note that the dietary supplementation of curcumin (Figures 1 and 2), the main active component in turmeric, is limited due to its low solubility in alkaline pH and susceptibility to hydrolysis when exposed to sunlight, resulting in poor absorption in animals [97]. However, studies on broiler chickens have shown that dietary supplementation of turmeric can lead to increased weight gain and improved feed conversion ratio [64].

Curcumin was found to have the potential to reduce stomach acid production, leading to an increase in blood sugar released from body cells. This decrease in sugar levels in body cells can induce hunger in chickens, resulting in a significantly higher feed intake and an increase in carcass weight within a shorter period of time. Additionally, since turmeric is readily available at a lower cost, incorporating it into the feed can help reduce overall feed expenses [134]. Durrani et al. [135] observed better feed efficiency in broiler chickens when turmeric was included in their diet at lower concentrations, specifically at 0.5%. This suggests that even at lower levels of supplementation, turmeric can positively impact feed efficiency in broiler chickens.

Turmeric powder (TRP) has shown positive effects on growth performance, as well as in reducing oxidative stress and improving the gut health of poultry [136,137]. Furthermore, the presence of curcumin, the active component in turmeric, in the chicken's diet may have potential health benefits for humans consuming the meat, including anti-inflammatory and antioxidant effects [138].

Therefore, incorporating turmeric powder into the diet of chickens can enhance the efficiency, health, and quality of the produced meat [139]. This also provides valuable insights into the potential impact of turmeric on human health.

Supplementing the basal diet with TRP in the range of 5 to 10 g per kilogram led to a significant reduction in abdominal fat in birds compared to the control group ($p < 0.05$). A study by Hosseini-Vashan et al. [140] demonstrated that supplementation of turmeric powder at 0.4 to 0.8 percent significantly reduced belly fat in birds during 28-day and 42-day fat sampling periods. Similarly, a study conducted in China showed that supplementation of 100 to 300 mg/kg of feeds significantly reduced the abdominal fat ratio ($p < 0.05$) compared to the control group [73]. Furthermore, adding 0.25, 0.5, and 0.75 percent of TRP to the diet of broiler chickens slaughtered at 49 days old resulted in a significant reduction in abdominal fat [141]. The mechanism behind fat reduction with TRP ingestion is attributed to its inhibitory effect on reactive oxygen species of enzymes, such as lipoxygenases, which play a role in adipogenesis processes [142]. The lipoxygenase pathway is known to contribute to adipose tissue inflammation in obesity-related animal models. Lipoxygenase isoforms are primarily produced by non-adipose cells and exhibit distinct translation patterns in subcutaneous and omental fatty tissues in humans [143].

Regulating effect on gut health can be attributed to curcumin's action on adipocyte death or glucose uptake from the blood [73,144]. The aforementioned mechanisms explain the significant decrease in abdominal fat observed with turmeric supplementation in broiler chickens' diets.

The effect of turmeric supplementation on liver weight in broiler chickens appears to be variable across different studies. Some studies have reported a slight but insignificant increase in liver weight with turmeric supplementation compared to the control group [68]. On the other hand, there are studies that have shown a significant reduction in liver weight with curcumin supplementation [15]. However, it is worth noting that the study by Yarru et al. [145] indicated that supplementation of TRP to aflatoxin-treated feeds improved liver weights in broiler chickens.

Turmeric, specifically its active compound curcumin, has been demonstrated to have hepatoprotective effects against various toxicants in mice, rats, and ducks, including carbon tetrachloride, aflatoxin B, and cyclophosphamide [146]. Curcuminoids have also shown a protective effect against aflatoxin B1.

The inconsistencies in the effect of turmeric on liver weight observed in different studies may be attributed to several factors, including variations in experimental conditions, turmeric dosage, duration of supplementation, and the presence of other variables that were not controlled or accounted for in the statistical analysis. It is important to consider these factors and conduct further research to better understand the impact of turmeric supplementation on liver weight in broiler chickens.

In previous research, turmeric has been extensively utilized in commercial broiler chickens and laying hens, yielding significant outcomes. Adding 0.75% turmeric to the feed increased BWG and enhanced the FCR of commercial broiler chickens, ultimately leading to increased gross profit [144]. Furthermore, incorporating turmeric into the diet has been shown to enhance the growth performance and egg production of poultry due to its natural antibiotic effects [68]. Moreover, turmeric supplementation improves antioxidant capacity, FCR, and carcass characteristics in broiler chickens [135]. Additionally, including 3% TRP in broiler chicken feed resulted in the lowest feed intake while simultaneously improving other performance measures [147]. All of the studies presented so far are summarized in Table 1.

Table 1. List of sources showing growth-promoting effects of spirulina and curcuma in broilers.

Drug Form	Dose	Effect	Source
Turmeric powder	1000 g of turmeric/kg	Enhanced feed utilization and improved weight gain	[118]
Spirulina platensis	1%	Increased body weight, decreased feed consumption ratios, improved blood parameters	[124]
Curcumin	200 mg/kg	Enhanced bird growth performance, behavioral patterns, and immunity	[137]
Turmeric powder	0.6 g/kg	Improved broiler performance index and net profit per bird	[148]
Spirulina platensis	0.5–1%	Improved broiler production performance and balancing of the redox status	[149]
Spirulina platensis phycocyanin	0, 0.25, 0.5, 0.75, and 1 g kg^{-1} diet	Enhanced growth-promoting, antioxidant, and anti-inflammatory properties	[150]
Spirulina plantesis	0.7–0.9 g/kg	Improved growth performance, blood parameters, and biochemical changes in serum and microbial load	[151]

3. Intestinal Morphology, Microbiota Modulation, and Immunomodulation

3.1. Effects of Spirulina on Intestinal Morphology, Microbiota Modulation, and Immunomodulation

The improvement in growth parameters observed in broilers may be attributed to the enhanced health of the birds, as evidenced by positive changes in the morphology of the small intestine. This includes increased villus length, higher numbers of goblet cells, and an enhanced absorption surface area, leading to improved nutrient digestibility and absorption. The phycocyanin component (PC) of spirulina has been shown to boost antioxidant enzyme activity and reduce the production of pro-inflammatory cytokines (IFN-γ and IL1β), contributing to a healthier gastrointestinal environment.

Spirulina has a beneficial impact on the gastrointestinal flora, promoting a healthier balance of microorganisms, and it also enhances the activities of digestive enzymes, leading to improved overall digestion of dry matter and nitrogen [122]. Additionally, it enhances the digestibility of nutrients, specifically amino acids, as well as protein synthesis [152], in addition to improving the utilization of apparent metabolizable energy [153]. Moreover, spirulina positively influences the composition of the intestinal microbial population by reducing the presence of harmful bacteria, such as *E. coli* and increasing the levels of beneficial lactic acid bacteria [154,155]. The presence of phycocyanin (PC) in spirulina stimulates the production of short-chain fatty acids and helps to reduce the presence of harmful pathogens in the intestines, thereby improving gut health [147].

The abundant essential amino acids found in spirulina play a crucial role in enhancing overall health status and body weight while also mitigating health disorders and mitigating the effects of heat stress. Additionally, phycocyanin (PC), a hydrophilic protein present in spirulina, helps regulate vascular colloidal osmotic pressure to maintain a balance with bodily fluids and promote optimal physiological functioning [156,157].

Phycocyanin (PC) exhibits antioxidant, anti-inflammatory, and immune-boosting activities [147,158]. Extracts derived from spirulina demonstrate antimicrobial effects that hinder the growth of various pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Klebsiella pneumonia* [159]. Moreover, spirulina possesses immunostimulant properties that enhance the secondary humoral response to SRBC antigens in broilers [160,161].

The supplementation of *Spirulina platensis* phycocyanin (SPC) led to a linear and quadratic increase in the activity of serum antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and total antioxidant capacity (TAC) [45,162,163]. Furthermore, SPC supplementation linearly reduces the levels of malondialdehyde (MDA), which can be attributed to its strong antioxidant activity [45,162,163]. This antioxidant activity is

believed to stem from the ability of spirulina extract to scavenge free radicals and chelate metal ions [164]. Mirzaie et al. [160] and Moustafa et al. [149] demonstrated that broilers fed diets containing spirulina exhibited higher activities of SOD and total antioxidant capacity, as well as lower levels of MDA, compared to those fed basal diets. Similarly, Park et al. [165] observed a linear increase in the enzyme activities of SOD and glutathione peroxidase (GPx) with increases in the spirulina supplementation levels (0.25%, 0.5%, 0.75%, and 1%) in broiler diets.

In the context of pro-inflammatory cytokines, one study showed that spirulina supplementation did not affect levels of IL1β, a potent pro-inflammatory cytokine involved in response to disease and infection [166]. The anti-inflammatory properties of spirulina may be attributed to its ability to inhibit the synthesis of proinflammatory cytokines, such as TNF-gamma, IL-6, and IL-1, as well as the activities of cyclooxygenase 2 (COX-2) enzymes and inducible nitric oxide synthase (iNOS) [167]. Additionally, SPC is present in spirulina and exhibits a strong anti-inflammatory effect [168].

The small intestine of broilers, especially the duodenum and jejunum, play a crucial role in the digestion and absorption of nutrients. A well-developed small intestine is associated with improved nutrient utilization and enhanced growth performance [165]. The development of the small intestine can be evaluated using morphometric measurements, such as villus height (VH) and crypt depth (CD), where longer villi and shallower crypts indicate the improvement of digestive efficiency via increased mucosal surface [165,169]. Additionally, the count of goblet cells is indicative of the condition of the small intestine [170].

The study demonstrated that supplementation with spirulina led to an increase in villus height, villus width, VH:CD ratio, and goblet cell count in the small intestine. These findings suggest a positive effect of SPC on gut health, nutrient utilization, and growth. These results are consistent with previous research conducted on broilers, in which it was also reported that SPC supplementation positively influences villus height, crypt depth, and goblet cell numbers in the intestine, ultimately improving nutrient absorption, FCR, and BWG [123,171]. Spirulina, when supplemented at different levels, exhibits an anti-inflammatory effect, as indicated by the decreased immunolabeling of caspase-3 and TNF-α. Interferon-α is an inflammatory cytokine produced during acute inflammation and plays a crucial role in the body's defense against cancer and infection. Caspase-3 is a lysosomal enzyme involved in protein degradation and is necessary for efficient cell apoptosis.

The anti-inflammatory properties of *Spirulina* spp., specifically SPC, may be attributed to its ability to downregulate the expression of pro-inflammatory cytokines, such as interleukin-2 (IL-2), IL-1β, interferon-γ (TNF-γ), and interferon-α (TNF-α) while increasing the expression of the anti-inflammatory cytokine IL-4 [172]. SPC acts as a COX-2 inhibitor and possesses hepatoprotective and anti-inflammatory activities [173]. Its hepatoprotective effect is linked to inhibited production of hepatocyte growth factor and TGF-β1, which prevent inflammatory infiltration [75]. Research conducted by Martinez et al. [174] demonstrated that SPC preparation can suppress TNF-α, IL-6, iNOS, COX-2, and neutrophil infiltration at the site of inflammation [175].

3.2. Effects of Curcuma on Intestinal Morphology, Microbiota Modulation, and Immunomodulation

Emadi et al. [176] conducted a study on broiler chickens and found that the inclusion of TRP at levels of 0.25%, 0.5%, and 0.75% in the diet did not have a significant effect on total protein and albumin concentrations at 21 days of age. Kumari et al. [99] reported no changes in the activity of liver enzymes, such as ALT, AST, and ALP, in the treatment group with TRP in broiler chickens. However, it is important to consider that factors such as chicken breed, level of turmeric inclusion, duration of the experiment, and environmental factors may have contributed to variations in results. Ekine et al. [117] observed an increase in AST and ALT levels with the inclusion of 250 g of TRP per 25 kg of feed, but this increase did not negatively impact the liver or muscle of broiler birds. Significant hepatic damage is typically indicated by AST and ALT levels exceeding 275 and 800 μL, respectively. In this

study, AST and ALT levels were not statistically influenced by dietary turmeric inclusion. Regarding alkaline phosphatase (ALP), it was observed to increase in the treatment groups (T2 and T3) compared to the control group (TC), which contrasts with the findings of Kumari et al. [99] and Mehala and Moorthy [100], where no changes in liver enzyme activities, including ALP, were observed with turmeric inclusion in the broiler chicken diet. However, ALP levels were lower in the treatment group T1, which aligns with the results of Emadi and Kermanshahi [176], where decreased ALT and ALP enzyme activities were observed in broiler birds fed turmeric at varying levels. The discrepancies between these studies may be attributed to factors such as different levels of turmeric inclusion and variations in the bioactive substances present in the turmeric plant, which depend on factors such as plant species, soil type, harvest season, and the preparation process. In avian species, an increase in ALP levels has been associated with increased osteoblast activity and various disease states, including traumatic, neoplastic, and infectious diseases.

Curcumin (Figure 1), a highly pleiotropic component found in turmeric, has been found to have significant effects on inflammatory responses. It exerts its anti-inflammatory effects by downregulating the activities of enzymes, such as lipoxygenase, cyclooxygenase-2, and inducible nitric oxide synthase [177]. Furthermore, curcumin can interact with various molecular targets involved in inflammation, leading to inhibitory effects on cytokines [79].

Studies have shown that curcumin has the ability to decrease the levels of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and various interleukins (IL-1, IL-1β, IL-6, and IL-8) [178]. It also inhibits the activation of nuclear factor-kappa B (NF-κB), a transcription factor involved in the regulation of inflammatory responses and reduces cell proliferation [84,177]. Overall, curcumin's anti-inflammatory properties are attributed to its ability to modulate various inflammatory mediators and signaling pathways, providing a potential therapeutic approach for managing inflammatory conditions.

Locally sourced turmeric is indeed known for its antioxidant properties. Antioxidants play a crucial role in protecting cells and tissues from oxidative damage caused by free radicals and reactive oxygen species. In the context of chicken eggs, maintaining oxidative stability is important to prevent lipid oxidation and preserve their quality. Turmeric, with its antioxidant function, has been found to help in this regard. A study by Laganá et al. [139] demonstrated that antioxidant diets containing turmeric can effectively inhibit yolk lipid oxidation and contribute to the preservation of egg quality.

Curcumin, the main bioactive compound in turmeric, was shown to have the ability to neutralize superoxide anion and hydroxyl radicals, which are highly reactive and can cause oxidative damage [73]. Moreover, the supplementation of broiler chickens with dietary turmeric rhizome extracts was found to significantly enhance the activity of SOD, an important antioxidant enzyme that helps neutralize superoxide radicals [73,179].

Curcumin exhibits antiviral properties against various viruses, including influenza. It demonstrated effectiveness against influenza viruses, altering cellular metabolism and serving multiple functions to hinder viral invasion. By binding to the viral envelope, curcumin renders the viral pathogens inactive, preventing them from infecting cells and causing harm. This suggests that curcumin has the potential to neutralize viruses before they can initiate infection and cause subsequent damage to cells [180].

Numerous studies have demonstrated the immunomodulatory properties of turmeric, highlighting its ability to enhance the body's defense against disease-causing microorganisms. Turmeric exhibits immediate antimicrobial effects against pathogens, acting as a natural antibiotic. Moreover, the bioactive compounds present in turmeric contribute to the reduction of infection-induced inflammation and stimulate an immune response in chickens. Turmeric, when incorporated as a phytobiotic, facilitates the healing of lymphocytes in lymphoid organs, thus providing a mechanism for cellular repair [78,84].

Furthermore, research has shown that the inclusion of TRP in broiler chicken feed can elevate the levels of immunoglobulins, such as IgM, IgA, and IgG, while significantly reducing the monocyte ratio [176]. Mehala and Moorthy [100] also reported an enhanced

immune response in broiler chickens with the dietary inclusion of turmeric. Additionally, when used as a natural feed additive, *Curcuma longa* has been found to serve as an immune enhancer for broilers against *Pasteurella multocida* infection [181].

The supplementation of turmeric in broiler chicken diets has been shown to have regulatory effects on their hematological parameters, as reported by Dono [78]. In Ross male broiler chickens, the addition of TRP to their feed resulted in an increase in hemoglobin, total cholesterol, and HDL cholesterol levels. Conversely, LDL cholesterol, VLDL cholesterol, and red blood cell counts were decreased. A significant decrease in blood albumin levels was also observed in the study [176].

Additionally, a study on Fayoumi broilers demonstrated that incorporating turmeric in their diets improved their ingestive behavior (drinking and feeding) and growth. Furthermore, it led to an increase in total serum protein, globulins, calcium, and phosphorus levels [182].

Curcumin has been demonstrated to have various positive effects on gut architecture and gut bacteria. It stimulates the release of enzymes, such as amylase, protease, and bile acids, in the stomach, promoting digestion and nutrient absorption [183] (Rajput et al., 2013). The gastrointestinal system of chickens, which houses diverse and complex microbiota, can benefit from adjustments in feed composition to include turmeric. Studies have shown that feeding broiler chicks phenolic compounds, such as curcumin, reduces gastrointestinal inflammation and enhances nutrient absorption [184]. In fact, supplementation with curcumin at a dosage of 200 mg/kg feed has been shown to improve overall metabolic efficiency, increase the absorption area of the small intestine through increased villus height, and reduce abdominal fat deposition [183].

Moreover, an increase in the height of intestinal villi and the villus height to crypt depth ratio has been linked to improved nutrient absorption rates [185]. The addition of TRP to the diet of hens was found to increase the abundance of the beneficial bacteria *Lactobacillus* spp. in their intestines [83]. However, it should be noted that in vitro studies have shown that high doses of turmeric extracts can inhibit the growth of *Lactobacillus*, while whole TRP at certain concentrations can completely suppress lactobacilli [186].

Supplementation of TRP has a significant impact on the length and weight of the small intestine ($p < 0.05$) [187]. The length of the small intestine increased significantly in broilers provided with 5–10 g compared with 0 or 2.5 g of TRP per kilogram in their basal diet. However, the weight of the small intestine decreased significantly with higher levels of TRP supplementation. It is noteworthy that while the length of the small intestine increased, its weight decreased with increasing TRP levels. This longer small intestine offers advantages, such as enhanced efficiency of fluid and nutrient absorption, as well as improved breakdown of ingested feed. The presence of villi and microvilli in the intestinal wall increases the surface area for absorption, which could contribute to the favorable growth of broiler chickens at an early age in addition to their recognized role as growth promoters. This finding was observed in a study conducted in 2023 [187].

The weight of the bursa of Fabricius in broiler chickens showed a significant increase with the supplementation of TRP in the basal diet compared to the control group ($p < 0.05$) [187]. The bursa of Fabricius plays a crucial role in the development of B-cells, which are responsible for producing antibodies. This finding is supported by the results of the study conducted by Naderi et al. [188], where the bursa of Fabricius exhibited a numerically larger mass in the turmeric and cinnamon-supplemented groups compared to the control and avilamycin-supplemented groups.

The supplementation of TRP in the basal diets did not have a significant effect on the weights of the pancreas, gall bladder, heart, and spleen in broiler chickens ($p > 0.05$) [187], which is consistent with the findings of previous studies. Mondal et al. [68] reported that supplementation of TRP at 0.5 to 1.5 percent did not have a significant impact on the heart weight of broiler chickens. Similarly, Hussein et al. [64] found that TRP did not significantly affect the relative weight of the pancreas in broiler chickens when supplemented at a rate of 0.25 to 0.5 percent. Qasem et al. [74] also reported that supplementation of TRP in the diet

of broiler chickens at a rate of 10–20 g per kilogram of basal feed did not have a significant effect on the relative weight of the pancreas. [147]. All of the studies presented so far are summarized in Tables 2 and 3.

Table 2. List of sources showing microbiota modulation effects of spirulina and curcuma in broilers.

Drug Form	Dose	Effect	Source
Spirulinaplatensis	1%	Decreased the numbers of coliform in the ileum and the caecum	[34]
Turmeric powder	1%	Increased the abundance of *Lactobacillus* spp. in the chicken intestines	[83]
Spirulina platensis powder	10 g/kg	Increased the levels of beneficial lactic acid bacteria	[155]
Spirulinaplatensis	1–2 g/kg	Increased the *Lactobacillus* spp. count	[189]

Table 3. List of sources showing antibacterial, antioxidant, and anti-inflammatory properties of spirulina and curcuma in broilers.

Drug Form	Dose	Effect	Source
Turmeric rhizome extract	300 mg/kg	Enhanced antioxidant capability, growth performance, and breast muscle weight ratio and reduced abdominal fat ratio	[73]
Turmeric rhizome extract	50 and 100 mg/kg curcumin	Improved antioxidant capability, high growth performance, increased breast muscle weight ratio, reduction in the abdominal fat ratio	[104]
Curcuma spp.		Increased anti-inflammatory activity and weight index of lymphoid	[117]
Curcuma longa (Turmeric)	0.5 and 1.0%	Increased both erythrocytic and total leukocytic counts in addition to bursa, thymus, and spleen weight	[138]
Curcumin powder	2.000 mg/kg	Significantly decreased absolute and relative abdominal fat weight and markedly decreased concentrations of plasma low-density lipoprotein cholesterol and plasma and hepatic triglyceride	[147]
Curcumin		Enhanced anti-inflammatory properties	[177]
Curcuma longa powder	1%	Immune enhancer	[181]
Spirulina powder	2 g Spirulina/kg feed	Improved gut integrity and immunity in broiler production	[171]
Spirulina platensis phycocyanin	0, 0.25, 0.5, 0.75, and 1 g kg^{-1} diet	Enhanced antioxidant and anti-inflammatory properties	[150]

4. Conclusions

Given the ban on growth-promoting antibiotics, research on gut mucosa-associated lymphoid tissue has been ongoing in order to develop pre- and probiotics that are best suited for stimulating the gut immune system. Following our review of literature sources, we can state that the addition of *Spirulina platensis* or curcumin positively influences both productive performances and immunity in broiler chickens, improving humoral and cellular immune responses and lymphoid organ development. The anti-inflammatory

effect of *Spirulina platensis* supplementation at different levels has been shown to decrease caspase-3 and TNF-α immunolabeling; reduce IL-1β, IL-2, and IL-4 interferon-γ expression; reduce IL-1β, IL-2, and IFN-γ; and increase the expression of anti-inflammatory cytokine IL-4. Spirulina inhibits the synthesis of cytokines IL-1, IL-6, and TNF-gamma and the activities of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) enzymes. Curcumin supplementation decreases the levels of TNF-α and IL-1β, IL-6, IL- 2, and IL-18.

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Review

Persistent Organic Pollutants (POPs): A Review Focused on Occurrence and Incidence in Animal Feed and Cow Milk

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Abstract: Persistent organic pollutants have particular ecotoxicological importance and they are amongst the most harmful groups of persistent pollutants. The complexity of persistent organic pollutants highlights the different sources of pollution from which they came and, depending on which, their profile could be characterized. In the first part of this review, the main characteristics of persistent organic pollutants were described, focusing on their complexity and toxic potential in relation to environmental elements. The second part of the review includes data related to the occurrence and incidence of persistent organic pollutants in different types of feed and cow's milk, focusing on the characteristic profile of pollutants as an indicator of the sources of pollution. Moreover, a description regarding the timing and duration of the contamination of feed and milk was carried out, evaluating the distribution of pollutants within the analyzed samples and highlighting those whose presence is predominant or whose residues persist in the environment for long periods. The review concludes that the identification of pollution sources associated with different proportions of organic pollutants found in different samples could represent a suitable solution for biomonitoring the potential contamination in a geographical area.

Keywords: persistent organic pollutants; feed; milk; environment; pollution

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1. Introduction

Environmental pollution represents a threat to human health, quality of life and the natural function of ecosystems [1]. Anthropic actions are the main causes of environmental pollution. Whether intentional or accidental, humans can generate various pollutants with direct or indirect negative impact on the ecosystem elements, such as the soil, water, air, plants, animals and even the human body [2,3].

Environmental pollution has been widely studied and the most important properties of the most prevalent pollutants were reported. The organic pollutants are qualified among the most harmful pollutants due to the eco-toxicological risks and their slow degradability; hence, their persistence [4,5].

Recent increases in industrial and technological activities have led to high emissions of pollutants. Generated from various developing productive sectors, persistent organic pollutants (POPs) have become a real threat for modern society due to their negative effects on the environment, animal welfare, health and the safety of animal products.

Synthesized mostly by anthropogenic sources, POPs are halogenated, lipophilic, organic compounds, with a very stable chemical structure, which are resistant to photolytic and biological degradation and which have a negative impact on the environment, flora, fauna and human health [5–7].

The character of POPs facilitates their accumulation in fatty tissues [8]. Most of the studies have focused on the biological behavior of POPs due to their ability to transfer and accumulate along the food chain [9,10] through bio-amplifying and volatilization, ending in more dangerous threats [11,12], especially if slow metabolic conditions occur [5,6,8,11,13–15].

Lately, high amounts of pollutants were released into the environment and have contaminated the natural substrates and the food chain [16]. A strong network of hazards was established between pollution, vegetal production, animal production and human consumers due to the possibility of pollutants' transfer along the food chain [17–20]. Food samples that have been tested were shown to be contaminated with several types of POPs [21]. Animal-originated food os one of the main vehicles of human exposure to POPs contamination [22–24].

Feed is particularly implicated in the transfer and occurrence of such contaminants in food, especially in dairy products. Studies of POPs in the food chain have recently focused on the correlations between feed production and food safety issues [25–28] or POPs contamination and effects on the human body [29–31]. In recent years, most research has included a study of the transfer of pollutants from feed to animals, and their accumulation and excretion in milk [32].

Considering the potential negative effects of pollutants in feedstuffs on animal yields and, subsequently on public health, assessing POPs contamination level from animal feed is really important to the safety of consumers [12,19,33–39].

This review aims to describe different classes of POPs as well as to discriminate between specific sources of pollution as it relates to the concentrations of POPs in feed and milk. Thus, the sources of POPs were analyzed, as were the contamination and the proportions of the most prevalent pollutants, as well as the methods used in their monitoring and quantification.

2. Characterization of POPs

Regulations addressing the persistent organic pollutants have aimed at reducing the consequences of pollution, eliminating existing pollutants or preventing potential contamination [40]. According to the European Chemical Agency [41], at least 12 types of organic substances, the so called "dirty dozen", were included, in 2001, under the Listing of POPs in the Stockholm Convention (Table 1). The Convention aimed to protect human health and the environment, especially by safely eliminating, reducing the production and decreasing use of the 12 dangerous substances and was signed, in the beginning, by 115 states. Currently, the Stockholm Convention is signed by 152 states and ratified by 184 states [41]. The list of banned or restricted organic pollutants currently reaches about 28 hazardous chemicals [42] and includes three categories of POPs distinguished by their originating anthropogenic process [12,40,43].

The most popular group of POPs is represented by the synthetic chemicals produced for agricultural usage in particular, which include organochlorine pesticides (OCPs) such as aldrin, dieldrin, dichloro–diphenyl–tetrachloroethane (DDT), heptachlor, mirex, chlordane, toxaphene and endrin. Some of them can be also used in dyes or plastics [5,44].

The second group of POPs refers to chemical compounds commercially produced for various industrial applications [45] or released from the chemical industry as secondary substances [46], including hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), perfluorinated compounds (PFCs) or brominated compounds (BFRs) [5,45,46].

Table 1. Characteristics of POPs listed in the Stockholm Convention.

Category	Type of POPs	Description	Source of Pollution	Ref.
OCPs	Aldrin	Quickly converted to dieldrin; Low toxicity to plants and high toxicity to animals and humans; contamination in dairy products and meat	Industrial activities Agricultural activities	[5,44]
	Dieldrin	High concentrations (transformation of aldrin into dieldrin); residues frequently found in air, water, soil, in the bodies of birds, mammals or humans (exposed through food, especially dairy products and meat); $t\frac{1}{2}$ = 5 years		
	Chlordane	Broad spectrum of action for crops; contamination by air; $t\frac{1}{2}$ = 1 year		
	DDT	$t\frac{1}{2}$ = 10 years (more than 50 % of the initial amounts can remain in the soil after 10–15 years after application)		
	Endrin	No accumulation in fatty tissues (compared to other organic pollutants), toxicity especially for aquatic animals; $t\frac{1}{2}$ in soil = 12 years	Industrial activities Agricultural activities	
	Mirex	$t\frac{1}{2}$ = 10 years		
	Toxaphene	$t\frac{1}{2}$ in soil = 12 years		
Industrial chemicals	ΣPCBs	Includes 209 different types of PCBs, of which 13 substances have particularly high toxicity, formed as a result of incomplete combustion from various industrial processes	Waste chemicals, plastic and rubber products or electrical equipment	[9,45–49]
	HCB	Residues of the pesticide production; residue of incomplete combustion	Chemical waste	[5]
Combustion chemicals	PCDDs /PCDFs	Highly toxic chemical compounds originating from industrial processes including ~210 polychlorinated aromatic chemical compounds: PCDDs/dioxins and PCDFs/furans $t\frac{1}{2}$ = ~7 years; the possibility of accumulation in animal and human tissues through contaminated food; low concentrations in the environment, but persistent and easily transferred	Chemical and industrial processes (herbicides/pesticides production, metalworking) Combustion processes (waste incineration) Natural events (volcanic eruptions, wildfires) Involuntary emissions from burning plastic, wood waste or agricultural waste contaminated with pesticides Vehicle emissions	[5,34,37,50,51]
	DL–PCBs	Include 12 compounds out of 209 types of PCBs, which are formed in combustion processes and have toxic properties and potential effects similar to PCDDs/PCDFs	Incomplete combustion (natural and anthropogenic) Incineration processes: Distillation of coal Emissions from transport Wood/forest vegetation burning Volcanic eruptions Oil exploitations Biomass burning	[52]
	ΣPAHs	Organic compounds with two or more condensed aromatic cores, with specific structures and variable toxicity		[5,38,53–55]

POPs—persistent organic pollutants; OCPs—organochlorine pesticides; DDT—dichlorodifeniltricloroetano; HCB—hexachlorobenzene; PCBs—polychlorinated biphenyls; PCDDs/PCDFs—polychlorinated dibenzodioxins/dibenzofurans; DL—PCB–dioxin-like PCBs; PAHs—polycyclic aromatic hydrocarbons; $t\frac{1}{2}$—half time. See reference [40] for POPs category symbol shape.

Another group of chemical compounds that are formed from incomplete combustion of organic matter represents the third category of POPs: dioxins/polychlorinated dibenzodioxins (PCDDs), furans/polychlorinated dibenzofurans (PCDFs) or polycyclic aromatic hydrocarbons (PAHs) [5,34,37,38,50–55].

Because of their toxic effects, 33 PAHs were described by the European Scientific Committee on Food as having a high risk for the environment. Moreover, Shafy and Man-

sour [54] focused their attention on some PAHs with higher toxic potential. Together with the Canadian Council of Ministers of the Environment [56], they classified 7,12 dimethylbenzoanthracene (DMBA) and benzo(a)pyrene (BaP) as two of the most toxic POPs.

Considering the increasing of amounts organic pollutants in the environment, several authors have focused their attention on describing the profile of POPs. Table 1 summarizes their works, highlighting the characteristics of the main categories of OCPs, the characteristics of chemical substances from industrial processes and combustion processes and also the specific sources of pollution identified in analytical studies. Manciulea and Dumitrescu [5] have classified the POPs into three categories according to the source of pollution; the three categories of POPs mentioned by these authors [5] include the same types of pollutants listed in the Stockholm Convention [12,40,43]: the OCPs, the industrial chemical pollutants and the combustion chemicals.

OCPs are persistent organic pollutants with broad spectrum of action, high chemical stability and toxic characteristics [57]. The characteristics of OCPs were underlined in a work conducted by Rusu et al. [35], who identified residual amounts of pesticides in former areas producing OCPs, but without current productive activity (production available up to 1990s). Thus, the occurrence of OCP residues in samples, in the absence of recent contamination, highlighted their long-lasting half–life. Other authors have also observed the presence of OCP residues in the environment even after their use was reduced or was even completely banned [58–60].

Amongst the causes of pollution, agriculture, together with the industrial production of agricultural substances or plastics, paints, rubbers or electronics, are the main sources of some of the most toxic OCPs [5,44] (Table 1). Recent data presented by Chavoshani et al. [61] have confirmed that intensive agriculture, over time, has negative effects on the environment. Even if, nowadays, many of the OCPs have been banned or limited, it is important not to forget about the contamination that has existed since different OCPs were permitted.

Almost two decades since the Stockholm Convention prohibited or limited the OCP usage in agriculture, studies are still being carried out to understand their residual state and the possible risks that may appear on the environment. Khuman et al. [59] reported a general trend of decreasing levels of OCPs in urban, agricultural and industrial soils. However, the study showed that historical OCPs and their breakdown products remain a threat to the environment; therefore, the agricultural activities are still included in the category of polluting factors.

The industrial chemical pollutants group of POPs includes chemical compounds such as HCBs or PCBs, i.e., pollutants that can be generally issued as residues from plastics production, but also residues from pesticides or other chlorinated organic compounds. In some cases, HCBs or PCBs occur as a consequence of incomplete combustion of chemical waste, rubber production, electrical equipment production or other industrial processes [5,45,62,63]. Chaukura et al. [64] stated that microplastics occurring in the environment as residues of various industrial processes are also important carriers of POPs, especially of OCPs, PCBs or PAHs.

PCBs are a group of chemical compounds included in the Stockholm Convention among the 12 POPs in the "dirty dozen" category [10]. PCBs can be released from electrical equipment or construction materials [9,65,66], but they can also be released from various industrial processes [67,68]. To limit the negative effects on the environment, the intentional production of PCBs for industrial application has been limited or even banned in some countries [10,69]. The environmental negative impact of PCBs persists through the products already manufactured before the ban in the form of polluting waste [70]. With respect to the negative contribution of PCBs from various industrial processes to environmental pollution, no clear measures to control were found.

For humans, PCBs are hazardous due to their high capacity for contaminating the food chain. Although they can also enter the human body through inhalation or dermal exposure [71], foodstuffs seem to be the main route for human exposure [72].

HCB is a chemical compound with persistent organic characteristics [73], whose conscious production and use have been limited or banned in most countries. However, HCB can be produced unintentionally in the processes of incomplete combustion of various chemical substances [74]. According to Starek-Swiechowicz et al. [75], HCB has high bioaccumulation potential especially for living organisms. Different amounts of HCB residues were detected in various substrates, such as fodder crops, in food (milk, fish, vegetables, meat), in blood or in adipose tissue [75,76].

Regarding combustion chemicals, they are highly toxic chemical compounds generated by incomplete combustion from industrial processes. According to Table 1, the characteristics of these POPs have been mentioned in various works [5,38,53–55].

3. Occurrence and Incidence of POPs

3.1. Milk

Due to its nutritional qualities, milk is one of the most important animal products [77–79]. The contamination of milk with POPs has been studied by various authors [80–83]. The literature describes pollutant profiles related to the pollution sources in some areas, the relationship between some pollutants categories and the associated pollution sources, the quantitative profile, the description of the methods and analytical techniques used or the investigation of the associated risk category (Table 2).

Table 2. Summary of POPs presence in milk and sources of pollution.

Location	Collected Area	Sample	POPs	Method	POPs Level (ng/g) Mean and/or Range (Min–Max)			Annex *	References
					●	▲	■		
Source of the milk: FARM									
France	Vicinity of a hazardous municipal waste incinerator	Raw milk collected from animals exposed to a 10-week long-term intake of contaminated hay	ΣPCBs ▲	GC –HRMS	-	2.22–3524.07	-	A, C	[84]
			PCDDs/TCDD ■		-	-	0.48	C	
			PCDDs/OCDD ■		-	-	2.31	C	
			PCDFs/TCDD ■		-	-	0.52	C	
			PCDFs/OCDD ■		-	-	0.16	C	
Calabria, Italy	Dairy farms near to Calabria region	36 milk samples (raw, pasteurized, semi-skimmed, whole)	ΣPAHs ■	HPLC –MS	-	-	5.42	A	[85]
Piedmont, Italy	No reported sources of pollution	Small and medium dairy farms	PCDDs/PCDFs ■	GC –HRMS	-	-	1.91	C	[37]
			DL–PCBs ■		-	-	5.18	A, C	
Source of the milk: MARKET									
Faisalabad, Pakistan	Urban area: dairy farms located near the cities	5 raw milk samples	α–endosulfan ●	GC –ECD	22.6–41.4	-	-	A	[86]
			β–endosulfan ●		4.06	-	-	A	
			DDE ●		1.52	-	-	A	
			γ–HCH ●		2.13	-	-	A	
Bacău, Romania	Villages around Bacău city, Comănești town, Târgu Ocna town, close to one of the greatest OCPs producers up to 1990	18 raw cow milk samples 18 pasteurized cow milk samples	α, β, γ–HCH ●	GS/MS	0.74–7.8	-	-	A	[35]
France	Dairy experimental station	Raw cow milk	ΣPAHs ■	GC–MS	-	-	0.08	A	[87]
Poland	Dairy farms located in unpolluted areas (no industry, no main roads, no chemical fertilizers) Negligible sources of pollution	Raw cow milk collected from animal exposed to contaminated feed (molassed sugar beet pellets)	PCDD/PCDFs ■	HRGC –HRMS	-	-	4.31	C	[34]
			DL–PCBs ■		-	-	0.71	A, C	
Punjab, India	Intensive dairy production, typical feeding management	Raw milk samples collected directly from the cans/ cooling tanks	γ–HCH ●	GC–MS	0.46	-	-	A	[33]
			DDE ●		0.83	-	-	A	
			Endosulphan sulphate ●		1.01	-	-	A	

Table 2. *Cont.*

Location	Collected Area	Sample	POPs	Method	POPs Level (ng/g) Mean and/or Range (Min–Max)			Annex *	References
					●	▲	■		
Italy	Different dairy farms from Province of Taranto, near industrial area	Raw milk	PCDD/PCDFs ■	HRGC –HRMS	-	-	0.28	C	[88]
			DL–PCBs ■		-	-	0.82	A,C	
			ΣPCBs ▲		-	2.53	-	A, C	
Catalonia region, Spain	Small local markets, supermarkets and big grocery stores	Commercial milk	ΣPAHs ■	HRGC /HRMS	-	-	0.47	A	[89]
Arkhangelsk Russia	Close to different urban areas from Northwest Russia	13 pasteurized milk samples purchased from supermarkets, shops and local markets; produced locally	ΣDDTs ●	GC	0.06–1.09	-	-	B	[90]
			ΣHCHs ●		0.04–0.22	-	-	A	
			ΣCHLs ●		0.01–0.02	-	-	A	
			Mirex ●		<0.01	-	-	A	
			DDE ●		1.4–17.0	-	-	A	
			HCB ▲		-	0.1–0.38	-	A, C	
			ΣPCBs ▲		-	0.17–1.1	-	A, C	
Qazvin, Iran	Marketed in urban area	7 pasteurized full–fat commercial milk samples	PCDDs/PCDFs ■	HRGC /HRMS	-	-	0.74	C	[91]
			DL–PCBs ■		-	-	0.13	A, C	
Sacramento, California	Local market area, California farms	23 commercial whole milk samples	ΣPCBs ▲	GC –MS/MS	-	142.8–172.4	-	A, C	[36]
Source of the milk: MARKET									
Tehran, Iran	Marketed in urban area	240 samples (pasteurized; sterilized)	ΣPAHs ■	MSPE /GC–MS	-	-	1.42	A	[92]
Tehren, Iran	Local market area	120 pasteurized cow milk samples, collected in 2 different seasons	ΣPCBs ▲	GC –ECD	-	18.92	-	A, C	[93]
			DL–PCBs ■		-	-	0.49	A, C	
China	Marketed in urban area/Traditional dairy farms	89 milk samples	ΣPAHs ■	GC–MS	-	-	8.85	A	[38]
Europe					-	-	9.38	A	
Australia					-	-	8.18	A	
Italy	Marketed in urban area (Southern Italy)	Whole milk	PCDD/PCDFs ■	HRGC –HRMS	-	-	1.19	C	[94]
			DL–PCBs ■		-	-	0.18	A,C	
Italy	Marketed milk	Cow milk	PCDD/PCDFs ■	GC –HRMS	-	-	0.155	C	[95]
			DL–PCBs ■		-	-	0.506	A,C	
Source of the milk: FARMS and MARKET									
* different regions: Egypt, Spain, Slovenia, Mexico	* cow milk of different origins: market zone—Egypt, Spain; dairy farms—Slovenia, Mexico.	Egypt—ns.; Spain – 94 pasteurized milk samples; Slovenia–174 raw milk samples; Mexico—355 raw milk sample	DDT ●	GC –ECD	15.9	-	-	B	[96]
			HCH ●		9.4	-	-	A	
			HCB ▲		-	1.6	-	A, C	
Cairo, Egypt	Urban region	18 milk samples from different sources (raw milk from farm, commercial and pasteurized milk)	ΣPAHs ■	GC/MS	-	-	0.37–1.01	A	[97]

● Organochlorine pesticides ▲ Industrial chemicals ■ Combustion chemicals

A—measures for elimination production and use
B—measures for restricting production and use
C—measures for accidental release

POPs—persistent organic pollutants; OCPs—organochlorine pesticides; Ind.Chem—Industrial Chemicals; Comb.—Pollutants from Combustion; HCH—hexachlorocyclohexane; DDE—dichlorodifenildicloroetano; DDT—dichlorodifeniltricloroetano; HCB—hexachlorobenzene; PCBs—polychlorinated biphenyls; PCDD/F—polychlorinated dibenzodioxins/dibenzofurans; TCDD/F—tetrachlorinated dibenzodioxins/dibenzofurans; OCDD/F—octachlorinated dibenzodioxins/dibenzofurans; DL—PCB-dioxin–like PCBs; PAHs—polycyclic aromatic hydrocarbons; GC—gas chromatography (HR/MS—high resolution/mass spectrometry; ECD—electron capture detector); HPLC/MS—high performance liquid chromatography; MS—mass spectrometry; Ns.—unspecified; CHLs—oxychlordane; cis—chlordane; trans—nonachlor. * Annex = Annexes from Stockholm Convention; this includes the list with the chemicals targeted by the Stockholm Convention: Annex A (Elimination); Annex B (Restriction); Annex C (Unintentional production). See reference [40] for POPs category symbol shape.

Most of the authors have analyzed fresh milk samples collected from farms of different sizes, with different levels of technology, located in areas with various sources of pollution [33–35,37,84–87]. Other authors have analyzed the samples of milk sold in different types of supermarkets in Egypt [96,97], Mexico [96], Spain [89,96], Russia [90], Italy [98], Iran [91–93], California [36], China or Australia [38].

For the investigation of pollutants, most of the authors have used gas chromatographic analysis coupled with mass spectrometry (GC–MS), high resolution-mass spectrometry (HRMS) or electron capture detector (ECD), and only a few authors have used high-performance liquid chromatography–mass spectrometry (HPLC–MS) [85] or HPLC with fluorescence detector [98,99].

In most of the samples, the pollutants from incomplete combustion prevailed, especially PAHs, PCDDs and PCDFs, with amounts from 0.08 to 26.6 ng/g. Some authors [33,35,86,90,96] also reported amounts of OCPs between 0.01 and 41.4 ng/g. The pollutants from industrial activity were identified only in a few cases [36,84,90,93] as being at risky levels for human health (0.09 to 3524.07 ng/g). In most of the studies, the pollutants belonged almost always to high-risk categories included in Annex A in the Listing of POPs in the Stockholm Convention (high toxic potential, regulated through measures for stopping their manufacturing and usage).

In most situations, the feed was implicated as the source of POPs in dairy animals, which ultimately resulted detectable levels of POPs in milk. Rusu et al. [35] investigated several types of milk samples (raw, pasteurized) collected from some farms in the northeast area of Romania, located close to one of the former greatest OCP producers in Romania (up to the 1990s). OCPs residues varied between 0.74 and 7.8 ng/g, suggesting long lasting persistence of OCPs in the environment and the transfer on the soil–fodder crops–grazing animals–milk chain.

Comparable results on the contamination of milk with POPs through feed was reported by Costera et al. [84] and Piskorska-Pliszczynska et al. [34]; Costera et al. [84] analyzed milk yielded by animals fed with hay produced in the vicinity of a municipal waste incinerator, and Piskorska-Pliszczynska et al. [34] analyzed molasses sugar beet pellets contaminated accidentally from different sources.

In both studies, the possibility of transferring organic pollutants such as PCDDs/PCDFs, PCBs and DL–PCBs following the long-term consumption of contaminated feed was followed. Costera et al. [84] blamed only the pollution from the municipal waste incinerator, while Piskorska-Pliszczynska et al. [34] have not identified important sources of pollution (excepting the accidental contamination) that could have modified the pollutant profile in milk. Without any exact sources of pollution, the conclusions presented by Piskorska-Pliszczynska et al. [34] have shown, therefore, that the presence of pollutants in milk (from 0.71 to 4.13 ng/g) can originated in previously contaminated feedstuffs, brought from outside the area.

Polder et al. [90] studied the contamination profile with POPs for 13 pasteurized milk samples purchased from supermarkets, shops and local markets close to different urban areas in northwest Russia. Variable amounts of OCPs, including DDT, hexachlorocyclohexane (HCH), chlordane, mirex or DDE and some pollutants from the processing of industrial substances such as HCB and PCBs, were identified. The authors emphasized the complexity of organic pollutants and how much the pollution sources associated with urban areas can influence the total amounts of POPs. Comparable results were obtained by Sajid [86] on samples of raw milk collected from farms located near cities. Important quantities of OCPs (1.52 to 41.4 ng/g) were quantified, along with high amounts of α, β–endosulphan and lower amount of HCH, dieldrin and DDE.

There were no reported sources of pollution in the study conducted by Desiato et al. [37] on milk samples collected from farms of different sizes in Italy. However, the presence of PCDDs/PCDFs (1.91 ng/g) and DL–PCBs (5.18 ng/g) was reported, and the atmospheric pollution was incriminated.

Other authors have reported OCP residues in milk, such as DDT and HCH (9.4 to 15.4 ng/g), HCB residues (1.6 ng/g) [96] and even the pollutants from incomplete combustion, especially PAHs (from 0.08 ng/g [92] to 5.42 or 5.48 ng/g) [85,99]; however, they do not mentions the pollution sources.

Considering that modern pollution sources are the industrial emissions and motor vehicle emissions, some authors have particularly studied the level of contamination of milk with PCDD/PCDFs, PCBs or DL–PCBs. In Europe, Italy is one of the countries that stands out, with numerous studies carried out on this topic [82,85,88,94,95,98].

The identification of pollution sources was only possible in a few studies run on locally produced milk by animals fed with known feedstuffs. In samples of marketed milk, the identification of pollution origin was almost impossible since no information was known about the provenience of the milk; this is collected from multiple farms then subsequently processed.

Regarding the proportions of POPs found, it is important to mention that all the levels of different types of POPs presented in the studies listed are not directly comparable with enforceable actions levels or with minimum tolerance levels in milk. The levels mentioned in almost all the papers are lower than enforceable action levels or minimum tolerance levels in feed established by different authorities (e.g., European Commission, FDA), but the efforts to reduce environmental pollution are very important and must be kept constant.

3.2. Animal Feed

Table 3 summarizes the predominant POPs reported by different authors in animal feed and the distribution per pollutant according to pollution sources, the pollutant category and its classification according to the degree of risk, the applied analytical method, and the quantitative profile of the identified pollutants.

Not many articles have focused on the presence of POPs in animal feed [33,34,84,100,101], especially due to the difficulties in studying this matrix, given either by the generally physicochemical characteristics of the feeds (for example, the low–fat content), or given by the lack of analytical methods and techniques applicable to the matrix.

However, a few authors have identified the presence of POPs in animal feed in different proportions, depending on the pollution profile of the analyzed area, through highly sensitive analytical techniques, such as GC–MS, GC–HRMS or high-resolution gas chromatography (HRGC) coupled with HRMS. All the levels of different type of POPs presented in the studies listed are not directly comparable with enforceable actions levels or with minimum tolerance levels in feed; in almost all the papers, the proportions founded are lower than the enforceable action levels or minimum tolerance levels in feed as established by different authorities (e.g. European Commission, FDA).

Reported according to the degree of risk of each pollutant category, according to the Stockholm Convention [42], the pollutants identified in most of the feed samples are not part of the high-risk categories and were particularly identified pollutants from Annex C of the listing of POPs. Only accidental release reduction measures are currently applied for these.

Nevertheless, despite numerous applied pollution prevention measures, the action taken will provide effective results in the long term due to the high residues persistence. In this context, recent works [33] reported the presence of some high-risk pollutants, such as OCPs, included in Annex A of the Stockholm Convention; they were banned from production and usage since 2001, suggesting an old contamination of the area.

The behavior of pollutants in the environment is complex and aims to clarify that the interaction between chemical compounds released into the environment and the substrates (soil, water, air, plants) is different. Not all substrates react similarly in contact with pollutants due to their different sensitivities and physical and chemical particularities [95].

Table 3. Summary of POPs presence in animal feeds and sources of pollution.

Location	Collected Area	Sample	POPs	Method	POPs Level (ng/g) Mean and/or Range (Min–Max)			Annex *	References
					●	▲	■		
France	Field located along motorways and airports, without any influence of other major sources of pollution	Grass collected from a field nearest a motorway and airport	ΣPAHs ■	GC–MS	-	-	25	A	[100]
Tarragona (Catalonia region), Spain	Urban area: chemical and petrochemical industries: a municipal solid waste incinerator, a hazardous waste incinerator, a PVC production facility, highways and roads with high traffic density	Feed sample collected near important sources of pollution	PCDDs/TCDD ■	HRGC /HRMS	-	-	-	C	[101]
			PCDDs/OCDD ■		-	-	1680	C	
			PCDFs/TCDF ■		-	-	210	C	
			PCDFs/OCDF ■		-	-	430	C	
France	Vicinity of a hazardous municipal waste incinerator	Contaminated hay	ΣPCBs ▲	GC –HRMS	-	52.2–79.8	-	A, C	[84]
			PCDDs/TCDD ■		-	-	60	C	
			PCDDs/OCDD ■		-	-	1033	C	
			PCDFs/TCDF ■		-	-	350	C	
			PCDFs/OCDF ■		-	-	1490	C	
Poland	Dairy farms located in unpolluted area (no industry, no main roads, no chemical fertilizers) (negligible sources of pollution)	Contaminated feed (molasses sugar beet pellets)	PCDDs/PCDFs ■	HRGC –HRMS	-	-	5.0–427	C	[34]
			DL–PCBs ■		-	-	20–100	A, C	
Punjab, India	Intensive dairy production, typical feeding management	Feed samples collected from the animal sheds	γ–HCH ●	GC–MS	2.73	-	-	A	[33]
			DDE ●		1.90	-	-	A	
			Endosulfan ●		2.95	-	-	A	
Poland	Marketed feed	Feeds of plant origin (sugar beet, pellets, dried alfalfa, dried apple)	PCDDs/PCDFs ■	HRGC –HRMS	-	-	3.43	C	[27]
			ΣPCBs ▲		-	0.11	-	A, C	

● Organochlorine Pesticides ▲ Industrial chemicals ■ Combustion chemicals

A—measures for elimination production and use
B—measures for restricting production and use
C—measures for release accidental eliberation

POPs—persistent organic pollutants; OCPs—organochlorine pesticides; Ind.Chem—Industrial Chemicals; Comb.—Pollutants from Combustion; HCH—hexachlorocyclohexane; DDE—dichlorodifenildicloroetano; PCBs—polychlorinated biphenyls; PCDDs/PCDFs—polychlorinated dibenzodioxins/dibenzofurans; TCDD/F—tetrachlorinated dibenzodioxins/dibenzofurans; OCDD/F—octachlorinated dibenzodioxins/dibenzofurans; DL—PCB–dioxin-like PCBs; PAHs—polycyclic aromatic hydrocarbons; GC–gas chromatography (HR/MS—high resolution/mass spectrometry). * Annex = Annexes from Stockholm Convention; this includes the list with the chemicals targeted by the Stockholm Convention: Annex A (Elimination); Annex B (Restriction); Annex C (Unintentional production). See reference [40] for POPs category symbol shape.

For the effective biomonitoring of different types of pollutants, the literature proposes the prediction of environmental factors that could favor the risk of contamination in order to render efficacy to control actions [102,103]. A methodological model for ranking the risks associated with pollution on livestock farms was developed by Battisti et al. [102], which is useful to estimate the probability of contamination from a certain area.

Monitoring the pollution sources and environmental factors that could increase contamination risks was also applied, without a mathematical model, in the study developed by Matei and Pop [104] to estimate the probability of contamination within a dairy farm. In addition to identifying environmental factors that may favor pollutant accumulation, Matei et al. [105] suggested that the particularities of the feed, and especially the fat content, can provide important clues about the presence of pollutants.

On the point sources of pollution, most articles mention in general the pollution from the chemical and petrochemical industry, but particularly the presence of PCDDs/PCDFs, PAHs and DL–PCBs from incomplete combustion from different industrial sectors for transport or waste burning [84,100,101]. The presence, in different proportions, of PCDDs/PCDFs and DL-PCBs was also confirmed by Piskorska-Pliszczynska et al. [34] and Bedi et al. [33], although the sources of pollution from industry and combustion were not specified.

Variable proportions of pollutants were found in animal feed, originating from incomplete combustion point sources (5–1680 ng/g), industrial point sources (52.2–79.8 ng/g) and from agriculture processes (1.90–2.95 ng/g) (see further Table 3).

Crepineau-Ducoulombier and Rychen [100] investigated the profile of contaminants in different feed samples collected from agricultural areas in the vicinity of a highway and an airport in eastern France, amd they analyzed samples of fodder exposed to pollution from transport emissions (release/evacuation of gasoline, diesel, kerosene). There were 16 types of PAHs listed by the U.S. Environmental Protection Agency as hazardous. The presence of these types of POPs in different proportions in all samples has led to the association of these types of pollutants with different point sources of pollution, such as transports emissions or other substances released from incomplete combustion. The same pollution sources were reported by Schuhmacher et al. [101] in the Catalonia region (Spain) for feed samples collected from an area with intensive road traffic, highlighting the presence of organic pollutants, such as PCDD/Fs, and compounds released from incomplete combustion, similar to PAHs. Schuhmacher et al. [101] also mentioned some important point sources of pollution of PCDD/Fs, such as the incomplete combustion from chemical industry factories and waste incinerators or plastic factories, whilst Crepineau-Ducoulombier and Rychen [100] did not identify such sources for PAHs, even they belong to the same group of POPs (combustion chemicals).

Costera et al. [84] investigated the presence of PCDD/Fs in hay samples collected from the vicinity of a municipal waste incinerator in France. The same source of pollution was reported by Schuhmacher et al. [101] for soil and vegetation samples taken from area with chemical and petrochemical industries. The GC–HRMS quantification results of PCDD/Fs were also in similar ranges—60–1490 ng/g PCDD/Fs in the hay samples [84] and 210–1680 ng/g in feed samples [101]. For similar pollution sources, Costera et al. [84] also observed the presence of different congeners of PCBs pollutants at various levels such as 52.2–79.8 ng/g, which was often associated with industrial activities [45] or waste processing activities [106].

The presence of different types of POPs within a small dairy farm located in Poland in an area without relevant pollution sources (no industry, no main roads, no chemical fertilizers) was reported by Piskorska-Pliszczynska et al. [34]. Levels of 5.0–427.0 ng/g for PCDD/Fs and 20.0–100.0 ng/g for DL–PCBs were identified in samples of sugar beet pellets, most likely contaminated prior to arrival in the farm, thus highlighting the possibility of accidental contamination during the feed manufacturing. The possibility of accidental contamination was also reported by Rychen et al. [106], so the pollution sources identified within an area are not always singular but can be reinforced by previously existing potential sources.

Accidental contamination of feed from unknown pollution sources was considered by Pajurek et al. [27] in a study focused on the official control of contamination with PCDDs/PCDFs, PCBs and DL–PCBs of different types of feed sold in Poland. Mean values of PCDDs/PCDFs levels of 3.43 ng/g and 0.11 ng/g for PCBs were measured. Moreover, in 2.8% of the analyzed samples, exceedances of contamination limits were reported in relation to feed and food safety regulations. Therefore, it is crucial to consider the origin of feedstuffs or feed to maintain traceability and to identify the cause of any possible contamination. Situations when feed is not produced locally and is purchased and transported from other areas [34] can have economic consequences and negative impacts on animals and their products.

Bedi et al. [33] analyzed concentrate and green fodder samples from 55 dairy farms in Punjab, India, via HRGC–HRMS detection techniques, and the presence of three types of pollutants—OCPs–HCH, dichloro–diphenyl–dichloroethane (DDE), endosulphan sulphate—was confirmed and quantified between 1.9–2.95 ng/g, without mentioning the sources of pollution in the studied area. In conclusion, only a few authors have focused on POPs' occurrence in feedstuffs and feed, and just a few solutions have been presented for the biomonitoring of POPs and for the identification of pollution sources. Therefore, more

studies on fodder in livestock grazing or in feed used in intensive farming should be encouraged.

4. List of Reported POPs

For the global problem of pollution, the data reported in the literature were compared with the main provisions of the Stockholm Convention on POPs. Moreover, pertaining to their amount and the different measures applied to eliminate, reduce or restrict them, the potential risk was assessed.

Figure 1 includes the POPs found in feed samples in relation to the listing of POPs in the Stockholm Convention.

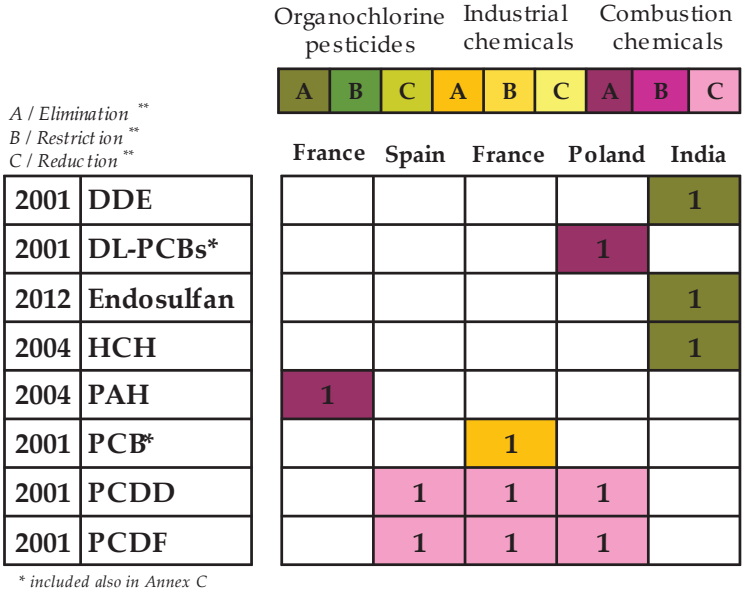

Figure 1. POPs presence in feed (including the number of reporting articles). Classification conducted in relation to the measures applied by the Stockholm Convention (**). The dates on the *Y* axis indicates the year when the chemical compound was included in one of the annexes of the Stockholm Convention. Some POPs are part of Annex A and also of Annex C (*).

Organic pollutants in feed have been investigated in relation to the Annex of the Listing of POPs in the Stockholm Convention. Out of eight pollutants reported as feed contaminants, six were considered to have high toxic potential (DDE, DL–PCBs, Endosulfan, HCH, PAH, PCB). Due to their toxic potential and persistence over years, these pollutants are regulated through measures to eliminate their production or use (substances in Annex A of the Stockholm Convention).

The presence of POPs included in Annex C of the Stockholm Convention on POPs (PCDDs; PCDFs; some PCBs congeners) was also reported in some studies on contamination [34,84,101]. Even if PCDDs/PCDFs were predominant, according to the classification in the Stockholm Convention, they have minimal potential risk and they are regulated through measures to reduce the use or accidental release. POPs from Annex B of the Stockholm Convention, for which restrictions in use and production are applied, were not identified in feed.

Figure 2 includes the POPs identified in milk samples in relation to their Listing in the Stockholm Convention. Thus, 14 types of POPs were reported, out of which 11 kinds are included in Annex A of the Stockholm Convention, highlighting a severe contamination of different types of milk with pollutants that threaten human health. Two types of POPs

included in Annex C of the Stockholm Convention were also identified and only one type of pollutant identified belonged to the Annex B.

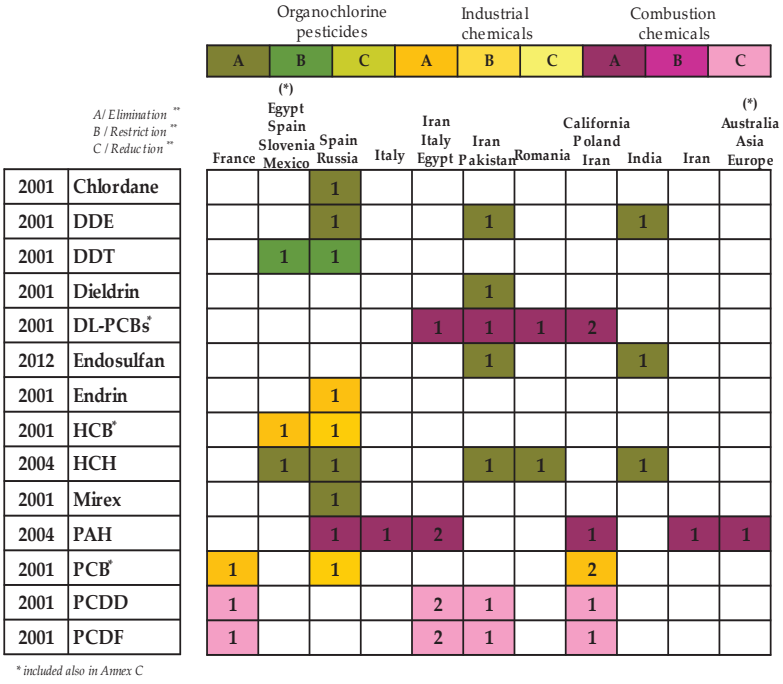

Figure 2. POPs presence in milk (including the number of reporting articles). Classification conducted in relation to the measures applied by the Stockholm Convention (**). The dates on the *Y* axis indicates the year when the chemical compound was included in one of the annexes of the Stockholm Convention. Some POPs are part of Annex A and also of Annex C (*).

The analysis of POPs in relation to the potential toxicity and to one of the three annexes of the Listing of POPs in the Stockholm Convention revealed, for both milk and feed, a worrying situation regarding their safety for humans and animals. Costera et al. [84] and Polder et al. [90] highlighted that the same sources of pollution can generate pollutants with higher toxic potential, namely, those comprised in Annex A or pollutants with lower toxic potential, such as those from Annex C or Annex B.

Desiato et al. [37] and Bedi et al. [33] mentioned the presence of pollutants included in Annex A of the Stockholm Convention in the absence of eligible sources of pollution, thus underlying the need for continuous monitoring, even in apparently risk-free areas.

With respect to this review, it is important to understand that the information presented is not a representative description of all the POPs. We must consider that not all the studies used the same analytical techniques and instrumentation, and not all looked for the same list of POPs, which makes it difficult to compare the results.

5. Conclusions

Important sources of pollution were identified, namely, industrial activity, particularly the incomplete combustion of various substances originating in transportation or waste management activities. In addition to the identified pollution sources, the presence of pollutants depends on the history of pollution in the study area, on the characteristics of pollutants, on the differences regarding urbanization and industrialization and on the environmental transfer of pollutants.

Considering the temporal dimension of feed and milk contamination, it was highlighted that despite numerous efforts to prevent and eliminate POPs, their imported residues persist in the environment and create adverse effects which persist for several years after taking measures and banning the dangerous POPs via law enforcement.

Further research should focus on developing more effective strategies to reduce or to eliminate POPs contamination, especially in feed, and subsequently to decrease animal and human exposure. Additional studies might be necessary to evaluate the response of the animal body to the action of POPs, focusing on the development of physiological measures to limit the transfer of POPs from the animal body to animal products.

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