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Feeding Strategies and Nutritional Quality of Animal Products

Edited by
Arabela Elena Untea, Mihaela Saracila and Petru Alexandru Vlaicu

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About the Editors

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Arabela Elena Untea (Ph.D. in Analytical Chemistry Sciences, University of Bucharest, Romania, 2013) is currently a senior researcher and the head of the Feed and Food Quality Department at the National Research and Development Institute for Animal Nutrition and Biology. Her research activity is mainly focused on feeds and animal-origin food with functional characteristics designed using nutritional approaches; bio-efficiency assessments of feed additives through laboratory experiments; studies addressing reductions in oxidative processes at the animal tissue level through nutrition; and in vitro studies on the bio-accessibility of some nutrients in the gastrointestinal tract of monogastric animals. She is the author and co-author of more than 120 papers published in peer-viewed international journals, two book chapters and two national patents. She has also participated in several EU-funded projects and acted as a scientific coordinator in 5 national projects and as a scientific team member in another 30 projects.

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Mihaela Saracila is currently working as a postdoctoral researcher at the National Research and Development Institute for Animal Nutrition and Biology, within the Feed and Food Quality Department. She holds a Ph.D. from the University of Agronomic Sciences and Veterinary Medicine of Bucharest focusing on the development of innovative nutritional solutions to ensure the performance and health of the digestive tract of broiler chickens reared under high-heat-stress conditions. In recent years, she has mainly focused on mitigating heat-stress-related oxidative stress in monogastric animals and maintaining their gastrointestinal health; improving the nutritional composition and oxidative stability of animal products using natural dietary sources of antioxidants; the development and/or optimization of analytical methods for the extraction of polyphenols; and the determination of hydrophilic compounds and antioxidant capacity. She is currently the project leader of a national project and has participated in more than 28 scientific projects. She has published a laboratory book dedicated to students (collaborator), a book chapter, one patent, eight national patent applications, and over 60 peer-reviewed scientific papers. In addition, she has won multiple awards at different scientific events.

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Petru Alexandru Vlaicu currently holds the position of postdoctoral researcher at the National Research and Development Institute for Animal Nutrition and Biology, in the Feed and Food Quality Department. He holds a Ph.D. focusing on the attainment of functional foods of animal origin via the use of different dietary feeding ingredients in livestock nutrition, which he obtained at the University of Agronomic Sciences and Veterinary Medicine of Bucharest. His research interests include, but are not limited to, assessing the nutritional quality of food and developing feeding strategies for monogastric animals, especially poultry. In addition, the recovery of co- and by-products or waste from the agro food sector in order to recover valuable bioactive compounds and nutrients, from the perspective of a sustainable and circular economy, is among his research interests. He has published more than 80 scientific papers in domestic and international peer-reviewed scientific journals. He has also obtained three national patent applications out of the ten deposited. His commitment to advancing knowledge in animal nutrition and food quality has been recognized through multiple awards obtained at different national and international events.

Preface

This Special Issue, entitled "Feeding Strategies and Nutritional Quality of Animal Products," delves into the complex interplay between the diets of livestock, health and the nutritional value of the products they yield. In our ever-evolving world, the dynamics of food production and consumption have become increasingly vital. As the global population continues to grow, so does the demand for high-quality, nutritious food, especially animal products.

The quality of animal-derived food products is linked to the animals' diets. What animals eat significantly influences the composition of their products, impacting not only their health, but also the nutritional quality of their products. Researchers worldwide have been exploring innovative feeding strategies, ranging from natural foraging to precision nutrition, to enhance the nutritional quality of these products, while ensuring sustainable and ethical production practices.

This Special Issue collates a diverse array of studies, encompassing both fundamental research and practical applications. Authors' contributors investigate the impact of various feeding strategies on the levels of essential nutrients, bioactive compounds, and fatty acid profiles in animal products. They also explore the potential health benefits and drawbacks associated with the various dietary strategies employed for livestock.

The articles collected in this Special Issue demonstrate the relationship between feeding strategies and the nutritional quality of animal products, and aim to contribute to the application of sustainable feeding strategies and nutritious food production, ultimately benefiting both human health and the environment.

Arabela Elena Untea, Mihaela Saracila, and Petru Alexandru Vlaicu
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Editorial

Feeding Strategies and Nutritional Quality of Animal Products

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Feeding strategies play a crucial role in determining the nutritional quality of animal products. The type and composition of an animal's diet directly influence the nutritional content of the products derived from them, such as meat, milk, and eggs. Various feeding strategies, including traditional and modern approaches, impact factors like the balance of macronutrients, essential vitamins, and minerals, as well as overall product quality and safety. Alternative feed sources containing bioactive compounds are being employed as agents that improve the health of animals and enhance the quality of animal products [1]. Incorporating high-quality and well-balanced feeds, optimizing grazing practices, considering supplementary nutrients, and even implementing sustainable and ethical feeding practices can lead to animal products with enhanced nutritional profiles and improved consumer satisfaction. It is evident that the careful selection and management of feeding strategies are essential to ensure the production of animal products that meet both human dietary needs and sustainability goals. Ongoing research and developments in animal nutrition continue to refine feeding strategies. Scientists are working to identify novel feed ingredients, optimize nutrient delivery, and develop precision feeding approaches that cater to the specific needs of animals at different stages of growth.

Among the papers included in this Special Issue, ten discuss different feeding strategies for ruminants and non-ruminant farm animals (nine research studies and one review paper). The research papers regarding the nutrition of ruminants focus on enhancing the diets of sheep and lambs, bioactive feed additives for dairy cattle, beef cattle, and newly weaned Nellore cattle, and nutritional strategies for the prevention of clostridial disease in cattle. The studies on non-ruminant feeding strategies not only center around the effects of microencapsulated probiotics on weaned piglets but also the effect of age at slaughter on the meat quality of male layer-type chickens. The exposure of piglets to lower concentrations of Zearalenone mycotoxin and a natural nitrite, as well as antioxidant sources for pork mince, are also considered. The scientific subject of the lone review paper mentioned above relates to obtaining valuable pigment additives for poultry nutrition.

The papers featured in this Special Issue explore three main topics, namely, animal health and well-being, growth performance, and product quality.

Animal health is a comprehensive concept that encompasses various factors that contribute to the overall well-being of animals, with a critical focus on gut health and the immune system. These aspects are interrelated and play a pivotal role in determining animal's resilience to diseases, growth performance, and the quality of animal products. Implementing appropriate feeding strategies is integral to maintaining optimal animal health and enhanced product quality.

For this topic, Barbosa et al. [2] explored the feasibility of substituting 0.018 g of lasalocid sodium with 1.500 g of dried and milled barbatimão bark or 0.300 g of barbatimão bark extracts (*Stryphnodendron* sp.) in the diets of feedlot lambs. The findings of this study highlighted the advantageous impact of the investigated feed additives on health status by reducing total cholesterol levels when compared with synthetic feed additives. These results require further investigation into the bioavailability of bioactive compounds derived

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from such extracts. In the context of dairy cows, the persistent challenge of clostridial disease remains troubling despite the global utilization of various vaccine methodologies. Probiotics and holistic supplements are gaining prominence as preventive measures against clostridiosis. An innovative approach that has proved effective in bolstering animal resilience, particularly against *Clostridium perfringens* β-toxin, involves the amalgamation of microorganisms and bioactive substances. Nekrasov et al.'s study [3] discusses improved metabolism and heightened antioxidant status in cows. This comprehensive strategy offers valuable insights into the evolution of bovine clostridia prophylaxis. The use of biofunctional additives has emerged as a practical facet within the scope of animal biosecurity measures against toxins, paving the way for their integration into animal diets. This study marks a significant step in unraveling clostridia prevention strategies, holding potential applications in enhancing biosecurity through animal diets. Batista et al. [4] aimed to assess diverse strategies for supplementing immunomodulatory feed additives at (10 g/100 kg BW/day of NUTRA) before and after transportation in weaned Nellore calves raised on pasture and the subsequent impact on physiological parameters and performance as they progressed through the growth phase. Their findings suggest that the specific conditions of the experiment, such as the short duration of transportation stress and the relatively stress-free grazing environment, could have mitigated the detrimental effects of stress on the animals. Consequently, the anticipated advantages of employing the immunomodulatory feed additive might have been addressed. The impact of microencapsulated *Lactobacillus acidophilus* and *Lactobacillus plantarum* supplementation, individually and in combination, was studied by Lefter et al. [5] on weaning piglets. Their findings revealed a remarkable reduction in diarrhea incidence that was particularly evident in the *Lactobacillus plantarum* and combined *Lactobacillus acidophilus* + *Lactobacillus plantarum* groups. Furthermore, the use of microencapsulated probiotics exhibited distinct advantages in terms of intestinal health. These results collectively showcase the potential to mitigate instances of diarrhea while concurrently ameliorating both intestinal structure and microflora composition. Bulgaru et al. [6] concentrated on the effects of two ZEN-contaminated diets—one below (75 ppb) and one above (290 ppb) the EU's recommended levels—on immune and oxidative responses in weaned piglets. Strikingly, exposure to ZEN at both concentrations exerted minimal effects on immune markers, oxidative stress, and colon inflammation. However, for a more comprehensive understanding of ZEN's influence on immune defense, broader studies on various organs are required. These findings contribute to the assessment of suitable ZEN concentrations in piglet feed and offer insight into the underlying mechanisms driving its effects.

However, achieving this intricate equilibrium is not a solitary endeavor. Instead, it hinges on the strategic application of prudent feeding approaches. The importance of these approaches cannot be exaggerated, as they are pivotal in nurturing animal welfare and enhancing the quality of resulting products. By tailoring diets to encompass the specific nutritional needs of animals, these strategies underpin the establishment and preservation of optimal health. Consequently, the animals are not only better equipped to endure potential health challenges but also thrive, achieving their growth potential and manifesting their genetic traits to the fullest, showing the profound correlation between the growth performance and nutritional quality of animal diets. This topic is also covered in the papers published in this Special Issue.

Arcos-Álvarez et al. [7] investigated the effect of olive oil treatment on various aspects related to lactating hair sheep, including their productive traits, and their treatment strategy displayed a positive outcome, as it did not exert any detrimental influence on the productive behavior, milk production, or chemical composition of lactating sheep. In feedlot lambs, Barbosa et al. [2] showed that substituting lasalocid sodium with dried and milled barbatimão bark or barbatimão bark extracts (*Stryphnodendron* sp.) demonstrated no detrimental effects on production performances. Similarly, Batista et al. [4], after supplementing immunomodulatory feed additives in weaned Nellore calves, found that their treatment

had no effect on animal performance. Likewise, in Lefter et al. study [5] on weaning piglets, probiotics supplements showed potential in enhancing production performance.

Feeding strategies have a direct impact on quality attributes in animal products. From influencing nutritional quality and tastes to determining fatty acid profiles, the nutritional composition of diets significantly shapes the sensory and nutritional characteristics of the final meat products, thus influencing consumer preferences and choices. The effect of animals' diets on product quality is a topic of continuous interest for researchers, farmers and producers, as shown in some of the papers published in this Special Issue.

According to Arcos-Álvarez et al. [7], including extra virgin olive oil in the diets of lactating sheep appears to offer a viable approach to mitigate saturated fatty acids while simultaneously elevating the levels of monounsaturated and polyunsaturated fatty acids. The utilization of extra virgin olive oil contributed to significant enhancements in specific fatty acid contents, including linoleic, linolenic, and eicosapentaenoic acids with potential positive impact on heart health. Soares et al. [8] presumed that the incorporation of 1.6 g/kg supplement dry matter of a blend of live yeast (*Saccharomyces cerevisiae* strains) and organic trace minerals, particularly chromium, could effectively replace the conventional monensin (30 mg/kg) and inorganic trace minerals during the finishing phase of beef cattle, and their study reports favorable effects on feed efficiency and carcass traits. In fact, the inclusion of a blend of live yeast and organic trace minerals exhibited notable benefits in terms of animal growth and meat color, all of which were attributed to elevated concentrate intake and heightened diet digestibility facilitated by the utilization of live yeast and organic minerals. The insights derived from the research of Popova et al. [9] contributed to understanding how the meat quality of slow-growing chicken male layer-type chickens differs according to meat portions, particularly regarding chicken breasts and thighs. In terms of nutritional constituents, advancing age was linked to a noteworthy reduction in intramuscular fat content in thighs, with a similar trend observed in breast meat. Collectively, the meat from nine-week-old male layer-type chickens displayed certain drawbacks regarding fatty acid profiles. This aspect unveils potential avenues for future studies to explore diverse feeding strategies or housing systems to enhance this particular trait. The impact of introducing varying levels of ethanolic hawthorn berry extract in conjunction with a consistent concentration of fermented parsnip juice on factors like lipid stability, heme pigment conversion, residual nitrite content, and the growth of spoilage bacteria in refrigerated pork mince was investigated by Predescu et al. [10]. Coupling fermented parsnip juice with hawthorn extract exhibits a notable inhibitory impact on spoilage bacteria that break down nitrogen compounds, leading to enhanced stability in unsaturated fatty acids that are prone to oxidation in minced pork. In this study, the interplay between the hawthorn extract and the fermented parsnip juice appeared to be pivotal in governing color and lipid stability.

In response to the growing emphasis on natural products within the food and feed markets, the demand for organic ingredients is on the rise. Current trends in livestock nutrition research are leaning toward feed formulation diets that incorporate natural and organic feed additives [11]. These additives encompass active elements that impact plants, animals, and microorganisms, yielding valuable biocompounds. Notably, yeast pigments offer a wealth of natural colors, coupled with a broad spectrum of nutritional and medicinal attributes, as noted by Grigore et al. in their review [12]. Achieving these optimizations hinges on strategic approaches involving strain genetic engineering and process development, often utilizing cost-effective organic substrates. According to Grigore et al. [12], investigations into the effects of value-added yeast pigment additives on livestock well-being, productivity, and product quality are essential in substantiating their nutritional and medicinal potential. It is equally important to consider the perspectives and preferences of consumers regarding purchasing animal products derived from microbial pigment additives, as well as the need for an enhanced understanding of these additives and their implications.

In summary, the presented works contribute towards solving issues regarding the development of "Feeding Strategies and Nutritional Quality of Animal Products" in differ-

ent livestock systems. The reported results addressing sheep, lambs, dairy cattle, weaned cattle, and piglets, as well as product quality, are of interest to specialists and scientists involved not only in research but also in daily farm support and management. The feeding strategies presented in the works published in this Special Issue address some important aspects in terms of animal health, production performance, and product quality.

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Article

Effects of Microencapsulated Probiotics on Performance, Organ Development, Diarrhoea Incidences, Blood Parameters, Intestinal Histomorphology and Microflora in Weaning Piglets

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Abstract: The study aimed to assess the effects of the dietary supplementation of microencapsulated *L. acidophilus* and *L. plantarum* and their combination on the growth performance, organ development, diarrhoea incidences, blood profiles, intestinal histomorphology and microflora in weaned piglets. For that, 160 piglets with an average body weight (BW) of 8.52 ± 0.15 kg were divided into four groups (40 piglets/group) and allotted to one of the four dietary treatments as follows: a basal diet (C diet) or a basal diet containing 1×10^8 CFU/g of *L. acidophilus* (LA diet), or a diet containing 3×10^8 CFU/g of *L. plantarum* (LP diet) and a diet with the combination of both bacterial strains (LA + LP diet) for 21 days. On day 14, probiotics significantly increased ADFI, while FCR was higher in the LA and LP groups than the C and LA + LP groups. No effects ($p > 0.05$) on visceral organs weight, intestinal pH and biochemical parameters among treatments were noticed. Treatments significantly lowered diarrhoea incidence compared to control. Villus width was greater ($p < 0.05$) in all small intestinal segments in piglets fed probiotics. In the jejunum and ileum villus length, crypt length, and total villi length were higher ($p < 0.05$), particularly in the LA + LP group. The probiotics, particularly the LA + LP group, modulated the cecal, jejunum and ileum microbial community structure and increased ($p < 0.05$) the amount of *Lactobacillus* spp. while decreasing the populations of *Escherichia coli* and *Staphylococcus*. Our results indicated that dietary supplementation of microencapsulated probiotics, particularly the combination of *L. plantarum* and *L acidophilus* strains, maintained growth performance, lowered diarrhoea incidence and beneficially altered the intestinal architecture and microbial populations of weaned piglets.

Keywords: *L. acidophilus*; *L. plantarum*; microencapsulated probiotics; weaning piglets; performance; microflora; piglets' health

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1. Introduction

In many commercial piggeries, weaning is an important source of production loss and a major stress factor [1]. This effect is generated by actual weaning practices, which involve abrupt separation of the piglets from the sow at a young age, rapid exposure to solid food, littermate, and adaptation to the new environment, among others [1,2]. Further, weaning causes dramatic shifts to the epithelial membranes of the small intestine, such as villus atrophy and crypt hyperplasia, which further leads to a decrease in the capacity for digestion and absorption of available nutrients [2–4]. Under these conditions, the intestine, this fertile environment for beneficial bacteria (such as *Lactobacillus* spaces) and pathogenic

microbes (i.e., *Escherichia coli*, *Salmonella typhimurium*) are affected, and the proliferation of harmful bacteria encouraged with the emergence of infectious diarrhoea [5]. Diarrhoea is a condition in weaned pigs. It can last several days and causes excessive secretion of water and electrolytes into the small intestine, which exceeds the absorptive capacity of the colon. Thus, weaning increases the intestinal oxidative stress in pigs reflected in changes in intestinal architecture and also impairs liver function by translocation of metabolites to the liver, altering the metabolic health status [2]. Frequently, to inhibit pathogenic complications and to prevent the decreasing of feed intake, growth retardation and mortality rate, ZnO or a variety of antibiotics are overused [1,2,6]. However, since it has been unsafe to use antibiotics as growth promoters or due to the main effect of ZnO as co-selects bacteria resistant to antibiotics or environmental implications, scientists have worked to find appropriate replacements [7]. This resulted in the introduction of probiotics as a live microorganism [8] that gives a health benefit to the host when administered in adequate amounts. Their potential modes of action significantly affect the gut's microbial diversity by lowering the luminal pH and bacteriocins production, inhibiting pathogenic strains adhesion and regulating the hosts' immune system [9]. Moreover, positive impacts on the piglets' gut health due to microbial metabolites that directly and indirectly ameliorated piglets' diarrhoea, growth and feed conversion ratio were noticed using *Lactobacilli* compounds (including *L. acidophilus, L. plantarum, L. reuteri, L. acidophilus* and *L. fermentum*) in a meta-analysis of *Lactobacillus*-based probiotics [10]. Furthermore, this new natural re-establishment of beneficial bacteria in the piglets' gut is a more efficient way to reduce the economic loss in this critical phase, as was previously reported [11]. In this context, several in vitro and in vivo studies have tested different lactic acid bacteria (LAB) as probiotics for weaned piglets as the main feed additive source on growth performances, health status, intestinal histomorphology and reducing diarrhoea incidences [12–14]. Among the beneficial bacteria, genus *Lactobacillus* can be considered one of the best candidates as feed additives in piglets' diets due to their high proliferation rate and their ability to colonize or be metabolically active in the intestine, including their potential to survive into stomach acid and throughout the specific digestion process [15].

Lactobacillus spp., the natural producers of nutrients, increase the immunological system and enhance the absorption of micronutrients [16]. They stimulate the generation of organic acids and amino acids and thus affect the physiological functions of animals, such as general health and growth [16]. Several studies have reported that some *Lactobacillus* spp. can change host intestinal microbiota by producing lactic acid and other microbial compounds, and they may prevent the colonization of pathogens via competitive exclusion [14,17,18]. Growing evidence indicates that *L. plantarum* and *L. acidophilus* were found to have probiotic effects on the gastrointestinal site like increased villus high and the villi/crypt ratio of the small intestine due to increasing averaged daily feed intake, average daily gain and the gain to feed ratio of weaning piglets [19]. However, according to some studies [20,21], probiotics, live bacteria, require an encapsulating process to optimize their survival ability and protect them from biological barriers, including stomach acid and bile salts, ensuring their safe delivery. Thus, microencapsulation is an important technological process which helps to delay the quick degradation of drugs in the upper gastrointestinal tract [17,21]. Moreover, spray drying provides a more favorable anaerobic environment for the probiotic bacteria and improves the storage properties. Further, the combination of *L. plantarum* and *L. acidophilus* can synergistically promote the growth of piglets as well as improve their resistance to pathogens by equilibrating the intestinal microflora populations [20]. For these reasons, microencapsulation is an alternative to improve viability during both processing and transit to the distal areas of the intestine.

To the best of our knowledge, there are no studies on the effects of a combined microencapsulated *L. acidophilus* and *L. plantarum* probiotics supplementation in weaning piglets' diets. Thus, we hypothesized that using a mixture of these two microencapsulated probiotics (1:1 ratio) may positively affect growth performance due to complementary effects of antimicrobial properties exerted by probiotics with potential benefits on the

intestinal health of weaning piglets. Therefore, this study aimed to assess the effects of the dietary supplementation of microencapsulated *L. acidophilus* and *L. plantarum* and their combination on the growth performance, organ development, diarrhoea incidences, blood profiles, intestinal histomorphology and microflora in weaned piglets.

2. Materials and Methods

2.1. Ethical Procedure

The study was conducted according to the experimental protocol approved by the Ethics Commission of the National Research-Development Institute for Animal Biology and Nutrition (protocol no. 699/02.2020) and complied with European Directive (2010/63/EU) and Law 43/04.2014 on the protection of animals used for scientific purposes.

2.2. Animals and Housing System

The experiment was conducted on 160 weaned piglets and lasted for 21 days. The hybrid Topigs piglets [♀Large White × Hybrid (Large White × Pietrain) × ♂Talent, mainly Duroc] with average body weight (BW) of 8.52 ± 0.15 kg, age 28 ± 3 days, were randomly divided into 4 treatments, balanced for sex (♀= ♂), weight and litter origin. Each treatment included 40 ear-tagged piglets distributed in four pens with 10 piglets each. Each pen was 175 m × 230 m × 80 m and contained an automatic stainless nipple steel drinker and feeder. The microclimate conditions in the piglets' houses were electronically monitored.

2.3. Probiotics

2.3.1. Strains Isolation, Characterization and Growth Conditions

Lactic acid bacteria (LAB) strains were isolated from healthy pigs' gut digesta (ileum content). After phenotypical and molecular identification [22], the strains were stored at –80 °C with 20% sterile glycerol as a cryoprotectant in Man Rogosa Sharpe broth (MRS, Oxoid CM0361).

2.3.2. Bioreactor Batch Fermentation and Spray Drying Process

Before the experiment, the LAB strain was revitalized in MRS broth to obtain the inoculum starter culture at a concentration of 10^{10} colony-forming units (CFU) per mL. The biomass production was performed in a 5-L bioreactor (BioFlo 320, Eppendorf, one unit, Hamburg, Germany) with a workload of 2 L at 37 °C, 24 h, 150 rpm, pH = 6.5 ± 0.2 as described elsewhere [23].

For the experimental design of drying, a Buchi-Mini Spray Dryer B-290 (BUCHI Labortechnik, Swiss-made, Flawil, Switzerland) was used to microencapsulate LAB strains. As carrier material, maltodextrin (24%, w/v) and glucose (4% w/v) were prepared in distilled water to improve the strains' viability during the process. The survival of powders was done in phosphate-buffered saline pH 7.0 (PBS, Dulbecco A; Oxoid Livingstone Ltd., London, UK) as explained by Dumitru et al. [23], and the final concentration was expressed as colony-forming units (CFU) per gram of spray-dried powder.

2.4. Diets

A basal diet without probiotics was used as control (C) and fed to one group of piglets. The other 3 groups were fed the control diet supplemented with different microencapsulated LAB containing 1% *L. acidophilus* (diet LA) (1×10^8 CFU/kg of feed), 1% *L. plantarum* (diets LP) (3×10^8 CFU/kg of feed) and their mixture (1:1, diet LA + LP). The supplemented probiotics have been mixed with the premix, and after, the premix was added to the basal diet. The ingredients and nutritional composition of the basal diet are shown in Table 1. The content of the feed follows the recommendations specified by the hybrid Topigs guide. Piglets had unrestricted access to food and water (drinkers nipple) throughout the experiment. The mash form feed was used.

Table 1. Ingredients and nutritional value of the basal diet.

Ingredients (g/kg as-Fed Basis)	Basal Diet
Ground corn	667.4
Mustard meal	20.0
Hempseed meal	10.0
Soybean meal	150.0
Corn gluten	30.0
Milk powder	50.0
Hempseed oil	25.0
DL-Methionine	2.2
L-Lysine HCl	4.4
Carbonate calcium	14.6
Monocalcium phosphate	14.3
Salt	1.0
Premix Choline	1.0
Vitamin-mineral premix *&	10.0
Phytase	0.1
Total	1000.0
Nutritional value	
Metabolizable energy (EM, MJ/kg) **	14.0
Crude protein (%)	172.5
Lysine (%)	10.5
Methionine + Cysteine (%)	7.3
Calcium (%)	10.1
Total Phosphorus (%)	7.8

* Vitamin-mineral premix provided per kg of diet: vitamin A, 10,000 IU; vitamin D3, 2000 IU; vitamin E, 30 IU; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B3, 20 mg; vitamin B5, 13.5 mg; vitamin B6, 3 mg; vitamin B7, 0.06 mg; vitamin B9, 0.8 mg; vitamin B12, 0.05 mg; vitamin C, 10 mg; Mn, 30 mg; Fe, 110 mg; Cu, 25 mg; Zn, 100 mg; I, 0.38 mg; Se, 0.36 mg; Co, 0.3 mg; antioxidant, 60 mg. & For experimental diets, probiotics were included in the vitamin-mineral premix. ** Metabolizable energy is a calculated value, while the others are determined values.

2.5. Growth Performance

The growth performances were monitored for 3 weeks (21 days) immediately after the weaning period. The performance parameters were determined as follows: from the first day after weaning up to 14 days, 15 to 21 days and overall period 0 to 21 days, respectively. Body weight (BW, kg) and feed intake (FI, g/day) were recorded and used to calculate average daily weight gain (ADG, g/day), average daily feed intake (ADFI, g/day), and feed conversion ratio (FCR, g feed/g gain) for each group.

2.6. Incidence of Diarrhoea Determination

The animals were monitored daily to identify the piglets with diarrhoea and mortality, and the observations were recorded. The faeces of every animal were examined visually. Diarrhoea incidence (DI%) was calculated using the appropriate formula [24].

$$\text{DI (\%)} = (\text{total number of pigs/total number of pigs with diarrhoea} \times \text{number of experimental days}) \times 100 \quad (1)$$

2.7. Blood Sampling and Analyses

At the end of the trial, 16 piglets/group were selected, and blood samples were collected from jugular venipuncture into 6 mL plain plastic tubes (anticoagulant-free) containing lithium heparin and a gel for plasma separation (Vacutest, Arzergrande, Italy). The samples were centrifuged at 3000 rpm for 15 min at 4 °C for plasma separation. Afterwards, the following plasma parameters: total protein (TP), albumin (ALB), bilirubin (BIL), blood urea nitrogen (BUN), uric acid (UA), creatinine (CRE), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), creatine kinase (CK), lactate dehydrogenase (LD), gamma-glutamyl transferase (GGT), glucose (GLU), triglyc-

erides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein (LDL), inorganic phosphorus (IP), magnesium (Mg), and calcium (Ca) were determined with a Spotchem EZ SP4430 (Arkray, Japan) chemistry analyzer and specific test kits were used.

2.8. Intestinal Sampling, Light Microscopy Examination and Intestinal Microflora Analyses

2.8.1. Sampling

After the weaning period of 21 days finished, on the last day of the experimental period 32 piglets (n = 8/group) were euthanised, following exsanguination according to Romanian Law 43/2014 for the handling and protection of animals used for experimental purposes. The contents of the ileum (n = 8/group) and cecum (n = 8/group) were aseptically collected in sterile plastic bags and quickly transferred on ice to the laboratory. One gramme of intestinal content (ileum and cecum) was homogenized with 7 mL Brain Heart Infusion (BHI) broth (Oxoid LTD, UK CM1135) supplemented with 2 mL sterile glycerol and immediately frozen at −20 °C until the analysis, according to the technique described elsewhere [25]. For histological analyses, fragments of the small intestine (middle portion of duodenum, first portion of jejunum and distal part of ileum) were collected, sampled, and transported for further histological analysis to the Synevovet Laboratory (Chiajna, Romania).

2.8.2. Light Microscopy

The duodenum (n = 8/group), jejunum (n = 8/group) and ileum (n = 8/group) sections (5 cm^2) were washed in 0.9% NaCl solution and fixed for 24 h in a 10% buffered (formaldehyde 4% aqueous solution, pH 7.2) formalin solution. Afterwards, parts from the duodenum, jejunum and ileum were embedded in paraffin, and 6 mm transversal sections were stained with the hematoxylin-eosin method. The villi length (VL), villi width (VW), and crypts length (CL) were measured in each section 10 times for each animal from each group using Leica DM3000, Tokyo, Japan microscope with a 40× objective, capturing images, using ×4, ×10, ×20, ×40, and ×100 lenses in succession. The ratios of villi length to the length of the crypt (V/C) and total villi together with crypt length (VCL) were calculated.

2.8.3. Microflora Analyses

After samples defrost, decimal dilutions in phosphate-buffered saline pH 7.0 (PBS, Dulbecco A; Oxoid Livingstone Ltd., London, UK) were performed to enumerate microbial populations. The sample was assessed for Lactic Acid Bacteria (LABs), *Escherichia coli* (*E. coli*; biotype β-haemolytic), *Salmonella* spp., *Clostridium* spp., *Enterococcus* spp., *Coliforms*, and *Staphylococcus* spp. The LABs were cultured on de Man Rogosa and Sharpe agar (MRS; Oxoid CM0361) and incubated in anaerobic conditions at 37 °C for 48 h (Oxoid jar with Anaerogen 2.5 L). *E. coli* biotype β-haemolytic was analysed, as reported by Dumitru et al. [26]. Briefly, it was inoculated 0.01 mL from 10^{-1} dilution on sheep blood agar [Trypticase soy agar (TSA) 5% (w/v)] and incubated at 37 °C for 24 h in aerobic conditions. *Salmonella* spp. was grown on *Salmonella-Shigella* agar (Oxoid CM0099), followed by aerobically incubation at 37 °C for 24 h. *Clostridium* spp. were cultured on Reinforced Clostridial agar (Oxoid CM0151) and incubated anaerobically at 37 °C for 48 h. *Enterococcus* spp. were enumerated on Slanetz-Bartley agar (Oxoid CM0377) incubated at 37 °C for 48 h in anaerobic conditions, according to Sorescu et al. [25] method. The *Coliforms* were cultured on MacConkey agar (Oxoid CM0007) and incubated aerobically at 37 °C for 24 h. Coagulase-positive *Staphylococci* were enumerated on Baird-Parker Agar (BPA; Oxoid LTD, UK) supplemented with egg yolk tellurite emulsion and incubated aerobically at 37 °C for 48 h. Every sample was repeated three times. The microflora enumerations were expressed as $\log_{10}$ colony-forming units (CFU) per gram.

2.9. Statistical Analysis

The results were expressed using the mean and pooled standard error of the mean (SEM). First, we used the Shapiro–Wilk test to check whether the data set was normally distributed. The data were analyzed using one-way ANOVA in IBM SPSS (version 27.0 for windows, SPSS Inc., Chicago, IL, USA) [27]. Means were compared using the Tukey test at 5% and 1% significance levels. The pen was used as an experimental unit for growth performance data and the piglets for other response criteria. The graphs were made in GraphPad Prism software, version 9 (GraphPad Software, La Jolla, CA, USA) and the values were determined to be significant when $p < 0.05$, between groups. The Principal Component Analysis (PCA) was performed using the corresponding function of the Matlab and Simulink (version 2020, MathWorks Inc Bartok B. ut 15/d 1114 Budapest Hungary) software package to determine the relationships between productions performances, diarrhoea incidence and intestinal health parameters corresponding to each experimental group.

3. Results

3.1. Effects of Microencapsulated Probiotics Supplements on Growth Performances of Weaning Piglets

The results regarding the effects of microencapsulated probiotics on the ADFI, ADG, and FCR are presented in Table 2. After the first two weeks of feeding diets supplemented with microencapsulated *L. acidophilus*, *L. plantarum* and their combination, the ADFI was significantly higher ($p = 0.041$) in experimental groups compared to the C group, while the FCR was significantly higher ($p = 0.003$) only in the LA and LP groups compared with both C and LA+LP groups. During the following week, no significant effect ($p > 0.05$) was noted for the production performances, as well as for the overall experimental period. However, the groups supplemented with LA, LP, and the combination of the probiotics (LA + LP) tended to perform better in terms of ADFI, ADG and FCR than the C group.

Table 2. Effects of dietary microencapsulated probiotics supplements on growth performance of weaning piglets.

Items	Treatments				SEM	*p*-Value
	C	LA	LP	LA + LP		
BW (at weaning, kg)	8.53	8.52	8.53	8.52	0.011	0.995
1 to 14 days						
ADFI (g/day)	202 ^{b}	210 ^{a}	215 ^{a}	217 ^{a}	0.013	0.041
ADG (g/day)	92.9	95.7	96.4	98.9	0.083	0.063
FCR (g feed/g gain)	2.15 ^{b}	2.33 ^{a}	2.23 ^{a}	1.99 ^{c}	0.026	0.003
15 to 21 days						
ADFI (g/day)	475	476	476	482	0.021	0.317
ADG (g/day)	341	365	358	359	0.071	0.353
FCR (g feed/g gain)	1.40	1.31	1.33	1.36	0.020	0.255
1 to 21 days						
ADFI (g/day)	263	288	286	289	0.014	0.351
ADG (g/day)	180	186	188	190	0.091	0.694
FCR (g feed/g gain)	1.55	1.64	1.59	1.61	0.018	0.745

$^{a, b, c}$ Means values with different superscripts in the same row differ at $p < 0.05$; SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; BW—body weight; ADFI—average daily feed intake; ADG—average daily gain; FCR—feed conversion ratio.

3.2. Effects of Microencapsulated Probiotics Supplements on Organs Development, Intestinal pH and Diarrhoea Incidence of Weaning Piglets

No significant differences were observed among treatments for any visceral organs weighed (Table 3). The pH measured in the intestinal sections tended to be lower but without significant effect ($p > 0.05$). Out of 21 days, all groups had piglets with diarrhoea during 10 days, but the diarrhoea incidence was significantly lower ($p = 0.001$) in the LP and LA + LP groups compared with the C group.

Table 3. Effect of microencapsulated probiotics supplements on organ development, intestinal pH and diarrhoea incidence of weaning piglets.

Items	Treatments				SEM	*p*-Value
	C	LA	LP	LA + LP		
Carcass yield, %	82.3	81.4	79.6	79.9	0.640	0.098
Liver, g	205.31	226.13	214.63	233.75	4.009	0.593
Spleen, g	21.63	19.58	21.04	19.70	1.249	0.937
Kidneys, g	45.0	45.65	46.33	47.00	1.212	0.960
Heart, g	47.75	50.00	48.00	50.75	1.234	0.818
SI length, cm	1025	1070.75	1086.50	1147.38	17.85	0.094
Intestinal pH						
Duodenum	6.35	6.16	6.10	6.21	0.264	0.337
Jejunum	6.51	6.24	6.30	6.47	0.195	0.061
Ileum	6.82 ^{a}	6.32 ^{b}	6.41 ^{ab}	6.28 ^{b}	0.014	0.023
Days with diarrhoea	10	10	10	10	-	-
Diarrhoea incidence	17.86 ^{a}	10.71 ^{ba}	9.52 ^{bc}	5.95 ^{c}	0.049	0.001

$^{a, b, c}$ Means value with different superscripts in the same row differ ($p < 0.05$); SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; SI—small intestine.

3.3. Effect of Microencapsulated Probiotics Supplements on Biochemical Parameters of Weaning Piglets

Table 4 shows the effect of microencapsulated probiotic bacteria on plasma parameters. No significant difference ($p > 0.05$) was observed in all treatments when lipid, protein, enzymatic and mineral profiles were determined in the experimental animals. From the lipid profile, the GLU parameter tended to be lower in LA and LA + LP groups, compared with the C group, while the HDL tended to be higher. From the protein profile, only the BUN parameter presented a tendency to increase in the LA and LP groups. Lastly, in the enzymatic profile, the AST parameters were slightly higher in the LP group and lowered in the LA + LP group than in the C group. No significant alteration ($p > 0.05$) or tendencies were noted for the mineral profile.

Table 4. Effects of microencapsulated probiotics supplements on plasma parameters of post-weaning piglets.

Items	Parameters	C	LA	LP	LA + LP	SEM	*p*-Value
Lipid profile	GLU, mg dL^{-1}	111.13	104.25	108.88	104.75	3.041	1.035
	TG, mg dL^{-1}	28.15	28.50	29.46	28.89	0.953	0.971
	TCH, mg dL^{-1}	93.00	92.88	92.38	92.01	1.790	0.998
	HDL, mg dL^{-1}	28.63	29.75	29.13	31.63	1.482	0.909
	LDL, mg dL^{-1}	58.87	58.57	58.54	56.63	1.030	1.000

Table 4. *Cont.*

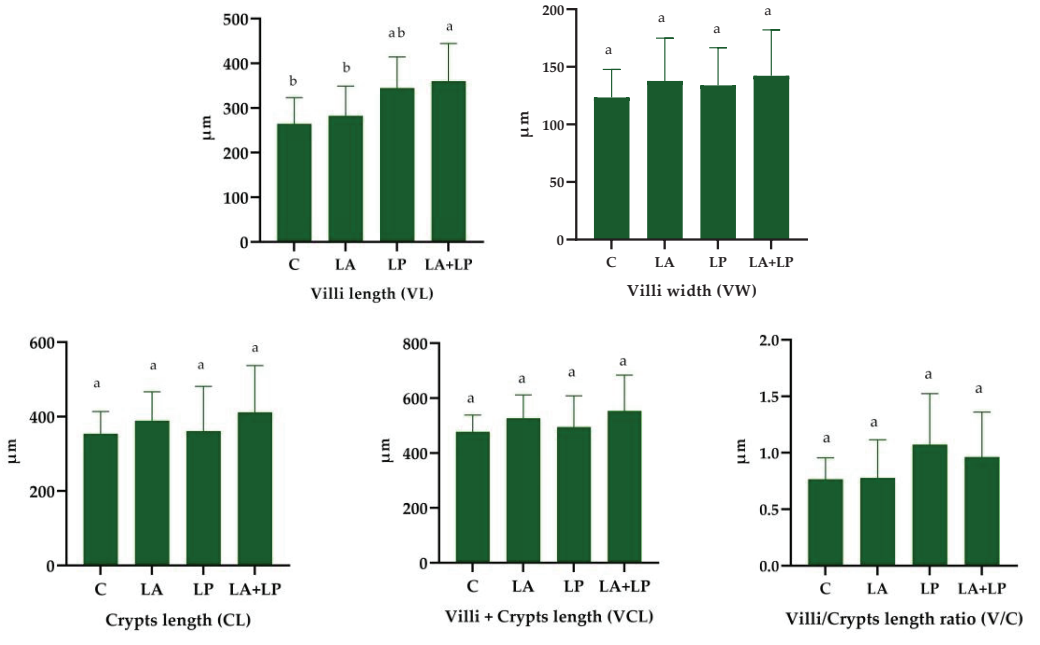
Items	Parameters	C	LA	LP	LA + LP	SEM	*p*-Value
Protein profile	TP, g dL^{-1}	4.76	4.88	4.70	4.80	0.101	0.951
	ALB, g dL^{-1}	2.81	2.88	2.76	2.81	0.063	0.811
	BIL, mg dL^{-1}	0.28	0.24	0.26	0.25	0.010	0.746
	BUN, mg dL^{-1}	16.69	17.75	17.72	16.96	0.622	0.856
	UA, mg dL^{-1}	0.53	0.53	0.54	0.51	0.011	0.941
	CRE, mg dL^{-1}	1.56	1.55	1.53	1.53	0.047	0.981
Enzymatic profile	ALT, U/L	27.75	28.19	28.25	27.50	1.090	0.818
	AST, U/L	25.75	25.25	26.00	24.13	1.168	0.949
	AP, U/L	1200	1120	1122	1222	52.33	0.314
	CK, UI/L	390.00	386.63	392.38	388.50	6.202	0.981
	LD, UI/L	1107.13	1110.63	1115.00	1105.8	34.18	1.000
	GGT, UI/L	45.38	45.63	46.50	46.13	1.921	0.997
Mineral profile	Ca, mg dL^{-1}	11.38	11.36	11.31	11.35	0.261	1.000
	Mg, mg dL^{-1}	2.28	2.29	2.23	2.30	0.053	0.948
	IP, mg dL^{-1}	6.13	6.08	6.04	6.09	0.164	0.998

SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; GLU—glucose; TG—triglycerides; TCH—total cholesterol; HDL—high-density lipoprotein cholesterol; LDL—low-density lipoprotein; TP—total protein; ALB—albumin; BIL—bilirubin; BUN—blood urea nitrogen; UA—uric acid; CRE—creatinine; ALT—alanine aminotransferase; AST—aspartate aminotransferase; AP—alkaline phosphatase; CK—creatine kinase; LD—lactate dehydrogenase; GGT—gamma-glutamyl transferase; Ca—calcium; Mg—magnesium; IP—inorganic phosphorus; n = 16/group.

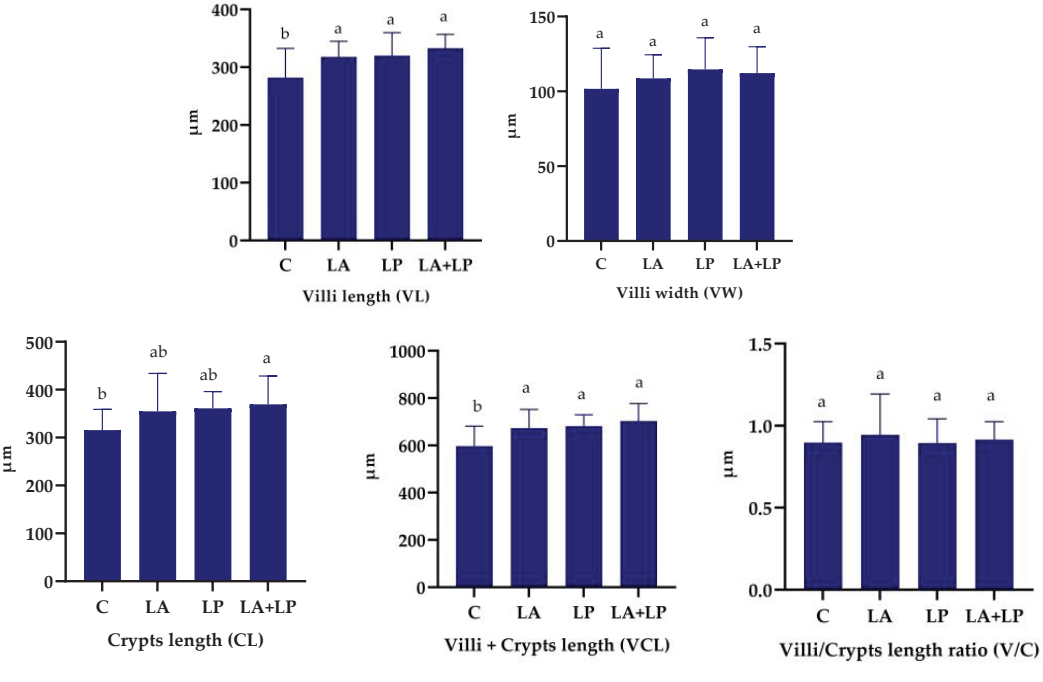
3.4. Effects of Microencapsulated Probiotics Supplements on Intestinal Histomorphology Measurements of Weaning Piglets

The effect of dietary microencapsulated supplements on VL, VW, CL, V/C ratio and VCL in the duodenum, jejunum and ileum segments of the weaned piglets are presented in Figure 1. From the obtained results, we observed that among the measurements made in the duodenum, only the VL was significantly ($p < 0.05$) affected. The LA + LP group had significantly higher ($p < 0.05$) VL compared with LA and C groups as shown in Figure 1A. In the jejunum segment, the VL was significantly higher ($p < 0.05$) in all groups of piglets fed with microencapsulated probiotics, while the CL was significantly higher ($p < 0.05$) only in the LA + LP group compared to the C group. This resulted in significantly higher ($p < 0.05$) VCL (Figure 1B) between the experimental group and the C group. Further, in the ileum segment, VL and CL were significantly higher ($p < 0.05$) in the LA+LP group compared with the C group (Figure 1C). The supplementation with LA resulted in significantly higher ($p < 0.05$) ileum VL compared to the C and LP groups, however, the V/C ratio was significantly higher ($p < 0.05$) only compared to the LA + LP supplemented group. The VCL in the ileum of piglets supplemented with microencapsulated probiotics was significantly higher ($p < 0.05$) only in the LA+LP group, compared to C and LP groups (Figure 1C).

The intestinal morphometry appearances of the duodenum, jejunum and ileum of the weaned piglets are illustrated in Figure 2. The structure of intestinal villi in the three microencapsulated probiotics supplementation groups was more complete and clearer than that of the control group. The measurement results in optical microscopy confirm that supplementation with LA, LP and their combination significantly increased VL of the duodenum but did not have much effect on the ileum and jejunum.

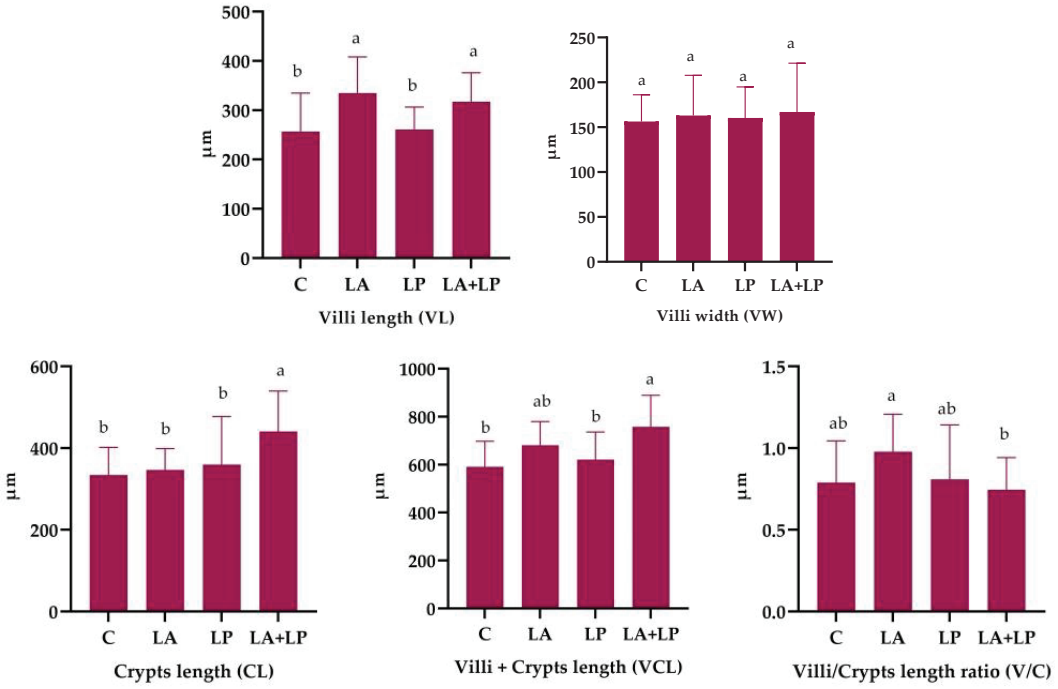


(A) Duodenum



(B) Jejunum

Figure 1. *Cont.*



(**C**) **Ileum**

Figure 1. Effects of microencapsulated probiotics supplements on intestinal histomorphology measurements in the duodenum (**A**), jejunum (**B**) and ileum (**C**) of the weaning piglets (n = 8/group). C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*. ^{a,b} superscripts withing each figure bars represents significant difference among the groups at $p < 0.05$.

3.5. Effects of Microencapsulated Probiotics Supplements on Intestinal Microbiota of Jejunum, Ileum and Caecum of Weaning Piglets

Figure 3 presents the results regarding the impact of microencapsulated probiotics on selected parts intestinal microflora of weaning piglets. In the jejunum part (Figure 3A), the *Lactobacilli* count was significantly higher ($p < 0.05$) only in LA and LA + LP groups compared to the C group. The *E. coli* was not detected in the LP and LA + LP ($p < 0.05$) groups, and the *Enterococcus* spp. was not influenced by the dietary supplements ($p > 0.05$). The *Coliforms* were significantly lower ($p < 0.05$) in the LA+LP group compared with all three (C, LA, LP) experimental groups. At the same time, the *Staphylococcus* was significantly lower ($p < 0.05$) only compared with C and LA groups. The *Clostridium* spp. was significantly higher in the LA group compared with the LP and LA + LP groups. Further, in the ileum intestinal segment (Figure 3B), the count of *Lactobacilli* spp. was significantly higher ($p < 0.05$) in the groups supplemented microencapsulated probiotics (LA, LP and LA + LP) compared to the C group. *E. coli* was significantly decreased ($p < 0.05$) only in the LA + LP group compared to the C group. The *Coliforms* were significantly decreased in the LA group compared with the other groups, while the *Clostridium* spp. was significantly decreased ($p < 0.05$) only in the LA + LP group. *Enterococcus* spp. was lowered in the LA+LP group compared with the C group, while *Staphylococcus* spp. parameter, both LP and LA+LP groups were significantly ($p < 0.05$) affected compared with the C and LA groups (Figure 3B). In the caecum segment, the count of *Lactobacilli* was significantly ($p < 0.05$) lower in the C group compared with all experimental groups. No significant

effect was observed for the *Coliforms* and *Enterococcus* ($p > 0.05$). The *E. coli* was significantly lower only in the LA + LP group compared with the C group, while the *Staphylococcus* was significantly lower ($p < 0.05$) compared with all other experimental groups (Figure 3C). *Salmonella* was absent in all cases.

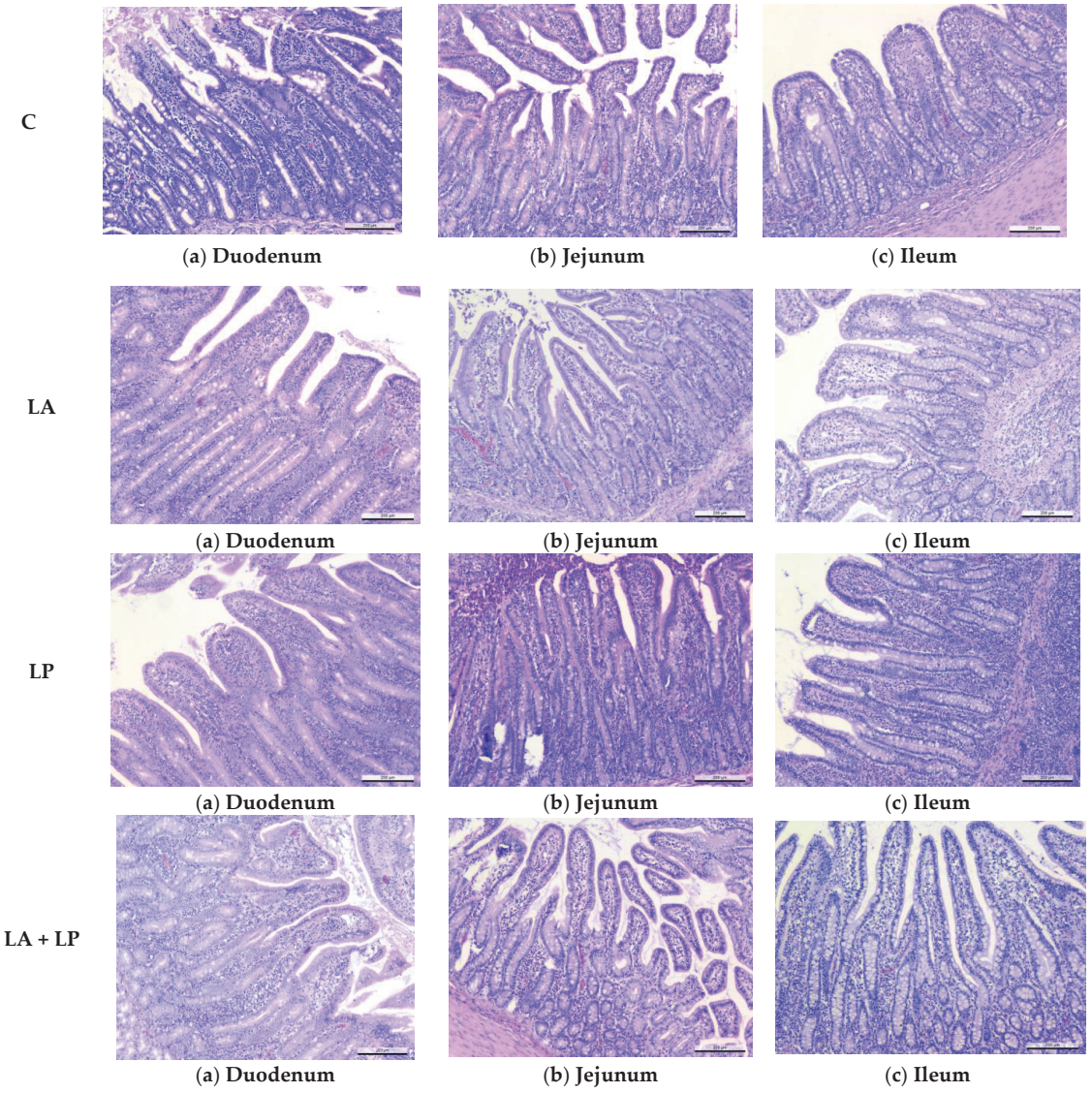


Figure 2. The intestinal morphometric appearances of the duodenum (**a**), jejunum (**b**) and ileum (**c**) of the weaned piglets (n = 8/group). The scale bars in each image indicate 200 μm. Villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth was measured from the crypt-villus junction to the base of the crypt. C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus;* LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*.

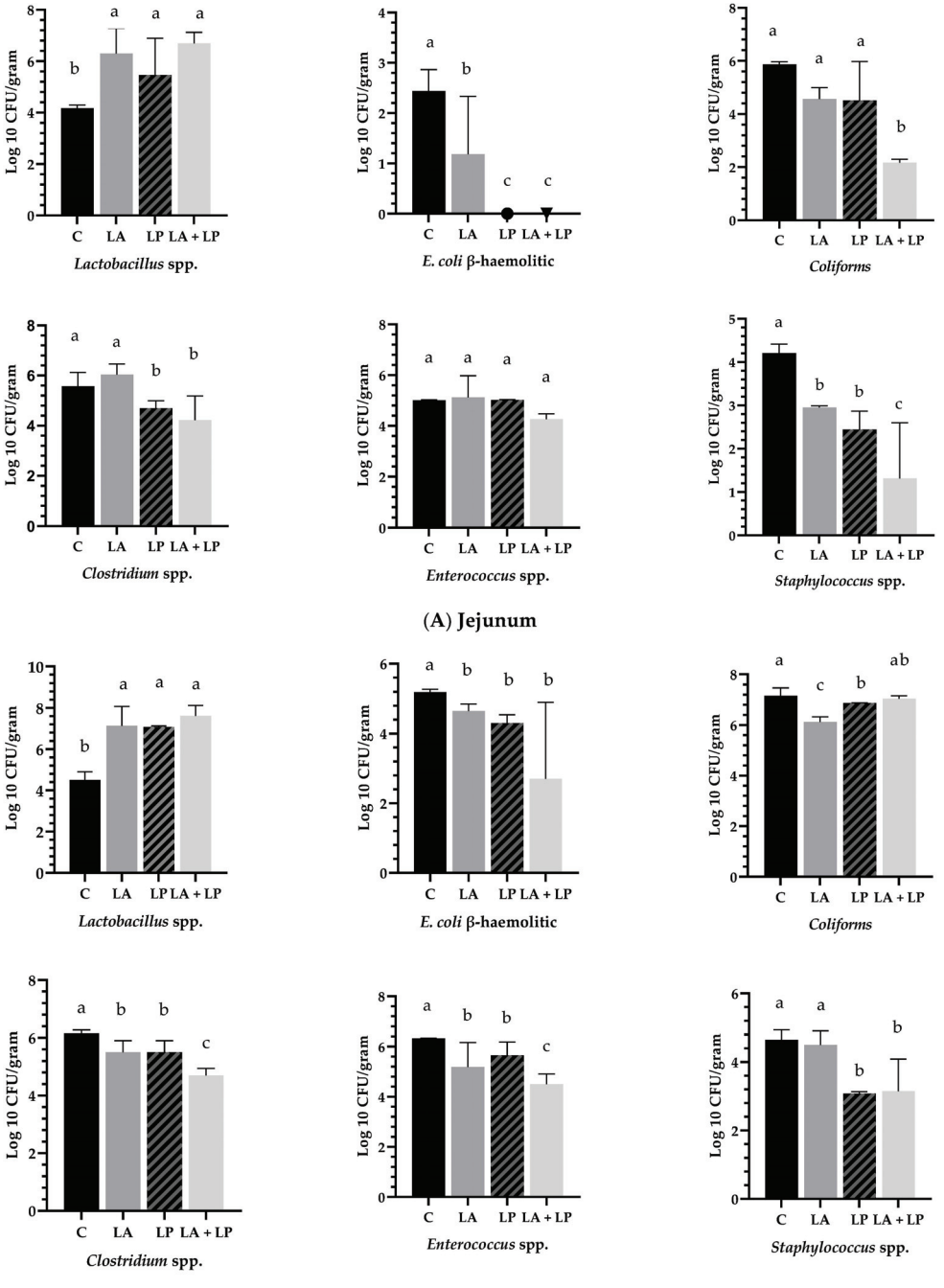


Figure 3. *Cont.*

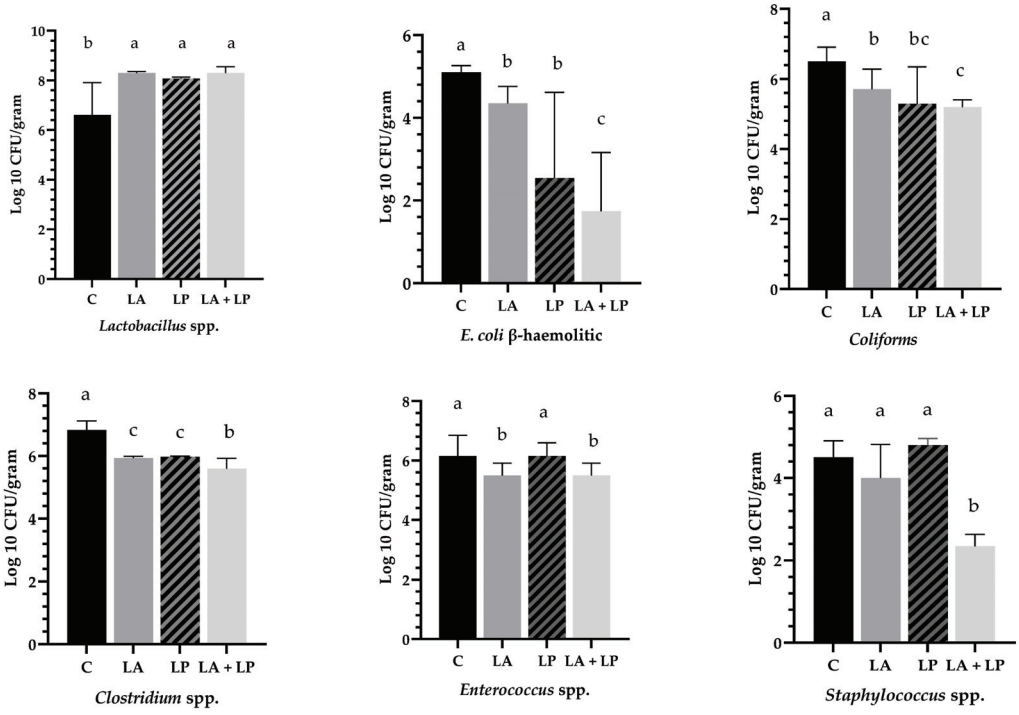
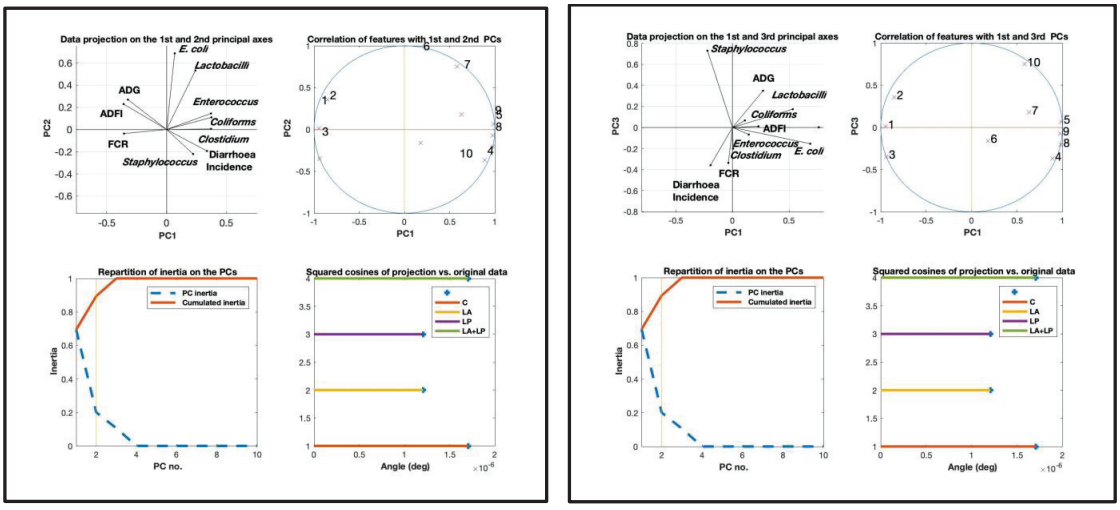


Figure 3. Effect of microencapsulated probiotic supplements on intestinal microflora in the jejunum (**A**), ileum (**B**) and caecum (**C**) of weaning piglets (n = 8/group). C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA+LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*. The results are expressed as (log_{10} CFU/g). $^{a, b, c}$ different superscripts within each figure represents significant difference among the groups at $p < 0.05$.

3.6. Principal Component Analysis (PCA)

The application of PCA enabled easier analysis and comparison of similarities between groups by lowering the number of variables. We considered a centred and normalized data version to obtain the PCA representation (Figure 4). The cumulated inertia of the PC1 + PC2 and the inertia of PC1 + PC3 dimensions showed that there are strong relationships between variables and suggests the number of dimensions that should be studied. The first and second components (PC1 and PC2) showed eigenvalues higher than 1 (6.91 and 2), covering 69% in PC1 and 20% in PC2 of the global variance of the data (Figure 4A). However, because the eigenvalue of PC3 was higher than 1 (1.02%), Cattell's scree plot confirmed that the usage of PC3 is also appropriate, showing a variance of the data of 10% (Figure 4B), and together explaining 99% of the data variation.



(**A**) (**B**)

Figure 4. Results of the principal component analysis (PCA), where: (**A**) is the graphical representation of the first and second principal components (PC1 and PC2), covering 89% of the global variance, correlation of features with first 2 PCs, inertia and angle degree. (**B**) is the graphical representation of the first and third principal components (PC1 and PC3), covering 79% of the global variance, correlation of features with first and 3rd PCs, inertia and angle degree. ADG—average daily gain; ADFI—average daily feed intake; FCR—feed conversion ratio.

4. Discussion

4.1. Effects of Microencapsulated Probiotics Supplements on Growth Performances of Weaning Piglets

The microencapsulated probiotics supplements used in this study showed positive effects on the ADFI, ADG and FCR during the first two-week period, while no significant modifications were observed for the overall period. Different studies have shown that probiotics are suitable substitutes for antibiotics in feed [28,29] because they can synergistically promote the growth of animals as well as improve their immune systems [30,31]. In previous research studies it was reported that utilization of *L. acidophilus* or *L. plantarum* did not affect the growth performances of weaned piglets [32,33], while others suggested that *L. plantarum* increased ADG in weaned pigs [29]. Moreover, it was reported that *Lactobacillus* (5×10^{10} CFU/kg) had positive effects on the growth performance of piglets by effectively improving barrier function [34] due to their probiotic effect. This can be explained by the exposure of the piglets to stress in connection with weaning, which produces major changes in the gut, and the time needed for adaptation to the new situation. In this regard, the efficiency of probiotics should be expected to be higher when the animals are confronted with stress during the first 2 weeks after weaning. When the most critical phase after weaning has passed and a normal gut function has been re-established, the impact of probiotic supplements should be expected to be less important for piglet performance [19]. In the present study, supplementation with microencapsulated *L. acidophilus* (1×10^8 CFU/kg of feed) or *L. plantarum* (3×10^8 CFU/kg of feed) had no significant influence on the overall production performances, which concludes other results where different concentrations of *L. acidophilus* (7.5×10^8 CFU/kg) or *L. plantarum* (2×10^8 CFU/g) were used [33,35]. However, the supplementation with the combination of microencapsulated *L. acidophilus* and *L. plantarum* significantly affected final production performances. This can be attributed to the variability in the viable counts of probiotics in the feed and the high survival rate of *Lactobacillus* colonizing in the intestine, which may produce organic acids and vitamins to improve growth performance. Probiotics also may exert their beneficial effects only when

their viable counts reach a certain quantity in the gastrointestinal tract. Literature data showed that combined probiotics supplementation with *L. reuteri* and *L. plantarum* did not affect ADG and FCR in weaned piglets [36]. However, the comparison of our results with those of others is complicated by the fact that different studies have used different bacterial strains and pigs of different ages, housing, and health status, but the utilization of combined microencapsulated probiotics needs further investigation. Moreover, in the current study, the piglets were fed microencapsulated supplements, while previous studies have used non-encapsulated administration methods. Although this aspect needs further investigation, we believe it leads to different results.

4.2. Effects of Microencapsulated Probiotics Supplements on Organs Development, Intestinal pH and Diarrhoea Incidence of Weaning Piglets

Feeding microencapsulated probiotics to the weaning piglets resulted in no modifications in the weight of the measured organs. This result was expected considering the young age of the animals and the fact that BW and ADG were similar among the groups. To our knowledge, literature data are lacking on reporting these results in weaning piglets, maybe because visceral organs do not present commercial interest for this category of piglet. Similarly, it has been reported that feeding piglets with tannic acids as antibiotics replacements for the development of the liver, spleen, kidney, heart and small intestine were not influenced [37]. For the intestinal pH determined in different intestinal segments it was observed that all experimental groups tended to have lower values, but only in the ileum, significant differences were observed in the LA and LA + LP groups compared to the C group. These results are very important because intestinal pH is one of the controlling factors for maintaining microbial balance and gut health in pigs. Weaning piglets have a limited capacity for secretion of hydrochloric acid and pepsin activity which further negatively influenced digestion and nutrient absorption of some nutrients, especially vegetal proteins. Therefore, a low pH is beneficial to prevent the growth of harmful bacteria, enhancing nutrient digestion and improving growth performance. Previously it was reported that lower gastric pH maintains a healthy gut because it hinders the passage of pathogenic bacteria into the small intestine [38–40], while a higher pH favours the introduction of food-borne pathogens to colonize the gut, resulting in the initiation of diarrhoea in piglets after weaning and decreased production performances [41]. In the study of [42], it was found that supplementing dietary probiotics bacteria to the weaning piglets increased the production of short-chain fatty acids, which helps to reduce the pH values and subsequently depress the growth of pathogenic bacteria. Literature data show that feeding microencapsulated *Enterococcus faecalis* for 21 days to weaning piglets' pH values were not different among treatments [43] but showed less incidence of diarrhoea. Similarly, tannin acids were ineffective in reducing pH in the intestinal segments [37]. Further, in this study dietary microencapsulated probiotics were shown to alleviate the detrimental effects of the weaning crisis by reducing diarrhoea, especially in the LP and LA+LP groups compared to the C group. Overall, the beneficial effects of the LAB strains are consistent with some recent findings using other probiotic strains, including *Enterococcus faecium* and multispecies probiotics such as *L. acidophilus*, *L. casei*, *Bacillus thermophilum*, and *E. faecium*, or microencapsulated *L. plantarum* or *Pediococcus acidilactici* which have been shown to reduce the severity of diarrhoea in weaning piglets [44,45]. However, to our knowledge, no other studies reported the effects of *L. acidophilus* and *L. plantarum* combined on organ development or reducing diarrhoea incidence in weaning piglets.

4.3. Effect of Microencapsulated Probiotics Supplements on Biochemical Parameters of Weaning Piglets

In the current study, supplementing weaning piglets with microencapsulated probiotic bacteria resulted in no significant effect on biochemical parameters. Only tendencies to decrease or increase some parameters were observed (Table 4). The fact that lipid, protein, mineral and enzyme levels were not significantly affected suggests that neither the oxidative challenge induced by weaning stressors nor the experimental diets induced circulating

abnormal metabolite levels. Similarly, Aiyegoro et al. [46] found no significant difference in biochemical parameters such as total serum protein, cholesterol and glucose among all treatment groups in piglets supplemented with different probiotics such as *L. reuteri*, *S. salivarius* or a combination of the same probiotics. These results suggest that the piglets without probiotics addition are more susceptible to infection and dehydration, as Busanello et al. [47] observed. Other authors [29–31] reported that probiotics could synergistically promote the growth of animals as well as improve their resistance to infections by stimulating the immune system.

Contrary to our results, multi-probiotic *Lactobacillus* supplementation has been reported to alter lipid fractions in pigs [48,49]. Although we did not find any studies following similar dietary supplementation on weaned piglets, it is difficult to confirm that probiotics derived from microencapsulated LAB contribute significantly to the immune system of the weaned piglets. The main reason behind this is that probiotics differ from antibiotics; they are not intended to eradicate invasive pathogens, only to protect them. Therefore, observed improvements or positive effects are often reported in sow or fattening-growing pigs [46,50] and are linked to the various applied situations.

4.4. Effects of Microencapsulated Probiotics Supplements on Intestinal Histomorphology of Weaning Piglets

Three critical components compose the gut ecosystem: the immune system, intestinal epithelial cells, and the microbial population. The nutrition, gender, background genotype, housing conditions, litter size, and age of the animals might all have a favourable or unfavourable impact on these three factors. Probiotics like *L. acidophilus* and *L. plantarum* or their combination may improve not only the integrity of intestinal epithelial cells but also the intestinal microbiome, which will further boost nutrient absorption and enhance animal development performance. In the present study, the supplementation of a microencapsulated *L. acidophilus* and *L. plantarum*, as well as their combination, improved the morphological parameters after 21 days of feeding in the weaning period of piglets, especially VL in the duodenum, and VCL in all segments with various changes for the other parameters. This result is benefic to the animals because, as it was previously reported, longer VL indicate a better absorptive capability of the small intestine and hence a healthy gut [51], while on the opposite, a reduction in VW has been associated with higher incidences of diarrhoea [41]. Additionally, the overall intestinal morphology, including VW, VL, CL, C/D, and VCL, represents the intestine's capacity to digest and absorb nutrients in relation to a healthy intestinal gut [52]. Wider villi are connected with an increased surface area capable of better absorption of nutrients accessible because intestinal villi are the main area for nutrient absorption [53]. The villus factory, or new epithelial cell production, is referred to as the crypts. Faster tissue turnover is typically associated with deeper and longer crypts to ensure villus renewal in response to natural sloughing or inflammation induced by infections or related toxins [54]. Moreover, the modifications we found in the current study regarding the effect of microencapsulated probiotics on the weaning piglets are correlated with improved growth performance, as presented in Table 2. Although there are fewer studies on the effect of microencapsulated LAB on the intestinal morphology of piglets, it has been reported that *L. plantarum* increased the VL and V/C ratio in the duodenum with no significant effect in the jejunum of the piglets [55]. Others [56] reported that combined chitosan and microencapsulated *Enterococcus faecalis* resulted in a tendency to increase VL but significantly affected the V/C ratio.

All in all, as observed in the present study and in agreement with the studies referenced above, microencapsulation of the LABs had a significant positive effect on intestinal morphology development, while the C group piglets had the shortest VL and higher incidences of diarrhoea. Nevertheless, comparing data on intestinal morphology from different experiments is difficult because of differences in the diets, breed, age, experimental conditions and as well as no known standards for the measurements of VL, VW, CL or V/C ratio [13]. Therefore, the positive effect on the intestinal morphology observed in the study

may be because the probiotics used were microencapsulated and presented better stability compared with other probiotics reported in the literature. This aspect leaves a door open for further studies to compare the parallel effect of these probiotics as microencapsulated products versus non-encapsulated ones in the weaning piglets' diets.

4.5. Effects of Microencapsulated Probiotics Supplements on Intestinal Microbiota of Jejunum, Ileum and Caecum of Weaning Piglets

The stressor factors, especially in the early weaning stage of piglets, induce changes in gut microbiota and epithelial barrier [57,58]; hence various studies have indicated that supplementation of probiotics could help to balance the bacterial community in weaned piglets [29,59]. In the current study, the microencapsulated probiotics used in weaning piglets showed some promising effects. The increased number of *Lactobacilli* in the intestinal segments represents a beneficial effect for weaning piglets, to defeat the overgrowth of pathogens. Moreover, the alteration of *E. coli* and *Streptococcus* spp. was favourable to creating an anaerobic environment for establishing other colonizers such as *Lactobacillus* and *Clostridium*. Previously, it was reported that in severely stressed animals the gastrointestinal microflora was significantly affected by decreasing total *Lactobacillus* populations during the neonatal and weaning period of untreated piglets [60]. Similarly, other studies conducted during the weaning transition of untreated piglets have reported a decrease in bacteria of the *Lactobacillus* group and a loss of microbial diversity, whereas *Clostridium* spp., *Prevotella* spp. or facultative anaerobes such as *Proteobacteriaceae*, including *E. coli*, were favoured [61–64]. Opposite to these studies, it was found that *Lactobacillus* strains with specific probiotic traits would decrease diarrhoea severity at various life stages and alleviate weaning stress syndrome [65], which is in line with our results. Moreover, the increased number of *Lactobacilli* in the intestinal segments is also reflected in the decreased number of *E. coli* and infections with *Salmonella* spp. It was reported that the administration of *L. plantarum* to newly weaned pigs appeared to inhibit the growth of opportunistic pathogens, as reported by other authors [66]. In a trial where *L. casei* combined with maltodextrin was given to the pigs, the count of *E. coli* in the jejunal mucosa was significantly inhibited [67]. As in our case, this inhibitory effect was probably caused by *Lactobacillus* addition which presents antibacterial substances and stimulates the host immunity system. However, besides the supplements used to alleviate the weaning crisis, the host's genetic background also plays a key role in driving the settlement of the gut microbiota, presenting a predisposing factor to piglet infections. Regarding the use of probiotic LABs in weaning piglets, recent studies have reported similar results in terms of improving intestinal health. Zhang et al. [68] reported that *L. reuteri* increased the beneficial species richness of the microbiota in the jejunum, colon, and cecum of weaning piglets. Orally administrated *L. casei* and *Enterococcus faecalis* or a combination of *L. casei* and *E. faecalis* at a ratio of 3:1 probiotic improved gut microbiota and increased microbial [24]. A greater villus height and abundance of *Lactobacillus* was detected in the intestinal segments of piglets supplemented with *L. salivarius* compared with the piglets from the control group, as reported in a recent study [1]. Different *L. plantarum* has been reported to beneficially modulate the intestinal microbiota in weaning piglets [69,70]; however, limited results are reported for *L. acidophilus* [34,71]. To our knowledge, no other studies reported the combined effect of *L. plantarum* and *L. acidophilus*. Although we obtained significant results from this probiotic supplement, further investigations are required.

4.6. Principal Component Analyses

The principal component analysis (PCA) was conducted in order to explore the relationship between dietary supplements and production performances, diarrhoea incidence and the intestinal microflora population of the weaning piglets (Figure 4). After mean-centering and scaling to unit variance, the PCA on these attributes explained about 99% of the variability in the data. The loading of PC1 and PC2 which occupied 89% of the data variance (Figure 4A), revealed that production performances and intestinal health as well

as diarrhoea incidence are oppositely correlated. These variables are positioned in opposite directions alongside the axis of PC1. From the same analyses, it was clear that the intestinal microflora population was strongly correlated with the diarrhoea incidence (Figure 4A), all these parameters being grouped on the same side of the PC1 axis, while the production performances were placed on the left side of the PC2 axis. However, by analyzing the data variance from PC 1 and PC3, which occupied about 79%, it was noted that the variables were differently distributed. The *Staphylococcus* was placed in the opposite direction (PC3) with ADG and *Lactobacilli*, with the total count of *E. coli* in the second dimension and diarrhoea incidence in the third dimension of PC1 (Figure 4B). These results revealed that the total count of *Lactobacilli* and *Coliform* might have an influence on ADG and ADFI. The values of *E. coli*, *Lactobacilli*, *Staphylococcus* diarrhoea incidence, ADG and FCR were higher in both plotting areas of PC1, PC2 and PC3. Overall, according to the PCA results, the three supplemented diets had a significant impact on the intestinal microflora population and diarrhoea incidence of the animals, with a low impact on production performances.

5. Conclusions

This study's findings revealed that microencapsulated probiotic bacteria such as *L. acidophilus*, *L. plantarum*, or their combination had no significant effect on production performances. However, the diarrhoea incidence was significantly decreased, especially in the *L. plantarum* and combination of *L. acidophilus* and *L. plantarum* groups. Further, the usage of the microencapsulated *L. acidophilus*, *L. plantarum* and their combinations were very effective in decreasing intestinal pH in the ileum segment and improving intestinal health by significantly increasing the beneficial bacteria such as lactobacilli and decreasing the counts of *E. coli* and *Staphylococcus* in the jejunum, ileum and caecum. In conclusion, the utilization of combined microencapsulated probiotics such as *L. acidophilus* and *L. plantarum* has been proven to be the most effective in weaned piglets' diet, in terms of reducing the diarrhoea incidence, improving the intestinal morphology and microflora.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Effects and Underlying Mechanisms of Zearalenone Mycotoxin at Concentrations Close to the EC Recommendation on the Colon of Piglets after Weaning

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Abstract: Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* fungi that contaminates food and feed, affecting both human and animal health. Among farm animals, the pig is a great consumer of grains and has a native sensitivity to mycotoxins. As the main route of contamination is oral, the intestine is the first defense barrier that plays an important role in the immune response being able to secrete effector molecules (cytokines). At the European level, there are no regulations regarding the amount of ZEN that can be present in the feed of piglets, only recommendations for piglets 0.100 mg ZEN/kg feed (100 ppb). In this study, the effects of ZEN in concentrations below (75 ppb) and above (290 ppb) EU recommendation on the level of some key markers involved in the oxidative and inflammatory response, as well as the mechanisms and signaling pathways through which ZEN could produce its toxicity, were monitored in the colon of weaned piglets. The exposure of the piglets to the lower concentration of ZEN (75 ppb) did not lead to changes in stress and inflammation markers or in the signaling pathways associated with these processes.

Keywords: zearalenone; piglets; weaning; inflammation; oxidative stress; signaling pathway

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1. Introduction

Mycotoxins are some of the most common natural contaminants in food or feed involving not only health issues but also enormous economic losses [1]. Fusariotoxins are among the most known toxins produced by molds of the *Fusarium* genus and the largest group, which includes over 140 known metabolites. The most common fusariotoxins are zearalenone, deoxynivalenol, nivalenol, T-2, HT-2 toxins, and fumonisins [2]. Farm animals are often affected by mycotoxins, particularly the swine species due to the high cereal content in the diet and their native sensitivity [3].

Zearalenone (ZEN) is a fusariotoxin belonging to the xenoestrogen class due to its structural similarity to β-estradiol and its affinity for estrogen receptors [4]. It was demonstrated that ZEN causes reproductive disorders, including hormonal and reproductive disorders, especially in pigs. Moreover, ZEN affects also other physiological systems: digestive, immune, and nervous system, which are manifested by diarrhea, vomiting, reduced appetite, leukopenia, etc. [5–7].

According to the European Commission (Recommendation EC/2006/576), the guideline values for ZEN as allowed limit in feed are 0.100 mg ZEN/kg (100 ppb) for piglets and young sows and 0.250 mg ZEN/kg (250 ppb) for mature sows and pigs [8]. These values are only recommendations and more toxicological data issued from in vivo studies are necessary in order to establish a regulation for the concentration of zearalenone that can be admitted in feed material and compound feed for swine. Moreover, there are studies on

pigs that reported significant effects at a concentration of 100 ppb ZEN in the feed [9] and others in which this concentration had no statistically notable effects [10].

In order to bring new scientific data leading to the establishment of a guideline value for ZEN in young pigs, the aim of this study was to evaluate in vivo the effects of two concentrations of ZEN, one below the EC recommendation (75 ppb) and the other above the EC recommendation (290 ppb) on piglets immediately after weaning.

Weaning is a difficult period for piglets due to the switch from sow's milk to solid feed, environmental changes, and an undeveloped immune and digestive system which predispose piglets to pathogen infections and digestive disorders including diarrhea [11]. The quality of the feed is of great importance in this period for the development of digestive and defense systems [12].

During the post-weaning period (1–2 weeks) the gut, including the colon are the most vulnerable tissue [13]. Currently, many studies on the exposure of weaned piglets to ZEN are performed at the small intestine level. However, the large intestine takes up undigested feed; therefore, at the level of the colon, the absorption of several substances can take place, excluding water and electrolytes [14]. Also, the colon is a gut segment with high microbial activity (active substrate hydrolysis and fermentation). As shown by Richards et al. [15] colonic microbiota produced fermentation products with protective function stimulating the immune response. But, at weaning, several defaults in the intestinal barrier function were observed which could be a starting point for inflammation, as well as water and electrolyte imbalance [16]. During this period, an increase in secretory activity and permeability was observed in the colon [17]. Moreover, diarrhea frequently occurred during the weaning period and is often associated either with the shifts in microbiota (suppression of several beneficial lactic bacteria) after the transition to solid feed or with colonic inflammation caused by enterotoxigenic *Escherichia coli* or other pathogens [18]. These pathogens increased the inflammatory cytokines in the colon, contributing to colonic inflammation. Zearalenone can be an additional pro-inflammatory factor that increases colonic inflammation if the feed that the piglets eat during this period is contaminated with ZEN. Bauer et al. [19] reported that feed type and quality had an important influence on gut microbiota. Reddy et al. [7], analyzing the colon content of three groups of pigs fed diets contaminated with zearalenone (800 ppb) and deoxynivalenol (8000 ppb), reported that the toxin-contaminated diets significantly affected the colon microbiota, especially *Lactobacillus*. Similarly, a previous study of our team found also that ZEN (290 ppb in the diet) decreased the populations of *Lactobacillus* and *Bifidobacterium* [20]. However, the effects of ZEN on the pig colon microbiota, and inflammatory and stress response are not completely understood [7]. Thus, the effect on innate (toll like receptors), pro-inflammatory and anti-inflammatory immune response (cytokines), oxidative/antioxidant response (lipid, protein, nucleic acids, and antioxidant enzymes), and the underlying signaling pathways (NF-kB, KEAP1, HO1, NQO1, Nrf2 etc.) were investigated in the colon of weaned piglets exposed to low (75 ppb) and for comparison to a high dose (290 ppb) of ZEN contaminated diet.

2. Materials and Methods

2.1. Toxin Preparation

Zearalenone purchased from FERMENTEK (Jerusalem, Israel) was dissolved in dimethyl sulfoxide (DMSO). The obtained solution was diluted in water at a ratio of 1:7 and then added to the basal diet until final concentrations of 75 μg/kg feed and 290 μg/kg feed were obtained. Considering the occurrence of mycotoxins naturally in feed, the presence of aflatoxin B1 (AFB1), deoxynivalenol (DON), ochratoxin A (OTA), total fumonisins (FBs: B1, B2, B3), T2/HT-2, and ZEN were analyzed by ELISA, using the VERATOX (Neogene, Lansing, MI, USA) kit according to the manufacturer's instructions, with a detection limit between 0.1 and 200 ppb (0.5 ppb AFB1, 100 ppb DON; 1 ppb OTA, 0.2 ppm FBs, T2/HT-2 25 ppb, 5 ppb ZEN). The concentration of all investigated mycotoxins was below the detection limit. The diets were also screened for contamination with bacteria (total bacteria

count, *Escherichia coli*, *Salmonella*) and with fungus (total fungi count) and the contamination levels were found to be under the EU limits accepted for pigs.

2.2. Experimental Design

The experiment was performed in vivo on 18 crossbred weaned piglets (TOPIGS-40) hybrid [female Large White × Hybrid (Large White × Pietrain) × male Talent, mainly Duroc]. Hybrid piglets were weaned at 27 days of age with a body weight of 11.25 ± 1.14 kg, randomly assigned to three groups (6 animals/group), control group (Control) fed with uncontaminated feed and two experimental groups: group experimental 1 (ZEN 75) fed the control diet artificially contaminated with 75 µg ZEN/kg feed below the concentration recommended by the EC and group experimental 2 (ZEN290) fed the control diet artificially contaminated with 290 µg ZEN/kg feed above the EC recommendation. The hybrid piglets were weaned at 27 days of age with a body weight of 11.25 ± 1.14 kg. The ingredients of the basal diet were described by Grosu et al., 2023 [20]. Briefly, the diet contained corn (68.46%), soybean meal (19%), vegetal milk (5%), corn gluten (4%), l-Lysine (0.31%), Methionine (0.10%), $CaCO_3$, (1.57), Ca $(H_2PO_4)_2$, mineral–vitamin premix (1%), choline premix (0.10%), and phytase (0.01%). The diets were screened for other mycotoxin contamination, and the levels were found to be under the EU limits for pigs. The experiment lasted 30 days and the animals had free access to feed and water. At the end of the experiment, animals were killed (electrically stunned) and colon samples were collected perfused with ice-cold saline solution and stored at −80 °C until further analysis. The experiment complied with the EU Council Directive 98/58/EC and Romanian Law 206/2004 which regulates treating animals used for experimental purposes. The experimental design was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, Romania (Ethical Committee no. 41/2023).

2.3. Quantification of Gene Expression

The effects produced by the low and the higher concentration of ZEN on the gene expression of several inflammatory cytokines, such as interleukin1-beta (*IL-1β*), tumor necrosis factor-alpha (*TNF-α*), interleukin-8 (*IL-8*), interleukin-6 (*IL-6*), interleukin-4 (*IL-4*), interleukin-10 (*IL-10*), interferon-gamma (*IFN-γ*); antioxidant enzymes superoxide dismutase (*SOD*), glutathione peroxidase (*GPx*), and catalase (*CAT*); Toll-like receptors *TLR 2*, *TLR 4*, *TLR 5*, and *TLR 9*; signaling molecules *TGFβ2*, *MYD88*, *IRAK1*, *TRAF6*, *TAK1*, *AKT*, *JNK 1/2/3*, *ERK 1/2*, and *p38α*; and transcription factors nuclear factor kappa b (*NF-kB*), activator protein 1 (*AP-1*), and nuclear factor erythroid 2-related factor 2 (*Nrf-2*), involved in inflammation and oxidative stress pathways were analyzed. The RNA extraction and transcription into cDNA were performed using the protocol described by Marin et al. [5]. Subsequently, 10 ng of cDNA sample was mixed with 0.3 µM primers of the genes of interest and 10 µL of SYBR Green qPCR Master Mix (Life Technologies, Carlsbad, CA, USA), and brought to the final volume of 25 µL with RNAse water free. The reactions took place in several steps: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C, completed with an elongation of 10 min at 72 °C. Data obtained were normalized with Norm Finder (x93NormFinderx94) software, and two reference genes were selected from a total of 6 housekeeping genes analyzed. Nucleotide sequences of the primers used in the experiment are shown in Table 1. The calculation method used was $2^{(-\Delta\Delta Ct)}$, and results were expressed as Fold Change (FC) compared to the control group.

Table 1. Nucleotide sequences of the primers.

Gene	Primer Sequence	Orientation	Tm (°C)	Amplicon Length (bp)
β-2 microglobulin	TTCTACCTTCTGGTCCACACTGA	5′-3′	50	162
	TCATCCAACCCAGATGCA	3′-5′	54	
GAPDH	ACTCACTCTTCTACCTTTGATGCT	5′-3′	49	100
	TGTTGCTGTAGCCAAATTCA	3′-5′	56	
Cyclophilin A	CCCACCGTCTTCTTCGACAT	5′-3′	54	92
	TCTGCTGTCTTTGGAACTTTGTCT	3′-5′	55	
β-actin	GGACTTCGAGCAGGAGATGG	5′-3′	60	230
	GCACCGTGTTTGCGTAGAGG	3′-5′	62	
HGPRT-1	TGGAAAGAATGTCTTGATTGTTGAAG	5′-3′	58.57	93
	ATCTTTGGATTATGCTGCTTGACC	3′-5′	59.66	
RPL 32	TGCTCTCAGACCCCTTGTGAAG	5′-3′	61.93	106
	TTTCCGCCAGTTCCGCTTA	3′-5′	59.63	
TNF-α	ACTGCACTTCGAGGTTATCGG	5′-3′	60	118
	GGCGACGGGCTTATCTGA	3′- 5′	60	
IL-8	GCTCTCTGTGAGGCTGCAGTTC	5′-3′	58	79
	AAGGTGTGGAATGCGTATTTATGC	3′-5′	54	
IL-6	GGCAAAAGGGAAAGAATCCAG	5′-3′	57	87
	CGTTCTGTGACTGCAGCTTATCC	3′-5′	61	
IL-1β	ATGCTGAAGGCTCTCCACCTC	5′-3′	62	89
	TTGTTGCTATCATCTCCTTGCAC	3′-5′	59	
IFNγ	TGGTAGCTCTGGGAAACTGAATG	5′-3′	54	79
	GGCTTTGCGCTGGATCTG	3′-5′	55	
IL-4	CAACCCTGGTCTGCTTACTG	5′-3′	52	173
	CTTCTCCGTCGTGTTCTCTG	3′-5′	52	
IL-10	GGCCCAGTGAAGAGTTTCTTTC	5′-3′	54	51
	CAACAAGTCGCCCATCTGGT	3′-5′	55	
NF-kB	CGAGAGGAGCACGGATACCA	5′-3′	55	62
	GCCCCGTGTAGCCATTGA	3′-5′	54	
CAT	CTTGGAACATTGTACCCGCT	5′-3′	62	241
	GTCCAGAAGAGCCTGAATGC	3′-5′	62	
GPx	GGAGATCCTGAATTGCCTCAAG	5′-3′	50	62
	GCATGAAGTTGGGCTCGAA	3′-5′	58	
SOD	GAGACCTGGGCAATGTGACT	5′-3′	62	139
	CTGCCCAAGTCATCTGGTTT	3′-5′	60	
Nrf2	CCCATTCACAAAAGACAAACATTC	5′-3′	57	72
	GCTTTTGCCCTTAGCTCATCTC	3′-5′	59	
AP1	CCCAAGATCCTGAAGCAGAG	5′-3′	62	136
	GATGTGCCCGTTACTGGACT	3′-5′	62	

Table 1. *Cont.*

Gene	Primer Sequence	Orientation	Tm (°C)	Amplicon Length (bp)
p38α	TGCAAGGTCTCTGGAGGAAT	5′-3′	52	109
	CTGAACGTGGTCATCCGTAA	3′-5′	52	
TGFβ2	CGATGATGATGTTGATGATGG	5′-3′	55	69
	GCAAGGCTTTCTTGTATTTTCTTG	3′-5′	58	
TAK1	TGCCCAAACTCCAAAGAATC	5′-3′	56	151
	TTTGCTGGTCCTTTTCATCC	3′-5′	56	
ERK1	CTACCTGGACCAGCTCAACC	5′-3′	60	85
	CACTGTGATCCGTTTGTTGG	3′-5′	60	
ERK2	TGACATTCAACCCTCACAAGA	5′-3′	42.86	198
	ATCTGTATCCTGGCTGGAATC	3′-5′	47.62	
JNK1	TGCTTTGTGGAATCAAGCAC	5′-3′	51	60
	TGGGCTTTAAGTCCCGATG	3′-5′	51	
JNK2	TATTATCGGGCACCAGAAGTC	5′-3′	51	97
	AACCTTTCACCAGCTCTCTCA	3′- 5′	53	
JNK 3	TGCCTATGACGCTGTTCTTG	5′-3′	58.27	180
	TGAAACTCCTCCAGCGTCTT	3′-5′	58.95	
HO-1	ATGTGAATGCAACCCTGTGA	5′-3′	57.71	89
	GGAAGCCAGTCAAGAGACCA	3′-5′	59.31	
NQO1	GTATCCTGCCGAGACTGCTC	5′-3′	59.97	134
	TAGCAGGGACTCCAAACCAC	3′-5′	59.31	
KEAP	ACGACGTGGAGACAGAAACGT	5′-3′	61.94	56
	GCTTCGCCGATGCTTCA	3′-5′	58.07	
Akt	AAGGCCACGGGCCGCTACTA	5′-3′	65.94	100
	GGAGGACGCGGTTCTCCGT	3′-5′	64.2	
MyD88	GCAGCTGGAACAGACCAACT	5′-3′	60	66
	GTGCCAGGCAGGACATCT	3′-5′	59	
MD-2	CCTTGTTTTCTTCCATATTTACTG	5′-3′	54	63
	CATCAGAGGAATTGCAGATCCA	3′- 5′	58	
IRAK1	CAAGGCAGGTCAGGTTTCGT	5′-3′	55	115
	TTCGTGGGGCGTGTAGTGT	3′-5′	58	
TRAF-6	CAAGAGAATACCCAGTCGCACA	5′-3′	50	122
	ATCCGAGACAAAGGGGAAGAA	3′-5′	48	
TLR2	TCACTTGTCTAACTTATCATCCTCTTG	5′-3′	59	162
	TCAGCGAAGGTGTCATTATTGC	3′-5′	59	
TLR4	GCCATCGCTGCTAACATCATC	5′-3′	60	108
	CTCATACTCAAAGATACACCATCGG	3′-5′	59	
TLR5	CCTTCCTGCTTCTTTGATGG	5′-3′	56	124
	CTGTGACCGTCCTGATGTAG	3′-5′	57	
TLR9	CACGACAGCCGAATAGCAC	5′-3′	59	121
	GGGAACAGGGAGCAGAGC	3′-5′	60	

Results for gene expression were validated through western blot and enzyme-linked immunosorbent assay (ELISA) techniques for the following proteins: Nrf-2 and NF-kB, as well as IL-1β, TNF-α, IL-8, and IFN-γ.

2.4. Quantification of Protein Expression by Western Blot

The phosphorylated form of protein expression of two important nuclear transcription factors Nrf-2 and NF-kB, key molecules which regulate inflammation and oxidative stress pathways was determined by Western Blot. Protein concentration in colon sample lysates in RIPA buffer was quantified using a specific kit (Pierce BCA Protein Assay Kit, Thermo Fischer Scientific, Waltham, MA, USA). The protein (30 µg) was separated by 10% SDS-page electrophoresis, transferred to the nitrocellulose membrane, blocked, and incubated with specific primary and secondary antibodies as described by Pistol et al. [21] using the same buffers, antibodies, and reagents, as well as the time of incubation. The immunoblotting images were developed using a MicroChemi Imager (DNR Bio-Imaging Systems Ltd., Neve Yamin, Israel) and the protein expression level was evaluated using GelQuant software (DNR Bio-Imaging Systems Ltd., Neve Yamin, Israel). The results of target protein expression were reported to β-actin housekeeping protein expression and reported as arbitrary units.

2.5. Quantification of Protein Concentration (ELISA)

Protein concentration for several pro-inflammatory cytokines, IL-1β, TNF-α, IL-8, and IFN-γ, was determined by using the ELISA technique and specific kits provided by R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions as described by Pistol et al. [21]. At the end of the reaction, the optical density was measured using a plate reader (SUNRISE TECAN, Grödig, Austria), and the protein concentrations were calculated by reference to a standard curve provided by the kit.

2.6. Assessment of Oxidative Stress

Oxidative stress was evaluated at three levels: lipid (TBARS), protein (protein carbonyl), and nucleic acids (DPA assay).

2.6.1. Lipid Peroxidation Measurement (Thiobarbituric Acid Reactive Substances-TBARS)

The lipid oxidation was determined from a 0.2-g colon sample homogenized in PBS (phosphate buffered saline), and thiobarbituric acid reactive substances (TBARS) were measured according to the protocol already described by Marin et al. [22]. Results were expressed as nmol/mL.

2.6.2. Protein Oxidation Measurement (Protein Carbonyl Analysis)

The protein oxidation was evaluated based on the detection of the reaction product between 2,4-dinitrophenyl hydrazine with protein carbonyls, known as the protein hydrazone using spectrophotometric methods. The protein concentrations from colon samples were determined by using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL, USA). The absorbances were determined at a wavelength of 370 nm using a microplate reader (Tecan, Sunrise, Vienna, Austria), the results being expressed in nmol/mg of carbonyl content.

2.6.3. DNA Fragmentation Using Diphenylamine (DPA)

To quantify DNA fragmentation in the colon samples, the DPA test was performed according to the method described by Ben Salah-Abbes et al. [23]. The tissue (0.05 g) was homogenized in 0.5 mL of lysis buffer (10 mM tris-HCl pH 8, 0.2% triton X-100, 1 mM EDTA) and centrifuged for 20 min at 4 °C and 10,000× *g*. The supernatant (S) was collected, the pellet (P) was resuspended in 0.5 mL of lysis buffer, and 0.5 mL of 25% trichloroacetic acid was added to both the supernatant and the pellet. The samples were incubated for 24 h at 4 °C. After incubation, the samples were centrifuged, the supernatant discarded,

and 80 µL of 5% TCA was added to the obtained precipitates and left for 20 min at 83 °C. The diphenylamine indicator (DPAi) was prepared using two solutions, the first with 1.5 g diphenylamine, 1.5 mL sulfuric acid, and 100 mL glacial acetic acid, and the second solution of 16 mg/mL acetic aldehyde. A volume of 160 µL of DPAi was added to each sample which was stored at room temperature for 24 h. At the end, the optical density (OD) was read at 600 nm using a microplate reader (Tecan, Sunrise, Vienna, Austria). DNA fragmentation was calculated according to the formula:

$$\% \text{ Fragmented DNA} = \frac{\text{OD(S)}}{\text{OD(S)} + \text{OD(P)}} \times 100$$

2.7. Assessment of Antioxidant Response

2.7.1. Determination of Total Antioxidant Status (TAC)

The Total Antioxidant Status was determined using a spectrophotometric method described by Taranu et al. [24]. The absorption of 20-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid cation (ABTS+)] was measured from colon samples at the wavelength of 732 nm, the results being expressed in mmol/L Trolox equivalents.

2.7.2. Determination of Antioxidant Enzyme Activity

The antioxidant enzymes Superoxide Dismutase (SOD) and Catalase (CAT) activity was assessed using Cayman kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instructions provided by the manufacturer as described by Chedea et al. [25]. The absorbances were read using the Tecan microplate reader (Sunrise, Vienna, Austria) at a wavelength of 450 nm.

2.8. Statistical Analyses

The results are represented graphically by mean ± standard error of the mean (SEM). A one-way ANOVA test was performed using GraphPad Prism (9.3.0) software, followed by Fisher's exact test, and the differences between the experimental groups were considered significant at a p-value < 0.05; p values between 0.05 and 0.1 being considered a trend. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05), *—significant (0.05 ≥ p-value > 0.01), **—very significant (0.01 ≥ p-value > 0.001) and ***—extremely significant (0.001 ≥ p-value > 0.0001).

3. Results

3.1. Effect of ZEN on Innate Immunity

Analyzing the gene expression of some TLRs involved in the innate immune response (Figure 1), it was observed that exposure to a higher concentration of ZEN leads to a significant increase in the gene expression level of TLR 4 (60% increased, p = 0.0236), while at the level of TLR2, TLR5, and TLR9, no change is observed. Moreover, along with the significant increase in TLR4 gene expression, the level of MYD88 gene expression increased significantly (48% increased, p = 0.0399), in the colon of piglets exposed to 290 ppb ZEN as compared with the control, suggesting that the TLR4/MYD88 signaling pathway could be involved in ZEN toxicity.

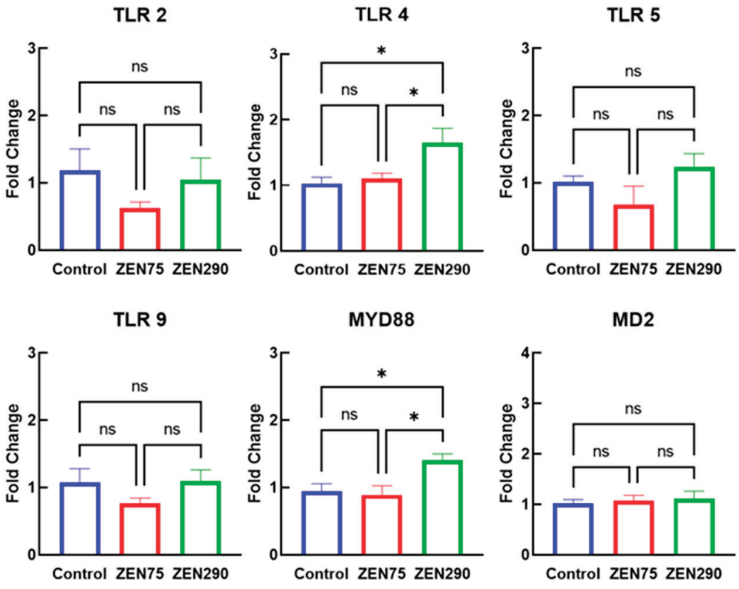

Figure 1. Effect of ZEN on gene expression of toll-like receptors TLR2, TLR 4, TLR5, and TLR9, and MYD88 and MD2 receptors in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05), *—significant ($0.05 \geq p$-value > 0.01).

3.2. Effect of ZEN on Oxidative Response

Regarding oxidative stress, at all three analyzed levels of lipid (TBARS), protein (protein carbonyl), and nucleic acids (DNA fragmentation), neither of the two concentrations of ZEN induced significant changes. However, in the case of protein oxidation, an increasing trend ($p = 0.069$) is observed for ZEN 290 (Figure 2).

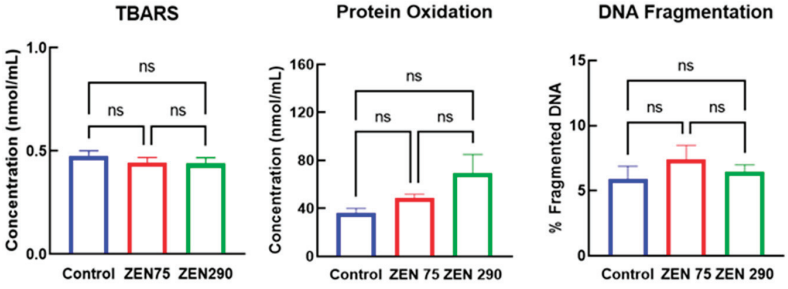

Figure 2. Effect of ZEN on lipid peroxidation (TBARS), protein oxidation, and DNA fragmentation in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05).

3.3. Effect of ZEN on Antioxidant Response

Regarding the total antioxidant status, a significant decrease was recorded in the case of ZEN 75 ($p = 0.025$) and ZEN 290 ($p = 0.006$). Knowing that the imbalance between antioxidants and oxidants plays an important role in oxidative status, the decrease in TAC indicates potential oxidative stress induced by ZEN exposure (Figure 3). Interestingly, the results on genes encoding for antioxidant enzymes CAT, SOD, and GPx showed that

both concentrations of ZEN below and above the EC recommendation did not significantly modify their expression in the colon of piglets fed the contaminated diet. Furthermore, the results obtained by qPCR were confirmed by enzymatic activity of CAT and SOD; ZEN did not induce any significant changes.

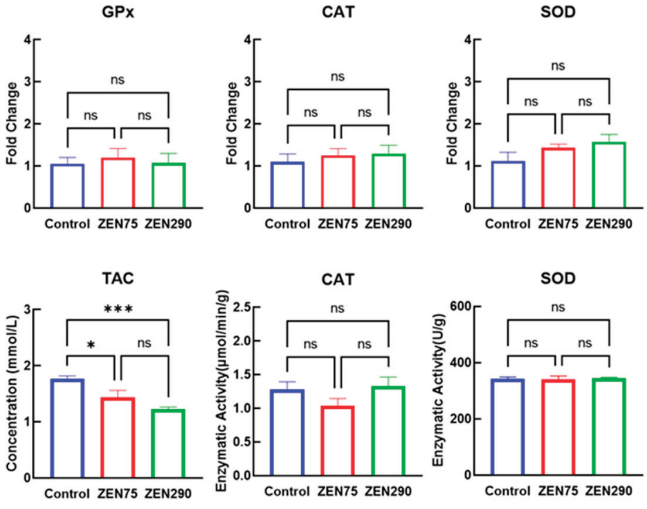

Figure 3. Effect of ZEN on total antioxidant capacity (TAC) and antioxidant enzymes CAT, SOD, and GPx in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value $\geq$ 0.05), *—significant (0.05 $\geq$ p-value > 0.01) and ***—extremely significant (0.001 $\geq$ p-value > 0.0001).

Analyzing the gene (Figure 4a) and protein expression (Figure 4b) of the Nrf2 signaling molecule responsible for the activation of antioxidant enzymes response, we did not observe a significant effect caused by any of the analyzed concentrations; however, in the case of ZEN 75, an increasing trend was observed in Nrf2 protein expression (p = 0.086).

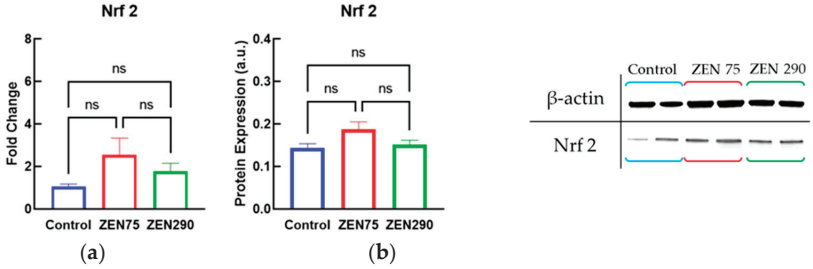

Figure 4. Effect of ZEN on (**a**) gene expression and (**b**) protein expression (histogram and protein detection image) of Nrf2 nuclear receptor in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value $\geq$ 0.05).

The gene expression of KEAP1, HO1, and NQO1 was also analyzed, knowing that they are key markers on the main regulatory KEAP1/Nrf2 pathway that triggers the intracellular defense against oxidative stress [26]. As in the case of Nrf2, no significant changes were observed regarding the gene expression levels of KEAP1, HO1, and NQO1 (Figure 5).

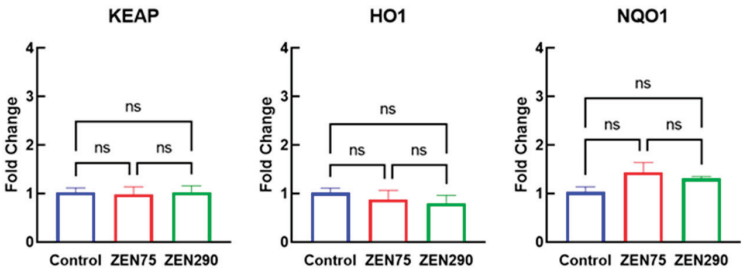

Figure 5. Effect of ZEN on gene expression of oxidative stress markers KEAP1, HO1, and NQO1 in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05).

3.4. Effect of ZEN on Pro-Inflammatory Response

The results obtained from the qPCR analysis showed that at the colon level, neither the concentration of ZEN 75 ppb, nor that of 290 ppb significantly affected the inflammatory response evaluated through the gene expression of the powerful inflammatory cytokines IL-1β, TNF-α, IL-8, IFN-γ, and IL-6 compared to the control group (Figure 6). Although a higher level of expression of these genes was recorded under 290 ppb of ZEN, the differences against the control group were insignificant.

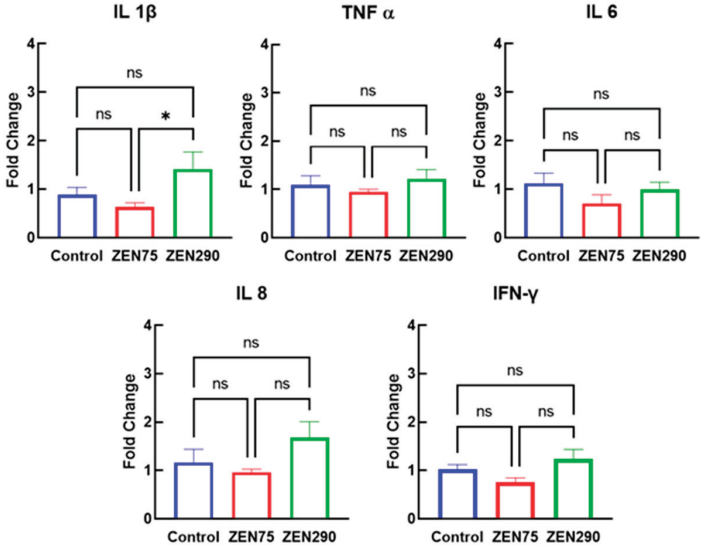

Figure 6. Effect of ZEN on gene expression of pro-inflammatory cytokines IL-1β, TNF α, IL 6, IL 8, and IFN-γ in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05), *—significant ($0.05 \geq p$-value > 0.01).

To validate the qPCR results obtained for the investigated pro-inflammatory markers, further analyses on the effect of the two concentrations of ZEN on their proteins were performed. The protein concentration determined by the ELISA method confirms the results obtained by qPCR for cytokines IL-1β, TNF-α, IL-8, and IFN-γ. No significant differences in protein concentration between the control and the two treatments were observed for the pro-inflammatory cytokines IL-1β, TNF-α, IL-8, and IFN-γ (Figure 7).

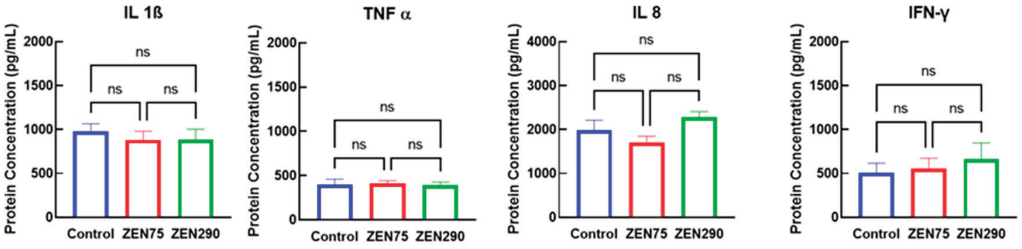

Figure 7. Effect of ZEN on the protein concentration of pro-inflammatory cytokines IL-1β, TNF α, IL 8, and IFN-γ in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05).

3.5. Effect of ZEN on Anti-Inflammatory Response

Furthermore, the effects of exposure to either ZEN 75 ppb or ZEN 290 ppb were investigated on the anti-inflammatory response expression in the colon through the assessment of cytokines IL-4 and IL-10 gene expression. Similarly, with the results obtained for pro-inflammatory cytokines, the gene expression level coding for the two interleukins IL-4 and IL-10 did not undergo statistically significant changes (Figure 8).

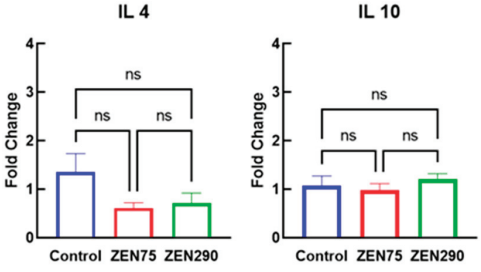

Figure 8. Effect of ZEN on gene expression of anti-inflammatory cytokines IL 4 and IL 10 in the colon of weaned piglets. The statistical significance was marked graphically as follows: ns—not significant (p-value ≥ 0.05).

3.6. Effect of ZEN on Molecules Involved in Signaling Pathways

The gene and protein expression of the NF-kB nuclear receptor, a key marker of the signaling pathway involved in inflammation and oxidative stress, did not undergo significant changes in 75 ppb ZEN group compared to the control, while in the case of 290 ppb ZEN group, a significant increase was observed for both genes (43% increase, $p = 0.028$, Figure 9a) and protein expression (24% increase, $p = 0.0013$, Figure 9b) of NF-kB when compared to control.

Figure 10 shows the gene expression of MAPKs analyzed in the colon of control and ZEN exposed piglets. A low concentration of ZEN did not induce significant changes compared to the control. However, exposure to ZEN 290 produces significant changes, increasing the gene expression level of *ERK1* (90% increase, $p = 0.0196$), *ERK2* (68% increase, $p = 0.0261$), *p38α* (72% increase, $p = 0.0131$), *JNK2* (67% increase, $p = 0.0062$), and *JNK3* (74% increase, $p = 0.0511$). Moreover, exposure to 290 ppb ZEN leads to a decrease in the gene expression level of the *NLRP6* inflammasome, which plays an important role in maintaining homeostasis at the gut level, while at the level of the *NLRP3* inflammasome, no change is observed due to exposure to ZEN.

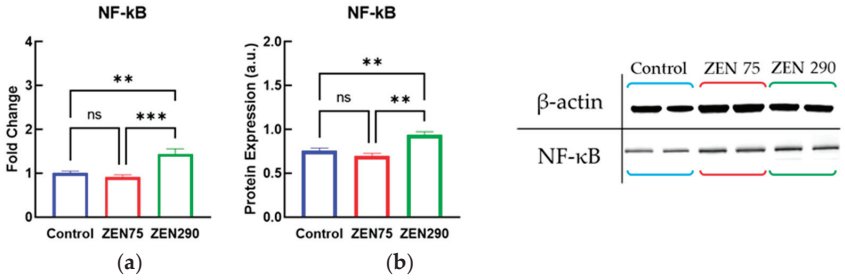

Figure 9. Effect of ZEN on (**a**) gene expression and (**b**) protein expression (histogram and protein detection image) of NF-kB nuclear receptor in the colon of weaned piglets exposed to a concentration lower (75 ppb) or above (290 ppb) EC recommendation in feed. The statistical significance was marked graphically as follows: ns—not significant (p-value $\geq$ 0.05), **—very significant (0.01 $\geq$ p-value > 0.001) and ***—extremely significant (0.001 $\geq$ p-value > 0.0001).

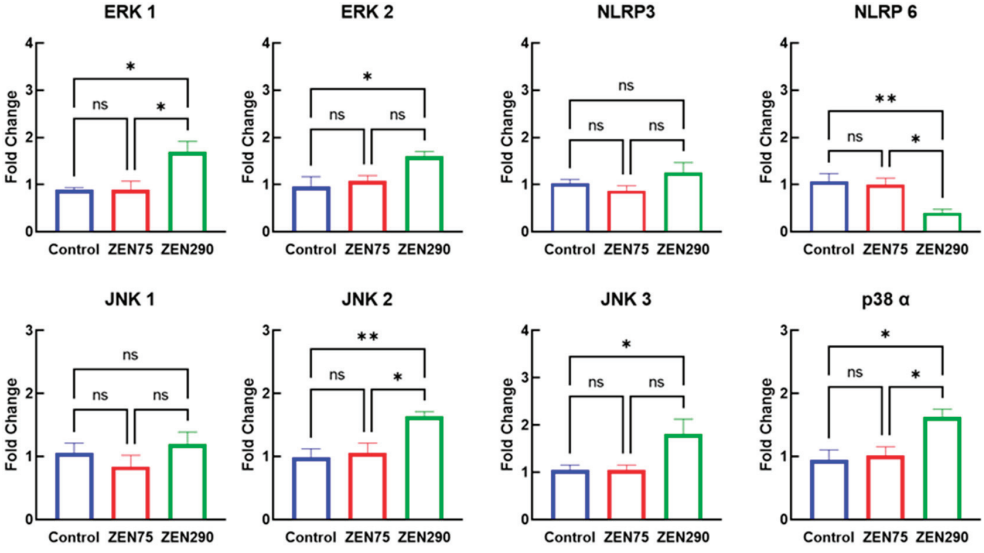

Figure 10. Effect of ZEN on gene expression of MAPKs ERK 1, ERK 2, JNK 1, JNK 2, JNK 3, and p38α, and inflammasomes NLRP 3 and NLRP 6 in the colon of weaned piglets. The statistical significance was marked graphically as follows: ns—not significant (p-value $\geq$ 0.05), *—significant (0.05 $\geq$ p-value > 0.01), **—very significant (0.01 $\geq$ p-value > 0.001).

Similar results were obtained in the case of the gene expression of other signaling molecules (Figure 11). As in the case of MAPKs, ZEN 75 did not induce significant changes, while ZEN 290 induced a significant increase compared with control in the gene expression level of IRAK1 (76%), TRAF 6 (67%), TAK 1 (64%), TGFβ2 (103%), AP1 (88%), and AKT (81%).

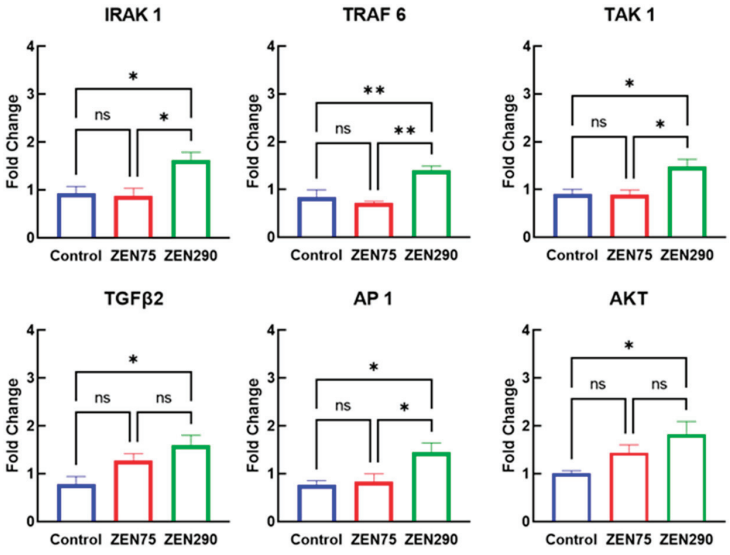

Figure 11. Effect of ZEN on gene expression of signaling molecules IRAK 1, TRAF 6, TAK 1, TGFβ2, AP1, and AKT in the colon of weaned piglets. The statistical significance was marked graphically as follows: ns—not significant (p-value $\geq$ 0.05), *—significant (0.05 $\geq$ p-value > 0.01), **—very significant (0.01 $\geq$ p-value > 0.001).

4. Discussions

Zearalenone is a fusariotoxin that frequently contaminates cereal crops and products derived from them. Its chemical structure not only gives it stability and temperature resistance but ZEN is also considered a xenoestrogenic compound due to its similarity to 17β-estradiol [4,27,28]. Considering the affinity for estrogen receptors, the main target of ZEN is the reproductive system, but toxic effects have been observed in the nervous, hepatic, and digestive systems [23]. Due to this toxicity, a regulation concerning the maximum allowed amount of ZEN in feed and food is necessary. Until now, at the European level, there are regulations only for grain and bakery products intended for humans (European Commission Regulation 1881/2006), with animal feed being only a recommendation (European Commission Recommendation 576/2006). For pigs, according to this document, the maximum level is 0.100 mgZEN/kg (100 ppb) feed for piglets, while for mature pigs and sows the level is 0.250 mg ZEN/kg feed (250 ppb) [29]. Considering the economic importance of pigs, and the health problems that ZEN-contaminated feed consumption can have on pigs, supplementary studies are necessary to establish if the EC recommendation might be taken as a norm (regulation) for the presence of ZEN in pig feed.

As the main route of exposure of piglets to ZEN is the oral one, its effects at the intestinal level are of major importance. Moreover, the intestine, colon included is one of the most sensitive organs for piglets during weaning. After the transition to solid feed diarrhea frequently occurred being associated either with the shifts in microbiota (suppression of several beneficial lactic bacteria) or with colonic inflammation caused by enterotoxigenic *Escherichia coli* or other pathogens (13). These pathogens increased the inflammatory cytokines in the colon contributing to colonic inflammation. Feed contaminated with mycotoxins could also aggravate the health of animals. That is why, in the present study, we investigated if a concentration of ZEN lower than the limit allowed by EC recommendation in the feed for piglets might produce toxic effects. A higher concentration of ZEN than the EC recommendation was used in the study for comparison. For this purpose, several key markers of innate and adaptative immune response were analyzed.

The innate immune response is responsible for recognizing and immediately combating foreign microorganisms, any disturbance of this system is directly correlated with inflammation [30]. Toll-like receptors, glycoproteins present on the cell surface or in the intracellular vesicles, play a very important role in recognizing and binding pathogens; they activate various defense mechanisms of the body, such as the synthesis of cytokines and chemokines [31,32]. No effect of feed with ZEN 75 ppb on TLRs was observed in our study. Only the exposure to the higher concentration of ZEN (290 ppb) leads to a significant increase in the gene expression level of *TLR 4* without modifying other TLRs such as *TLR 2*, *TLR 5*, and *TLR 9* (Figure 1). Studies on pregnant rats have shown that ZEN can induce oxidative stress and inflammatory response by increasing the level of inflammatory cytokines mediated by TLR4 [33]. Also, studies on cell intestinal porcine line IPEC 1 in vitro showed that combined exposure to ZEN and *Escherichia coli* lead to the upregulation of *TLR 2*, *TLR 3*, *TLR 4*, *TLR 6*, and *TLR 10* [34].

The activation of TLRs leads to the recruitment of adapters molecules such as MyD88, IRAK, or TRAF 6. The activation of IRAK or MYD88-dependent signaling pathway triggers complex signaling cascades mediated by MAPKs (p38α, ERK1/2, JNK1/2/3), the process leading to the activation of transcription factors such as AP-1 and NF-kB, key modulatory factors of cytokine production [35]. In the case of co-exposure of Zebra fish to ZEN (200, 400, 800 μg/L) and DON (4000 μg/L), an increase in the expression of *TLR4*, *MYD88*, and *NF-kB* was also observed, leading to an increase in the level of ROS and antioxidant enzymes (CAT, SOD, and GPx). Although there are in vitro and in vivo studies related to TLR4-mediated ZEN toxicity, very few are carried out on pigs or pig cells. As mentioned in our study, only ZEN at 290 ppb produces an increase in the gene expression level of *TLR 4*. Moreover, in the case of ZEN290, the gene expression of *MYD88* is also increased, suggesting a potential mechanism mediated through the TLR 4-MYD88 signaling pathway.

Thus, oxidative stress is produced by the accumulation of reactive oxygen species (ROS) that have toxic effects, causing the oxidation of proteins, nucleic acids, and lipid membranes which can be correlated with TLR4-mediated pathways [36]. A decrease in the level of antioxidant enzymes was observed in the small intestine. Studies carried out on gilts showed that their exposure to a very high concentration of ZEN (1040 ppb) led to a significant decrease in the antioxidant enzymes CAT, SOD, and GPx activity in the ileum, duodenum, and jejunum of piglets [37]. It is known that ZEN can induce oxidative stress at the level of several other organs. Tests were performed in vitro on porcine Granulosa cells with induction of oxidative stress by low doses of ZEN (15 μM, 30 μM) while the mRNA level of the antioxidant enzymes SOD, CAT, and GPx decreased [38]. Studies carried out on weaned piglets show that exposure to ZEN (1.1 to 3.2 mg/kg feed) led to a decrease in the activity of the antioxidant enzymes SOD and GPx [39], enzymes that counteract oxidative stress. Similar effects were also observed in the spleen of weaned piglets that received feed contaminated with ZEN at 316 ppb, registering a decrease in the gene expression level of *SOD*. However, at the level of other organs, such as the liver, a totally opposite effect was observed, with the expression of CAT and GPx being increased [40]. In our study, at the level of the colon, a very important organ during the weaning period, neither ZEN 75 ppb nor ZEN 290 ppb produced oxidation at the protein, lipid, or nucleic level. Also, the level of gene expression of antioxidant enzymes was not significantly modified either by ZEN 75 ppb or by ZEN 290 ppb. However, this concentration produced a decrease in the total antioxidant capacity and a tendency to increase protein oxidation.

Oxidative stress is very closely associated with the inflammatory response, a complex process of defending the organism against toxins, viruses, bacteria, and other traumatic factors [41], mediated by proteins such as cytokines, bacterial lipopolysaccharides, and other chemical mediators [42]. Although cytokine secretion is generally specific to immune system cells, intestinal epithelial cells can also produce these mediators of the inflammatory response. The cytokines that can be synthesized by intestinal epithelial cells are IL 1, IL 10, IL 15, and TGFβ, but other cytokines such as TNF α, CCL20, IL 1β, IL 6, and IL8 are also expressed. Recent in vitro and in vivo studies show a dual character of ZEN, which can be

both pro and anti-inflammatory depending on the tissue type, concentration, and exposure time [27]. In pigs, it has been observed that the gene expression of certain pro-inflammatory cytokines increases in the case of bacterial infections [43]. During the weaning period, a transitory period of inflammation was observed in the intestine, in which an increase in the level of TNF α, IL β, and IL 6 is produced due to the change in diet [44]. Similarly, the increase in gene expression of pro-inflammatory cytokines IL-1β, TNFα, IL-6, IL-8, and IFN-γ was observed in young sows' colon, kidneys, and pancreas exposed to ZEN 100 ppb [10]. Also, a pro-inflammatory activity was reported in the case of daily exposure of weaned gilts to oral pills containing 5, 10, and 15 μg ZEN/b.w. where an increase in the level of pro-inflammatory cytokines IFN-γ, IL-1α, IL-1β, IL-2, IL-6, and TNFα was observed in the ileum after 14, 24, and 48 days of exposure.

In vitro studies on the porcine cell line IPEC 1 showed that ZEN in concentrations of 10–100 μM induced an increase in the synthesis of IL 8 and IL 10 cytokines after 24 h [45]. By contrast, at the systemic level an opposite effect was observed when pig PBMCs (peripheral blood mononuclear cell) were exposed 48 h to 5 and 10 μM ZEN, a decrease in IL 8 synthesis being registered [46].

Moreover, the exposure of pregnant sows to ZEN produced effects not only at the maternal level, but also affected the immunity of the newborn piglets. A study by Liu et al. [47] showed that an experimental diet with 270 μg ZEN/kg feed produced inflammation in the intestines of sows, but also increased the synthesis of pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, and IL-6 in the jejunum of their piglets. Transmission of the inflammatory effect from mother to piglets can also occur during lactation; studies have shown that feeding piglets with milk from sows exposed to 300 ppb ZEN led to intestinal inflammation [48]. While studies show that there are inflammatory effects at the intestinal level when pigs are exposed to ZEN, the concentrations used within them are higher than those recommended by the European Commission of 100 ppb ZEN/kg feed for piglets and 250 ppb ZEN/kg feed for pigs and sows. The results obtained from this study show that the concentrations close to the recommended limit did not produce any significant changes, the exposure of piglets to 75 ppb and 290 ppb ZEN did not lead to major changes in the level of gene expression or protein concentrations of some inflammatory markers such as IL-1β, TNFα, IL-6, IL-8, IFN-γ, IL-4, and IL-10.

Taking into account the obtained results, we analyzed further if the mechanisms involved by ZEN in inflammation and oxidative stress are affected. As said before, one of the key factors that could be involved is the nuclear transcriptional factor NF-kB, which modulates a multitude of processes including oxidative stress, inflammation, innate immune response, cell proliferation, or apoptosis. NF-kB has an antagonistic activity with Nrf2; in conditions of oxidative stress the activity of NF-kB increases, while that of Nrf2 decreases, leading to an increase in pro-inflammatory cytokine synthesis. The NF-kB signaling pathway can also be inhibited, influencing protein oxidation and lipid peroxidation [49]. The activity of NF-kB can be mediated also by transforming growth factor-beta 2 (TGFβ-2), affecting the activity of the AP-1 transcriptional complex [50]. Concerning the intestinal epithelium, in vitro studies on the IPEC-J2 cell line showed that ZEN (6 μg/mL or 8 μg/mL) led to an increase in p38α gene expression, without inducing effects at the level of JNK1/2 and ERK1/2 [51]. In the case of the exposure of piglets to 316 ppb ZEN, a tendency to decrease the gene expression of NF-kB was observed in the spleen, a key organ in immunity, while other transcriptional regulators AP-1 registered a slight increase [52]. Another study shows similar effects, a decrease in the level of NF-kB in the liver of weaned piglets when experimental diet was contaminated with 250 ppb ZEN [53]. Moreover, the same authors reported a decrease in the gene expression level of TAK1/p38α, suggesting the involvement of MAPKs in the signaling pathways of ZEN. The results obtained in this work also suggest that ZEN modulates NF-kB and AP-1, as well as MAPKs such as ERK1, ERK2, p38α, JNK2, and JNK3.

Our results show that at the colon level of weaned piglets ZEN, at a higher concentration (290 ppb) than the EC recommendation, leads to an increase in the level of NF-kB,

AP-1, and TAK 1, but also at the level of MAPKs, suggesting that ZEN toxicity could be mediated by these molecules, while the exposure to lower concentrations did not induce significant changes.

Another potential mechanism for inducing intestinal inflammation is the activation of the NLRP3 inflammasome. Exposure of intestinal porcine enterocyte cell line (IPEC-J2) cells and mouse peritoneal macrophages from Balb/c mice to ZEN 8 μg/mL led to an increase in IL-1β synthesis, a mechanism presumably mediated by the activation of the NLRP3 inflammasome [54]. However, in the present study in the colon of piglets, exposure to 75 ppb and 290 ppb ZEN did not induce changes in the gene expression of the *NLRP3* inflammasome but led to a significant decrease in the *NLRP6* inflammasome in 290 ppb ZEN. Studies show that the NLRP6 inflammasome plays an important role in the Intestinal microbiome, but also in infectious and inflammatory gastrointestinal diseases. NLRP6 attenuates NF-κB activity, but a decrease in NLRP6 can have a negative effect, stimulating the activation of both the signaling pathway modulated by NF-κB and MAPKs [55]. The results obtained in this study suggest the same effect; simultaneously with the significant decrease in the gene expression of *NLRP6*, an increase in the level of NF-kB, and of MAPKs *ERK1*, *ERK2*, *p38α*, *JNK2*, and *JNK3*, was also observed in the colon of ZEN 290 group. By contrast, ZEN at the concentration below the CE recommendation (75 ppb) has no effect on these signaling molecules.

5. Conclusions

The exposure of weaned piglets to a concentration of ZEN below the EU recommendation (ZEN 75) did not induce significant changes in the studied markers at the colon level. Our results show no effect on oxidative stress (protein oxidation, lipid oxidation, antioxidant enzymes activity) and on inflammation markers (IL-1β, TNF-α, IL-8, IFN-γ, IL 4, IL 10), or on the innate immunity markers (TLR2, TLR 4, TLR5, TLR9, MYD88, MD2). Moreover, excepting the increase in gene expression of *TLR4*, *MYD88*, and decrease in antioxidant capacity, the concentration of ZEN above EU recommendation (ZEN 290) did not induce significant changes in innate immunity, oxidative and inflammatory response.

Similarly, the exposure of piglets to ZEN 75 ppb did not induce any changes on the nuclear receptor's genes of *NF-kB*, *AP1*, and *Nrf2*, or MAPKs (*p38α*, *ERK1/2*, *JNK1/2/3*), *NLRP3* and *NLRP6* inflammasome, as well as on other signaling molecules such as *IRAK1*, *TRAF 6*, *TAK 1*, *TGFβ2*, and *AKT*. By contrast, the results obtained when piglets were exposed to ZEN 290 ppb showed that ZEN induced modulation on most molecules involved in signaling pathways involved in inflammation ad oxidative stress. However, more studies are needed at the level of all the organs implicated in immune defense that can be affected by ZEN in order to obtain a complete image necessary to establish a clear guideline regarding the allowed concentration of ZEN in the feed for piglets and to elucidate the underlying mechanisms produced by zearalenone.

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Extra Virgin Olive Oil: Does It Modify Milk Composition of Hair Sheep?

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Abstract: The aim of this study was to determine the effect of olive oil addition on the production, chemical composition, and fatty acid profile of sheep's milk. Twenty-four lactating ewes with a live weight of 34.6 ± 4.61 kg were used. The animals were randomly distributed into four treatments (n = 6) with dietary addition of 0%, 2%, 4%, and 6% (dry matter basis) olive oil for 45 days. Milk samples were taken every 7 days for fatty acid (FA) and chemical analyses. A decrease ($p < 0.05$) in dry matter and crude protein intake was observed with 4% oil inclusion. Milk production and milk components were similar between treatments. The kilograms of meat from weaned lambs linearly increased as the oil inclusion increased. Milk C4:0 to C17:0 decreased with 2% olive oil. The monounsaturated and polyunsaturated FA content in the milk increased with the oil inclusion. There was an increase in the milk's linoleic acid, linolenic acid, and eicosapentaenoic acid content with 2% olive oil. Overall, the addition of 2% extra virgin olive oil is recommended to improve milk's FA profile without negative effects on animal performance.

Keywords: olive; atherogenic; milk; sheep; lipids; small ruminants; supplementation

1. Introduction

In the tropical regions of Mexico, sheep production is of socioeconomic importance and represents an activity of relevance in rural areas, as sheep can adapt to different regions and climates [1]. In these regions, lactating hair sheep feeding is mainly based on grazing, and during drought periods, the supply of nutrients from these forages is low (<7% of CP and NDF >70%) [2]. These factors reduce animal productivity (low milk production and low pre- and post-weaning weight gain) [3]. In this sense, when the feeding of these animals is of poor quality, milk saturated fatty acids (SFAs), mainly lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0), can increase, and these FAs are associated with high cholesterol blood levels, generating cardiovascular problems and hypertension in humans [4–6]. In addition, due to rumen biohydrogenation, most dietary unsaturated

fatty acids (UFAs) are converted to saturated fats upon entering the rumen, which limits the UFA content in milk and dairy products [7].

In the last few years, strategies have been sought to reduce the amount of SFA and increase UFA in milk; these include adding oilseeds (i.e., linseed, soybean, and safflower oil) rich in linoleic and linolenic acid to ruminant diets [8–11]. This is because during the rumen biohydrogenation process, some by-products are generated, such as α-linolenic acid (ALA), linoleic acid (LA), rumenic acid (conjugated linoleic acid; CLA), and vaccenic acid (VA; t11-18:1), and these can promote anti-inflammatory and anticancer processes and prevent heart problems [5,12–14].

There are studies on the use of oleic acid-rich lipids, such as the silage of olive pastes, olive oil, calcium salts of olive oil, and lampante olive oil [9,15–17], added to the diet of dairy sheep and dual-purpose breeds. However, no studies related to dietary extra virgin olive oil and its effects on the fatty acid profile and composition of milk have been conducted in hair sheep from the Mexican tropics. Therefore, the objective of this study was to determine the effect of the addition of olive oil on the productive traits, milk composition, and milk fatty acids of lactating hair sheep.

2. Materials and Methods

2.1. Localization

The study was conducted at Rancho San Francisco, located at 21°14′48″ N and 89°02′35″ W longitude at five masl in the municipality of Dzidzantun (Yucatán, Mexico). The average temperature was 26 °C with 980 mm of rain during the experimental months, and the extremes of relative humidity were between 66% and 89% [18,19].

2.2. Animal Handling and Experimental Design

The animals were treated according to the guidelines and regulations for animal experimentation of the Academic Division of Agricultural Sciences, Universidad Juárez Autónoma de Tabasco (project ID PFI: UJAT-DACA-2015-IA-02). Twenty-four ewes (Pelibuey × Katahdin) with a live weight of 34.6 ± 4.61 kg were used. In a completely randomized experimental design [20], the animals were divided into four groups (n = 6). Animals were housed with their lambs in individual cages (2 × 3 m) and provided with feeders and drinkers. The study lasted 45 days.

2.3. Experimental Diets

The basal diet (offered at 0800 h) consisted of ground sorghum, ground corn, soybean meal, cane molasses, soybean husk, minerals, vitamins, and chopped fresh Taiwan grass (*P. purpureum*; offered at 1800 h), using only the stems to reduce the nutritional variation throughout the experimental phase, at a ratio of 80:20 (concentrate:forage) (Table 1).

The concentrate was formulated according to the AFRC recommendations [21] for sheep with an average weight of 45 kg and a milk yield of 1.74 kg/d; the protein and total fat contents were 4.5% and 7.0%, respectively. The concentrate had a metabolic energy of 10.0 MJ and 13% crude protein per kg DM (Table 2). The dietary treatments had increasing levels of 0, 2, 4, and 6% of the DM of extra virgin olive oil (Sesajal®, Guadalajara, Jalisco, Mexico).

2.4. Handling and Feeding

At the beginning of the study, the sheep were weighed and dewormed with 5% Closantel® (Wyeth LLC, Madison, NJ, USA) and given an ADE vitamin supplement (Vigantol ADE® intramuscularly; 1 mL/10 kg BW). The offered diets were adjusted to allow a minimum rejection of 10% to estimate voluntary intake. The animals were fed twice daily, at 0800 and 1500 h.

Table 1. Formulation and chemical composition (g/kg DM) of the concentration and forage offered to lactating hair sheep.

Ingredient	Concentrate (g/kg)	*P. purpureum* (g/kg)	Olive Oil (g/kg)	Total
P. purpureum	0	200	0	
Ground sorghum	181	0	0	
Soybean meal	198	0	0	
Molasses	100	0	0	
Wheat bran	300	0	0	
Mineral blend	20	0	0	
ADE vitamins	1	0	0	
Total	800	200		1000
Chemical composition (g/kg)				
DM	847	283	0	
OM	919	953	0	
CP	161	31	0	
EE	43	19.2	999	
NDF	447	693	0	
ADF	245	470	0	
ME (MJ/kg DM)	10.61	7.6	37.7	
Fatty acid composition (g/kg)				
C16:0	0.00	2.44	6.69	
C18:0	0.26	0.27	4.40	
C18:1	9.39	0.33	695	
C18:3	1.62	10.71	2.20	
C20:0	6.03	0.36	1.60	
∑SFA	6.29	3.07	12.69	
∑MUFA	9.39	0.33	695	
∑PUFA	1.62	10.71	2.20	

SFAs (saturated fatty acids) = C16:0 + C18:0 + C20. MUFAs (monounsaturated fatty acids) = C18:1. PUFAs (polyunsaturated fatty acids) = C18:3. DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; ADF = acid detergent fiber; NDF = neutral detergent fiber; ME= Metabolizable energy.

Table 2. Chemical composition (g/kg DM) of dietary treatments with increasing levels of olive oil.

Component	% Olive Oil			
	0	2	4	6
DM	734.2	722.6	711.4	700.6
OM	925.0	911.2	897.1	883.4
CP	135.0	132.9	130.8	128.8
EE	38.2	53.4	68.0	82.2
NDF	496.2	488.4	480.8	473.5
ADF	290.0	285.4	281.0	276.7
ME (MJ)	10.0	10.4	10.9	11.3
Fatty acid profile (g/kg DM)				
C16:0	0.01	0.11	0.22	0.32
C18:0	0.01	0.08	0.15	0.21
C18:1	0.32	11.3	21.84	32.11
C18:3	0.10	0.13	0.16	0.19
C20:0	0.21	0.23	0.25	0.27
∑SFA	0.23	0.42	0.61	0.80
∑MUFA	0.32	11.25	21.84	32.11
∑PUFA	0.10	0.129	0.16	0.19

SFAs (saturated fatty acids) = C16:0 + C18:0 + C20; MUFAs (monounsaturated fatty acids) = C18:1; PUFAs (polyunsaturated fatty acids) = C18:3 (Tsiplakou et al., 2017); DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; ADF = acid detergent fiber; NDF = neutral detergent fiber; ME= Metabolizable energy.

2.5. Weight Gain and Feed Conversion

The animals were weighed every eight days with a Torrey® scale with an accuracy of 0.100 kg. The weight gain of the sheep was determined by the difference between the final weight (FW) and the initial weight (IW) divided by the days between weightings (g/day). The feed conversion was calculated by the difference between the intake (kg) and weekly weight change of the sheep (kg).

2.6. Production and Chemical Composition of Milk

The daily milk production was determined by manual milking from the first week of the study until day 45; a total of 6 samplings were obtained. The lambs were separated from the mothers one day before the mother's milk sampling starting at 1900 h, for 12 h, starting the milking 0700 h. Before milking, the sheep received 3 IU of oxytocic intramuscularly (Pisa, Mexico). The teats of the animals were disinfected with an iodized solution, and after 30 s, they were dried with paper towels. The daily milk yield (DMY, kg) of the sheep was calculated using the milk obtained over a period of 12 h multiplied by 2.

Samples of 100 mL of milk were taken from each sheep milked weekly, of which 5 mL was taken for protein and fat analysis, analyzed in duplicate by infrared methodology with the aid of an automatic milk analyzer (Lactoscan LS—60, Milkotronic Ltd., Nova Zagora, Bulgaria), and the rest of the milk sample was kept frozen. In addition, the milk yield with 6% fat correction (FCM) and energy (ECMY) was calculated according to the formula [22]:

$$FCMY = (0.28 + 0.12F) \times MY$$

where F = fat percentage. The energy-corrected milk yield (ECMY, kg) was calculated according to the formula:

$$ECMY = (0.071 \times F + 0.043 \times P + 0.2224) \times MY$$

where P is the protein percentage.

The protein correction was calculated according to the formula proposed by Pulina et al. [23]:

$$FPCM\ 6.5,\ 5.8\%,\ kg/d = [milk\ (kg/d)\ (0.25 + 0.085\ fat\% + 0.035\ protein\%)].$$

2.7. Fatty Acid Profile

The composition of fatty acids in the milk was carried out in the environmental engineering laboratory of the Faculty of Engineering of the Autonomous University of Yucatan (UADY). Milk samples were taken using 1 mL of the sample, which was stirred for one minute in a vortex to homogenize the sample. The total fat was extracted with a mixture of methanol chloroform (2:1) and quantified by gravimetry using the method of Folch et al. [24]. Total fat concentrations are expressed in g/100 g of milk. The fatty acids were derived using boron trifluoride and extracted with hexane [24]. The derivatized extracts were injected into a Hewlett Packard gas chromatograph 5890 Series II, which used a Supelco SP 2560 column, 100 m long × 0.25 mm internal diameter [24]. Peaks were identified by means of Supelco reference standards (FAME).

2.8. Chemical Analysis

The DM determination of the forage samples was performed in a forced air oven at 55 °C for 48 h (constant weight) (#7.007) AOAC [25]. The N content was realized (CP, N × 6.25) by combustion using a LECCO CN-2000 series 3740 (LECCO, Corporation, Saint Joseph, MI, USA) (#2.057) AOAC [25]. MO was determined by incineration in a muffle at 600 °C for 6 h, and the contents of FND and FDA were determined as suggested by Van Soest et al. [26].

2.9. Statistical Analysis

The data were subjected to analysis of variance in a randomized design [20] using the PROC GLM procedure of the SAS [27]. The means of the treatments were compared with Tukey's test with an alpha of 0.05:

$$Y_{ij} = \mu + \tau_i + E_{ij}$$

$$i = 1, 2, 3, \ldots, t$$

$$j = 1, 2, 3, \ldots, n$$

where Y_{ij} = variable response in the j-th repeat of the i-th treatment, μ = general mean, τ_i = effect of the i-th treatment, and E_{ij} = random error.

Milk production and weight gain data were subjected to a PROC MIXED time repeated measures analysis of the SAS [27]. Tukey's test was performed when a significant treatment effect was detected ($p < 0.05$). Additionally, a response surface analysis was performed to evaluate the linear, quadratic, and cubic effects of the treatments (0, 2, 4, and 6% olive oil in the ration) [20].

3. Results

3.1. Voluntary Intake

The dry matter intake (DMI) and crude protein (CP) intake were affected ($p < 0.05$) by the addition of olive oil; these levels were reduced with the addition of 4% olive oil in the ration, whereas for 0%, 2%, and 6% olive oil, they were similar with a cubic effect. A linear effect ($p < 0.0001$) was found for ether extract (EE) intake as the levels increased (0, 2, 4, and 6% of the DM). The same pattern was observed when DMI, OM, and CP were expressed in $g/kg^{0.75}$ (Table 3).

3.2. Productive Behavior of Sheep and Lambs

No differences were found in the productive behavior of lactating sheep when adding extra virgin olive oil to the diet (Table 4). On the other hand, differences ($p < 0.03$) were observed in the intake of concentrated feed as oil levels (0%, 2%, 4%, 6% of DM) were increased (Table 4). No differences ($p > 0.05$) were found in the growth of lambs, but a linear trend ($p < 0.02$) was observed in the kg of meat weaned per sheep as the oil was increased in the ration (Table 5).

3.3. Production and Chemical Composition of Milk

Milk production and milk components were similar between treatments, but quadratic effects were detected in milk production and corrected milk production for protein, fat, and energy, and except for the percentage of milk fat, the non-fat solids as well as daily fat and protein yields in our study showed no changes (Table 6).

3.4. Fatty Acid Profile in Milk

The addition of extra virgin olive oil at levels of 0, 2, 4, and 6% caused changes ($p < 0.05$) in the fatty acid profile of the milk of hair sheep (Table 7). The oil concentrations used showed linear and quadratic effects ($p < 0.05$); mostly, short- and medium-chain fatty acids (C4:0 to C17:0) showed a reduction when 2% of the DM of olive oil was added. Long-chain fatty acids with greater than 17 carbons increased ($p < 0.05$) with 2% oil inclusion, and this level seemed to improve the quality of the milk fat (Table 7). According to our data (Table 7), the control treatment (0% olive oil) resulted in the highest levels of saturated fatty acids; this content showed a linear effect ($p < 0.0001$), in which the concentration decreased as the amount of olive oil added increased. The saturated fatty acid with the highest presence was C16:0. Monounsaturated fatty acids showed linear ($p < 0.01$) and

quadratic ($p < 0.001$) effects. In this sense, the percentage of polyunsaturated fatty acids increased as the addition of olive oil in the diet increased; this was related to the high concentration of oleic acid contained in olive oil, which caused the decrease in the activity of the enzyme Δ^9-desaturase, which participates in de novo synthesis and the synthesis of saturated fatty acids and possibly increases the microbial activity in the rumen (Table 7).

In this study, an increase in various fatty acids, such as oleic, linoleic, and linolenic acids, was found with 2% olive oil ($p < 0.05$), while an increase in eicosapentaenoic acid was observed as more olive oil was integrated into the diet ($p < 0.05$), as shown in Table 7. These fatty acids are relevant because they have various health benefits, and the human body cannot synthesize them.

The milk of hair sheep had quadratic ($p < 0.004$) and cubic ($p < 0.001$) effects in the atherogenic index (AI) analyses, while for the thrombogenic index (TI), a lower concentration was found in the 2 and 6% olive oil groups compared with the control group.

Table 3. Feed intake from hair sheep fed with different levels of olive oil inclusion.

Items	% Olive Oil						Contrast		
	0	2	4	6	SE	*p*-Value	L	Q	C
LW (kg)	38.42	37.88	38.08	36.01	0.93	0.26	0.09	0.41	0.47
MDW (kg)	15.40	15.25	15.26	14.68	0.28	0.27	0.08	0.43	0.55
TDMI (kg/d)	2.08	1.96	1.96	2.06	0.07	0.45	0.79	0.11	0.93
DMI (% PV)	5.44 ^{a}	5.04 ^{ab}	4.89 ^{b}	5.32 ^{ab}	0.12	0.01	0.37	0.001	0.58
OM (kg/d)	1.93	1.77	1.73	1.78	0.06	0.13	0.08	0.10	0.93
CP (kg/d)	0.29 ^{a}	0.27 ^{ab}	0.26 ^{b}	0.27 ^{ab}	0.008	0.04	0.03	0.06	0.89
EE (kg/d)	0.08 ^{ab}	0.12 ^{b}	0.166 ^{ab}	0.214 ^{a}	0.004	<0.0001	<0.0001	0.38	0.86
NDF (kg/d)	1.02	0.93	0.90	0.94	0.034	0.26	0.166	0.14	0.96
ADF (kg/d)	0.59	0.54	0.53	0.54	0.02	0.37	0.22	0.19	0.97
DMI (g/kg$^{0.75}$)	135.24 ^{a}	125.04 ^{ab}	121.11 ^{b}	130.30 ^{ab}	3.33	0.02	0.21	0.004	0.65
OM (g/kg$^{0.75}$)	124.98 ^{a}	115.60 ^{ab}	111.97 ^{b}	120.50 ^{ab}	3.12	0.02	0.22	0.005	0.64
CP (g/kg$^{0.75}$)	19.10 ^{a}	17.52 ^{b}	16.95 ^{b}	18.16 ^{ab}	0.42	0.004	0.07	0.001	0.68
EE (g/kg$^{0.75}$)	5.32 ^{b}	7.95 ^{b}	10.83 ^{b}	14.46 ^{a}	0.20	<0.0001	<0.0001	0.01	0.59
NDF (g/kg$^{0.75}$)	65.55	60.86	58.99	63.61	2.004	0.10	0.39	0.02	0.68
ADF (g/kg$^{0.75}$)	37.80	35.19	34.10	36.83	1.31	0.20	0.49	0.04	0.70

$^{a, b}$ Separate literals in the same column indicate statistical differences ($p < 0.05$); SE, standard error of the mean; LW, live weight; MDW, metabolic body weight; TDMC, total dry matter intake; DMI, dry matter intake; OM, organic matter; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber. L, linear contrast; Q, linear quadratic; C, cubic contrast.

Table 4. Productive parameters from hair sheep fed with different levels of olive oil inclusion.

Items	% Olive Oil						Contrast		
	0	2	4	6	SE	*p*-Value	L	Q	C
IW (kg)	33.05	34.74	35.98	34.78	2.17	0.82	0.52	0.52	0.84
FW (kg)	41.20	39.75	40.86	40.84	3.47	0.99	0.99	0.84	0.81
TWP (kg)	8.15	5.01	4.88	6.06	1.68	0.51	0.41	0.22	0.82
DMI (kg/d)	2.08	1.96	1.96	2.06	0.07	0.45	0.80	0.11	0.93
CC (kg/d)	1.76 ^{a}	1.60 ^{ab}	1.56 ^{b}	1.60 ^{ab}	0.05	0.03	0.02	0.06	0.87
FC(g/d)	492.81	469.88	461.94	490.13	20.98	0.67	0.86	0.23	0.82
OOC (g/d)	0.00 ^{d}	46. 62 ^{c}	93. 98 ^{b}	138. 21 ^{a}	2.59	<0.0001	<0.0001	0.65	0.74

$^{a–d}$ Separate literals in the same column indicate statistical differences ($p < 0.05$); SE, standard error of the mean; IW, initial weight; FW, final weight; TWG, total weight gain; DMI, dry matter intake; CC, concentrate consumption; FC, forage consumption; OOC, olive oil consumption; L, linear contrast; Q, linear quadratic; C, cubic contrast.

Table 5. Productive traits from hair sheep lambs fed with different levels of olive oil inclusion.

Items	% Olive Oil						Contrast		
	0	2	4	6	SE	*p*-Value	L	Q	C
BW (kg)	3.74	4.58	5.48	5.76	0.52	0.06	0.01	0.60	0.78
KW (kg)	13.04	16.90	16.62	19.63	1.68	0.10	0.02	0.81	0.34
WGL (kg)	9.30	12.32	11.13	13.87	1.41	0.19	0.07	0.92	0.22
DWG (kg/d)	0.21	0.27	0.24	0.31	0.03	0.20	0.07	0.87	0.23

SE: standard error; PIC: birth weight; KW: kilograms weaned; WGL: weight gain in lactation; DWG: daily weight gain; L: linear contrast; Q: linear quadratic; C: cubic contrast.

Table 6. Effect of dietary inclusion of olive oil in hair sheep on the estimated milk production and milk chemical composition.

Items	% Olive Oil						Contrast		
	0	2	4	6	SE	*p*-Value	L	Q	C
DMP (g/d)	1158.78	1314.40	1032.80	1292.00	85.53	0.07	0.75	0.54	0.01
CPC (g/d)	1108.15	1256.61	1058.01	1284.96	91.53	0.21	0.41	0.66	0.05
FC (g/d)	1129.68	1284.61	1079.26	1310.44	70.77	0.20	0.41	0.67	0.05
EC (kcal/d)	1015.46	1004.61	1071.96	1019.18	22.33	0.14	0.44	0.35	0.05
Chemical composition of milk (g/100 g)									
Fat	6.41	6.30	6.87	6.42	0.21	0.22	0.51	0.41	0.06
Protein	5.10	4.98	5.20	5.08	0.08	0.27	0.63	0.92	0.05
Non-fat solids	12.17	11.93	12.02	12.04	0.08	0.24	0.42	0.12	0.27
Daily performance g/d									
Fat	72.26	82.29	71.86	85.81	6.40	0.30	0.29	0.76	0.12
Protein	60.49	67.87	60.74	72.29	5.63	0.36	0.26	0.71	0.18

SE, standard error of the mean; DMP, daily milk production; CPC, protein correction; FC, fat correction; EC, energy correction; L, linear contrast; Q, quadratic linear; C, cubic contrast.

Table 7. Effect of dietary inclusion of olive oil in hair sheep on milk fatty acid profile (g/100 g FA).

Fatty Acid (g/100 g Fatty Acids)	% Olive Oil					Contrast			
	0	2	4	6	SE	*p*-Value	L	Q	C
C4:0	1.02	0.78	1.04	0.66	0.14	0.21	0.24	0.65	0.07
C6:0	0.68	0.63	0.70	0.52	0.05	0.06	0.06	0.21	0.08
C8:0	9.31 ^{a}	7.92 ^{a}	8.30 ^{a}	5.84 ^{b}	0.50	<0.0001	<0.0001	0.28	0.04
C10:0	9.31 ^{a}	7.92 ^{a}	8.30 ^{a}	5.84 ^{b}	0.50	<0.0001	<0.0001	0.28	0.04
C12:0	0.46 ^{ab}	0.45 ^{ab}	0.65 ^{a}	0.31 ^{b}	0.06	0.006	0.38	0.01	0.01
C11:0	0.19 ^{ab}	0.19 ^{ab}	0.24 ^{a}	0.13 ^{b}	0.02	0.03	0.30	0.04	0.04
C13:0	3.66 ^{a}	3.31 ^{ab}	2.89 ^{ab}	2.63 ^{b}	0.22	0.008	0.0007	0.85	0.82
C14:0	0.44 ^{a}	0.47 ^{ab}	0.66 ^{b}	0.37 ^{b}	0.06	0.01	0.94	0.01	0.02
C14:1	0.09 ^{a}	0.09 ^{a}	0.06 ^{b}	0.09 ^{a}	0.007	0.004	0.15	0.04	0.004
C15:0	1.45 ^{ab}	1.19 ^{BC}	1.56 ^{to}	1.06 ^{c}	0.09	0.001	0.06	0.22	0.001
C15:1	0.35 ^{a}	0.29 ^{ab}	0.25 ^{b}	0.27 ^{b}	0.02	0.005	0.001	0.07	0.70
C16:0	14.98 ^{a}	14.63 ^{a}	13.74 ^{a}	11.53 ^{b}	0.50	<0.0001	<0.0001	0.06	0.72
C16:1	1.10	0.95	1.18	0.86	0.29	0.87	0.71	0.76	0.49
C17:0	0.96 ^{a}	0.69 ^{b}	0.66 ^{b}	0.62 ^{b}	0.07	0.004	0.001	0.10	0.45
C17:1	0.40 ^{a}	0.29 ^{ab}	0.35 ^{ab}	0.27 ^{b}	0.03	0.03	0.02	0.76	0.03
C18:0	9.58 ^{a}	8.59 ^{a}	9.61 ^{a}	7.45 ^{b}	0.29	<0.0001	<0.0001	0.04	0.002
C18:1cis-9	19.11 ^{c}	23.36 ^{ab}	20.44 ^{bc}	27.02 ^{a}	1.09	<0.0001	<0.0001	0.28	0.0009
C18:1 1n9t	0.07	0.08	0.06	0.09	0.01	0.20	0.41	0.17	0.14
C18:2n6c	9.74 ^{b}	10.86 ^{b}	15.31 ^{ab}	16.95 ^{a}	1.48	0.001	0.0002	0.85	0.35
C18:2n6t	2.42	1.97	2.45	2.55	0.28	0.46	0.48	0.33	0.29
C20:0	0.19 ^{ab}	0.21 ^{ab}	0.17 ^{b}	0.24 ^{a}	0.01	0.01	0.08	0.09	0.03

Table 7. *Cont.*

Fatty Acid (g/100 g Fatty Acids)	% Olive Oil					Contrast			
	0	2	4	6	SE	*p*-Value	L	Q	C
C18:3n6	0.21 ^{a}	0.17 ^{ab}	0.16 ^{b}	0.16 ^{b}	0.01	0.01	0.003	0.19	0.92
C20:1	0.13	0.11	0.08	0.11	0.01	0.17	0.23	0.11	0.26
C18:3n3	11.45 ^{a}	12.67 ^{a}	8.87 ^{ab}	10.73 ^{b}	0.54	<0.0001	0.01	0.55	<0.0001
C21:0	1.33	0.68	0.86	1.63	0.43	0.35	0.60	0.08	0.90
C20:2	0.11	0.10	0.11	0.12	0.01	0.74	0.57	0.39	0.69
C22:0	0.16	0.19	0.16	0.22	0.02	0.24	0.19	0.45	0.16
C20:3n6	0.13	0.11	0.11	0.14	0.01	0.46	0.67	0.12	0.91
C22:1n9	0.19	0.22	0.20	0.27	0.03	0.35	0.13	0.62	0.39
C20:3n3	0.07	0.06	0.05	0.07	0.007	0.25	0.77	0.11	0.20
C23:0	0.07	0.06	0.05	0.08	0.008	0.14	0.74	0.07	0.14
C20:4n6	0.08 ^{b}	0.10 ^{b}	0.09 ^{b}	0.14 ^{a}	0.008	0.0001	0.0002	0.05	0.03
C22:2	0.07	0.06	0.05	0.07	0.007	0.17	0.51	0.08	0.19
C24:0	0.35	0.48	0.32	0.89	0.17	0.07	0.06	0.20	0.17
C20:5n3	0.15 ^{b}	0.18 ^{ab}	0.22 ^{ab}	0.27 ^{a}	0.03	0.04	0.004	0.76	0.91
C24:1	0.03	0.03	0.02	0.03	0.004	0.36	0.54	0.42	0.13
∑ SFA	56.72 ^{a}	53.02 ^{ab}	50.13 ^{b}	43.61 ^{c}	1.73	<0.0001	<0.0001	0.42	0.56
∑ MUFA	13.28 ^{a}	11.70 ^{ab}	13.13 ^{a}	11.51 ^{b}	0.44	0.001	0.01	0.65	0.001
∑PUFA	29.77 ^{c}	35.08 ^{bc}	36.60 ^{b}	45.06 ^{a}	1.81	<0.0001	<0.0001	0.39	0.18
AI	0.09 ^{ab}	0.09 ^{b}	0.13 ^{a}	0.06 ^{b}	0.01	0.0003	0.16	0.004	0.001
TI	0.17 ^{a}	0.14 ^{ab}	0.18 ^{a}	0.10 ^{b}	0.01	0.006	0.06	0.11	0.007
Δ 9-desaturase index	0.21 ^{a}	0.19 ^{a}	0.12 ^{b}	0.20 ^{a}	0.01	0.005	0.21	0.01	0.01

$^{a, b, c}$ Different literals in the same column indicate statistical differences ($p < 0.05$). SE: standard error of the mean. L, linear contrast; Q, quadratic linear; C: cubic contrast. ∑SFA = (C4:0 + C6:0 + C10:0 + C8:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:20 + C23:0 + C24:0); ∑MUFA= (C14:1 + C15:1 + C16:1 + C17:1+ C18:1 1n9t + C18:1cis-9 + C20:1 + C22:1n9 + C24:1); ∑PUFA = (C18:2n6t + C18:2n6c + c18:3n6 + C18:3n3 + C20:3n6 + C22:1n9 + C20:3n3 + C20:4n6 + C22:2 + C20:5n3) [22]; AI: atherogenic index, (12:0 + 4(C14:0) + C16:0/(n − 6 + n − 3) + C18:1 + ∑MUFA); TI: thrombogenicity index, (∑C14:0 + C16:0 + C18:0)/(0.5 × C18:1) + (0.5 × ∑MUFA) + (0.5 × n − 6) + (3 × n − 3) + (n − 3/n − 6) [28]; Δ 9-desaturase index: C14:1/(C14:0 + C14:1) [28].

4. Discussion

4.1. Voluntary Intake

Voluntary intake in animals is one of the most important variables in productive behavior and can be affected by multiple factors. In this sense, feed intake is related to the success of the mother and lamb production (pre-weaning weight gain) [29,30]. In sheep fed diets with canola oil, DMI was higher when using 5%DM, and the crude protein passage rate was lower. Similar results were observed in this experiment when 4% olive oil was used. This reduction was also observed with flaxseed oil at up to 9% DM, which could be explained by a reduction in rumen fiber digestibility [31].

Fiorentini et al. [32] evaluated different lipid sources (palm oil, linseed oil, protected fat, and soybean oil) in Nellore steers with 4.5%, where the highest intakes of DM, OM, and CP were recorded in animals fed an oil-free diet. However, in this study, the intake of DM, OM, and CP decreased as the amount of olive oil increased or had a linear increase in EE, which could indicate that the reduction in DMI was probably due to fiber digestion problems [31]. This coincided with what was reported by Vargas-Bello-Pérez et al. [9], which indicated that dry matter intake and digestibility decreased as olive oil increased; this could be due to a regulatory response indicating a higher energy intake, which reduces voluntary intake.

4.2. Productive Behavior of Sheep and Lambs

In this study, it was observed that the levels of oil used did not affect the productive parameters in the sheep and their offspring, which was similar to other studies carried out on sheep in which different oils were used in the diets; in some cases, over 6% of the DM was offered [11,33–35]. However, there are reports in sheep and goats in which levels greater than 6% of the DM of oil were added to the diet without generating changes in productive behavior [36,37], as observed in dairy sheep of the Spanish Assaf breed that had

6% soybean oil added to their diets; this was similar to what happened in this trial for hair sheep in a tropical region.

Martínez-Marín et al. [38] added 4.8% sunflower oil to the diets of dairy goats of the Malagueña breed without observing an effect on voluntary intake. In this regard, Parente et al. [39] evaluated various oils at 3% inclusion in the diet (canola, sunflower, and castor oils) and reported a lower feed intake in the groups that had oil without finding effects on lambs, while the sheep showed a weight gain of over 1 kg during the experiment; these findings were similar to the results found in this study in sheep and lambs. This is because a decrease was found in the DMI without affecting the productive indicators, which are related to the amount of energy added to the ration, because the lactation stage requires a greater energy demand to avoid weight loss during this period, which would ensure post-weaning ovarian reactivation.

4.3. Production and Chemical Composition of Milk

In this study, the addition of olive oil to the sheep diet did not affect the production or chemical composition of milk at the different levels offered (0, 2, 4, and 6% olive oil); this finding is different from that of Gómez-Cortés et al. [17], who observed an increase in milk production in dairy sheep of the Assaf breed with 233 g/d with the addition of 6% olive oil, with reductions in the percentage of fat and protein at this level. By contrast, Gallardo et al. [40] did not report changes in milk production or protein content; these findings were similar to those observed in this study, except for the reduction in fat content. Antongiovanni et al. [15], using the technique of calcium salts of free fatty acids obtained from olive oil, observed that the fat and protein content was increased without modifying milk production. However, Vargas-Bello-Pérez et al. [9] developed diets for lactating sheep (cross with Finnish Landrace, Border Leicester, Poll Dorset, and Merino) with 36 and 88 g/d of lampante olive oil; no effects of these dietary treatments on milk production and milk chemical composition were reported, which agrees with the results obtained in this study. These changes found in sheep in milk production and fat and protein yield may depend on the genetic potential of the sheep to increase milk production as they consume greater amounts of nutrients because studies have been conducted in milk-producing sheep [41].

4.4. Milk Fatty Acid Profile

Short-chain fatty acids are related to low-density cholesterol levels in the blood, causing cardiovascular problems in humans [5,6]. Due to this, new mechanisms are being sought to modify the proportion of fatty acids in milk and meat, which is why different vegetable oils, such as soybean, linseed, sunflower, and safflower oils, have been used to change milk fatty acid profiles [10,22,42–44], as they can increase the milk contents of MUFA and PUFA.

The high concentration of C18:1 in olive oil was reflected in sheep milk as concentrations increased; in this sense, other PUFAs (C18:2n6c, C18:3n3, and C18:1cis-9) increased with 2% olive oil inclusion. This could be explained by the fact that when olive oil fatty acids were biohydrogenated, the increased fatty acid elongation led to an increased production of polyunsaturated fatty acids.

These effects were observed in this study when adding extra virgin olive oil. For their part, Martini et al. [16], when supplementing Massese sheep with calcium soaps of olive oil, observed less than five percent reductions in saturated fatty acids, while unsaturated fatty acids increased, with considerable amounts of CLA observed in the milk. In this study, CLA precursors, such as stearic acid, were observed. Gomez-Cortez et al. (41), by including three percent olive, flaxseed, and soybean oils, observed a decrease in saturated fatty acids and an increase in the concentration of unsaturated acids. In this study, we observed that with small concentrations of olive oil, changes could be generated in the fatty profile of the milk; the inclusion of 2% of olive oil in the diet of lactating hair sheep decreased saturated fatty acids and increased polyunsaturated acids.

So far, studies related to supplementation strategies with vegetable oils have used less than 5% inclusion in the diet; this is because it can affect voluntary consumption, which could reduce the production, composition, and portion of milk fatty acids in ruminants [45,46]. There have been a few studies with levels greater than 5%, such as a study performed by Gómez-Cortes et al. [17] that obtained results similar to those of this study, with the addition of 6% oil resulting in a reduction in unsaturated fatty acids. The reduction in saturated fatty acids in milk may be related to the negative effects of high oil levels on de novo synthesis because saturated fatty acids are synthesized in the mammary glands [11,47,48], and most MUFAs and PUFAs, such as linoleic acid, linolenic acid, and eicosapentaenoic acid, are generated in the rumen through incomplete desaturation, which occurs by the biohydrogenation process.

Similarly, it has been observed that when the bioavailability of medium- and long-chain fatty acids increases because of higher dietary intake, de novo synthesis decreases the concentrations of short-chain fatty acids in milk [17]. This is similar to this work because there were increases in polyunsaturated fatty acids and a decrease in Δ 9-desaturase activity with extra virgin olive oil. However, a diet rich in oleic acid should have increased C18:0 concentrations due to biohydrogenation and increased Δ^9-desaturase activity [6,7,17].

According to Vargas-Bello-Pérez et al. [9], AI was reduced as lampante olive oil was added to the diet; [17] conducted tests with 6% olive oil in the diet, yielding similar effects to those observed when 6% extra virgin olive oil was added in this study. Vargas-Bello-Pérez et al. [9] used lampante olive oil in smaller quantities than those used in this study, and no differences in IT were found; in this study, this index was lower with 2 and 6% extra virgin olive oil. C14:0 was the most atherogenic one, with approximately four times more potential to increase cholesterol than 16:0 [28]. In this study, with C14:0, it was possible to decrease by 7% when 6% extra-virgin olive oil was added to the diet.

5. Conclusions

The addition of extra virgin olive oil did not generate negative effects on the productive behavior, milk production, or chemical composition of lactating sheep, but it did generate changes in the fatty acid profile. Therefore, it is feasible to add extra virgin olive oil to reduce saturated fatty acids; increase the levels of monounsaturated and polyunsaturated fatty acids; increase the contents of acid linoleic, linolenic, and eicosapentaenoic acids; and reduce the atherogenicity index.

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The Strategic Use of an Immunomodulatory Feed Additive in Supplements for Grazing Young Nellore Bulls Transported after Weaning: Performance, Physiological, and Stress Parameters

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Abstract: The objective of this study was to evaluate four different feeding strategies using an immunomodulatory feed additive for newly weaned Nellore cattle, before and after road transport, on their physiological parameters and performances during the growing phase of pastures. In total, eighty-four young Nellore bulls (initial BW = 174 ± 11 kg; 7 ± 1 months of age) were blocked by their initial body weights 42 days before road transport (d −42) and randomly assigned to one of the four supplementation strategies. The treatments were: (1) Control (CON): no immunomodulatory feed additive (NUTRA) supplementation; (2) NUTRA pre: the inclusion of NUTRA only in the pre-transport period (d −42 to d 0); (3) NUTRA post: the inclusion of NUTRA for 42 days, only in the post-transport period (d 0 to d 42); and (4) NUTRA growth: the inclusion of NUTRA during the whole experimental period (d −42 to d 210). On d 0, the calves were transported on dirty roads in a commercial livestock trailer for 200 km (8 h). There was no effect of the treatments on the animal performance or the physiological parameters in their plasma. However, there were effects on the day of the blood sampling for all the parameters. The highest concentration of cortisol was observed on d 3 post-transport (129 ng/mL) and this decreased over time (22.4 ng/mL; d 210). On the other hand, their glucose peaked at unloading, with lower concentrations on d 7 and d 14. Their total protein concentrations increased from d 0 to d 7. The immunomodulatory feed additive supplementation at 10 g/100 kg BW/day did not modulate the physiological responses in their plasma and did not influence the performance of the Nellore bulls during the growing phase of their pastures.

Keywords: *Bos indicus*; NutraGen; stress; supplementation; weaned calves

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1. Introduction

Long-distance transport is frequent and often inevitable within the beef industry. This is mainly due to the geographical separation of cow–calf regions, growth phases, and finishing operations [1,2]. One of the most widely recognized stressors in beef cattle production is transport [1,3]. In weaned calves, it has been shown that transport is perceived as an acute stress that increases their serum cortisol concentrations [4] and alters their energy and protein metabolism [5]. Therefore, transport may change and impair animal growth rate, which leads to a greater susceptibility to diseases [6,7]. Thus, nutritional strategies that prevent these stress-related physiological disorders caused during transport may be advantageous to the well-being, health, and productivity of beef cattle [7].

Feed additives with immunomodulatory properties have been used to improve the health and productivity parameters in dairy cattle and sheep during stressful periods [8–10]. Lippolis et al. [11] reported a lower plasma cortisol concentration and enhanced innate immunity in newly weaned feedlot steers that were supplemented with an immunomodulatory feed additive after road transport. Under the conditions of thermal stress, dairy cows supplemented with an immunomodulatory feed additive showed positive changes in their energy, protein, and mineral metabolism, in addition to an increased forage intake, milk yield, and milk fat [12]. Moreover, Wu et al. [13] reported a reduced incidence of mastitis, a lower body weight loss during early lactation, and a lower somatic cell count in the milk of transition cows that were supplemented with an immunomodulatory feed additive compared to non-supplemented animals.

Previous studies have suggested the potential benefits of immunomodulatory feed additive supplementation for animals under stressful conditions. However, to our knowledge, studies evaluating the effects of this immunomodulatory feed additive supplementation for grazing calves transported after weaning, especially for Nellore cattle, are scarce. A supplementation with the feed additive evaluated in this study, several weeks before the stress or immune challenge, has been recommended [14]. Therefore, more research is needed to evaluate the benefits of an immunomodulatory feed additive supplementation for weaned Nellore cattle under grazing conditions.

We hypothesized that an immunomodulatory feed additive supplementation during pre- and post-transport would decrease the plasma cortisol concentrations of the cattle, changing their metabolism and improving the animal performance during the growing phase. The objective of this experiment was to evaluate different immunomodulatory feed additive supplementation strategies, pre- and post-transport, for weaned Nellore calves that were maintained on a pasture, and their effects on the physiological parameters and performances during the growing phase.

2. Materials and Methods

2.1. Local and Climate

The experiment was conducted at the Agência Paulista de Tecnologia dos Agronegócios (APTA), Colina, SP, Brazil (20°43′5″ S, 48°32′38″ W). During the pre-transport period, the temperature ranged from 11.8 °C to 29.5 °C and the total rainfall was 38.8 mm, distributed over seven days. During the post-transport period, the temperature ranged from 16.9 °C to 32.4 °C and the total rainfall was 984 mm, distributed over 96 days.

2.2. Experimental Period

The animals were evaluated from August 2018 to April 2019. The experimental period was divided into pre- and post-transport during the growing phase. The pre-transport period lasted 42 days (beginning on d −42; Figure 1) and the post-transport period lasted 210 days (beginning on d 0), which was divided into five periods of 42 days each (d 0, d 42, d 84, d 126, d 168 and d 210; Figure 1).

2.3. Experimental Area

The experimental area was composed of *Urochloa brizantha* cv. Marandu and was divided into 12 paddocks (2.2–2.4 ha each). All the paddocks were equipped with water tanks (1000 L) and feeders (27 linear cm/animal) for the supplementation. A urea-based fertilizer was applied at a rate of 90 kg/ha of nitrogen during the experimental period and was divided into two applications of 45 kg/ha in February and March 2019.

2.4. Animals

In total, eighty-four non-castrated Nellore calves (weaning BW = 184 ± 19 kg; 7 ± 1 months of age) were used in a randomized complete block design (blocked by initial BW). The animals belonged to the same herd and were managed similarly until the beginning of the experiment. The calves were weaned two weeks before the beginning

of the experiment and were allowed to keep fence line contact with the dams for seven days, according to the recommendations by Taylor et al. [15]. This interval was considered to be a transition period between the weaning and experimental procedure to reduce any changes in the animal behavior due to weaning [16].

Prior to the experimental period, all the animals were vaccinated against clostridial diseases: *Clostridium chauvoei*, *Clostridium botulinum* types C and D, *Clostridium septicum*, *C. novyi*, *C. perfringens* types B, C, and D, and *C. sordelli* (Poli-Star®; Vallee SA, Minas Gerais, Brazil). They were also administered an anthelmintic: Eprinomectina 5% (LongRange®; Boehringer Ingelheim, São Paulo, Brazil) at 1 mL/50 kg BW, and they were treated against ectoparasites using Fipronil (Topline® Pour-on; Boehringer Ingelheim, São Paulo, Brazil) at a concentration of 1 g/100 mL. The same protocol was repeated on d 42, along with a vaccination against foot-and-mouth disease (Ourovac® Aftosa; Ourofino Saúde Animal Ltda., Cravinhos, São Paulo, Brazil).

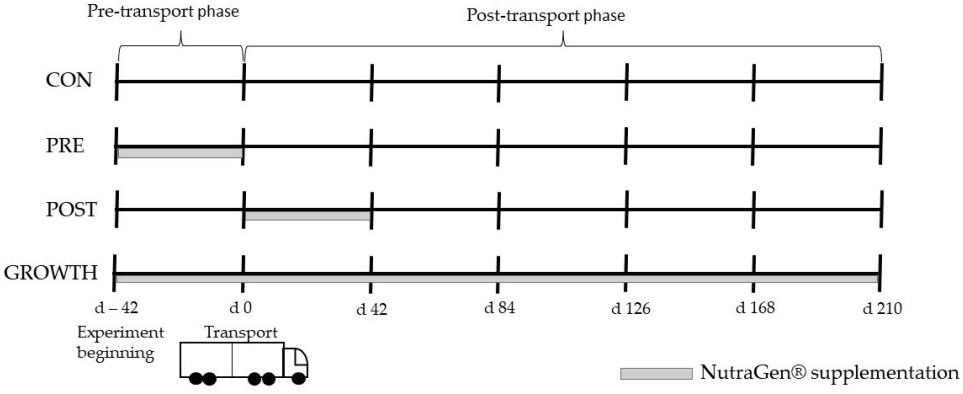

Figure 1. Schematic representation of experimental period and different strategies of inclusion of an immunomodulatory feed additive (NutraGen®; Phibro Animal Health, Guarulhos, São Paulo, Brazil) added to supplements at 10 g/100 kg BW/day for Nellore calves submitted to post-weaning transport.

2.5. Experimental Design and Treatments

A total of four treatments were evaluated in a randomized complete block design. The animals were randomly distributed into 4 paddocks per block and 7 animals per paddock. The blocks were established based on their BWs (high, medium, and low) and the paddock was considered to be the experimental unit. The treatments were based on different supplementation strategies with an immunomodulatory feed additive (NutraGen® (NUTRA); Phibro Animal Health, Guarulhos, São Paulo, Brasil) during the pre- and post-transport periods in the growth phase (Figure 1): (1) Control (CON): no immunomodulatory supplementation during the entire experiment; (2) NUTRA pre: the inclusion of the immunomodulatory feed additive for 42 days, only in the pre-transport period (d −42 to d 0); (3) NUTRA post: the inclusion of the immunomodulatory feed additive for 42 days, only in the post-transport period (d 0 to d 42); and (4) NUTRA growth: the inclusion of the immunomodulatory feed additive during the entire experiment.

2.6. Supplementation and Immunomodulatory Feed Additive

From d −42 to d 42, all the animals received a dry season protein energy supplement (crude protein = 300 g/kg; total digestible nutrients = 650 g/kg), and from d 43 to d 210, the animals received a rainy season protein energy supplement (crude protein = 200 g/kg and total digestible nutrients = 650 g/kg). The supplementation was provided daily at 09:00 at 3 g/kg of the BWs (Table 1).

Table 1. Ingredient and nutrient composition of supplements.

Item	Protein-Energy Supplement [1]	
	(d −42 to d 42)	(d 43 to d 210)
Ingredients (g/kg)		
Corn	167	484
Sorghum	50.0	147
Soybean meal	429	125
Corn gluten meal (21% CP)	150	-
Rice bran	100	100
Slow-release urea	10.0	5.0
Urea	4.0	21.0
Sodium chloride	35.0	36.0
Mineral mixture	48.7	75.8
Vmax2® [2]	6.6	6.6
Composition as feed (g/kg)		
Dry matter	89.0	89.6
Crude protein (minimum)	30.0	20.0
Protein equivalent from NPN	12.5	8.13
Total digestible nutrients (minimum)	65.0	65.0

NPN = non-protein nitrogen. [1] Provided daily at 3 g/kg of body weight per animal. In both supplements, the macro and micro mineral guaranteed levels were: calcium (minimum) 15 g/kg, calcium (maximum) 25 g/kg, cobalt (minimum) 8.7 mg/kg, copper (minimum) 100 mg/kg, sulfur (minimum) 5000 mg/kg, fluorine (maximum) 70 mg/kg, phosphorus (minimum) 7000 mg/kg, iodine (minimum) 5.30 mg/kg, magnesium (minimum) 1000 mg/kg, and manganese (minimum) 68 mg/kg. [2] Guaranteed analysis of Vmax2® Phibro = 20 g of virginiamycin per kg of product.

The immunomodulatory feed additive was added to the supplements at 10 g/100 kg of the BWs. The inclusion level of NUTRA was based on previous studies [10,17]. The additive contained a mixture of active dry *Saccharomyces cerevisiae*, dried *Trichoderma longibrachiatum* fermentation extract, niacin supplement, vitamin B12, riboflavin-5-phosphate, D-calcium pantothenate, choline chloride, biotin, thiamine monohydrate, pyridoxine hydrochloride, menadione dimethylpyrimidinol bisulfite, folic acid, calcium aluminum silicate, sodium aluminum silicate, diatomaceous earth, calcium carbonate, rice hulls, and mineral oil. The additive was mixed with the supplement every three days in a horizontal ribbon mixer for 5 min.

2.7. Road Transport

On d 0, all the animals were transported for 8 h over 200 km of unpaved roads. The transport was conducted over three different days to allow for the sampling (observing the supplementation period of 42 days pre-transport). Animals of the same blocks were transported in the same commercial livestock trailers, with dimensions of 7.40 m long × 2.40 m wide × 1.90 m high, allowing for a density of 1.6 animals/m^2 and 339, 296, and 270 kg of BW/m^2, respectively, for the high-, medium-, and low-BW animals, who were weighed before transport.

At around 06:00, the animals were removed from the paddocks, mixed, taken to the corral, and weighed. At 07:00, the animals were loaded and transported throughout the day. There was a one-hour break at around 12:00. At the end of the day, the animals were unloaded, weighed, and their blood samples were taken. After the sampling, the animals were placed into the same paddocks, maintaining the same groups of animals prior to the transport.

2.8. Grazing Method

A continuous grazing system with a variable stocking rate was used to maintain the same forage sward height for all the treatments (around 25 cm) [18]. This sward height corresponded to the 95% light interception for *Urochloa brizantha* cv. Marandu, in which the productivity parameters were highest [19]. Put-and-take steers were used to control the sward height [18] after the post-transport period, when the pasture parameters were characteristic of the rainy season, i.e., there were higher rates of green leaf accumulation [20]. These animals came from the same herd as the experimental animals and were kept in an adjacent area with access to the supplementation throughout the experiment.

2.9. Quantitative and Qualitative Evaluation of the Pasture

The forage mass was estimated at the beginning of the experiment (d −42) and every 42 days (d 0, d 42, d 84, d 126, d 168, and d 210), using the double sampling method [21]. The quantitative and structural components of the forage sward were evaluated at medium heights. The forage samples were separated into green leaf, dead leaf, green stem, and dead stem in the pre-transport period (dry season), and green leaf, green stem, and dead material in the post-transport period (rainy season). After the separation, the forage components were weighed and oven-dried at 55 °C for 72 h to obtain the partial DM and the proportion of each component in the forage sward (Table 2).

Table 2. Characteristics of the forage sward grazed by young Nellore bulls during growth phase, subjected to different immunomodulatory feed supplementation strategies in the pre-and post-transport periods.

Item	Pre-Transport					Post-Transport				
	Treatments [1]				SEM	Treatments [1]				SEM
	CON	Pre	Post	Growth		CON	Pre	Post	Growth	
Quantitative characteristics (n = 12)										
Sward height (cm)	21.3	21.4	16.4	18	2.74	25.2	24.7	22.7	23.1	1.85
Forage mass (kg DM/ha)	4888	4356	3509	3585	705	4030	3767	3526	3502	337
Green leaf (% DM)	8.66	11.5	7.53	13.1	3.2	42.1	45.3	48	48.8	3.88
Green stem (% DM)	9.85	8.33	5.95	6.56	1.49	22.4	21.2	21.2	23.9	1.21
Dead leaf (% DM)	33.5	28.3	33	28.1	2.33	-	-	-	-	-
Dead stem (% DM)	48	51.9	53.5	52.3	3.95	-	-	-	-	-
Dead material (% DM)	-	-	-	-	-	35.5	33.5	30.7	27.3	4.66
Forage allowance (kg DM/kg BW)	8.15	7.3	5.6	5.86	1.14	4.11	4.02	3.79	3.67	0.35
Forage allowance kg (GLDM/kg BW)	0.29	0.25	0.17	0.24	0.06	1.7	1.82	1.86	1.76	0.16
Stocking rate (AU/ha)	1.22	1.22	1.2	1.19	0.06	2.14	2.01	1.88	1.97	0.1
Qualitative characteristics (g/kg) (n = 12)										
Crude protein	70.8	65.9	73.7	72.1	4.80	123	110	120	117	5.60
NDF	699	702	689	703	10.6	623	632	617	620	5.50
ADF	333	331	334	338	5.40	274	274	271	264	5.50
Lignin	42.3	41.9	42.7	44.9	1.90	30.7	30.6	28.7	29.2	1.00
IVDMD	676	677	684	670	9.70	807	804	812	816	5.60

GLDM = green leaf dry matter; IVDMD = in vitro dry matter digestibility. [1] CON: no immunomodulatory supplement; NUTRA pre: inclusion of NutraGen® (10 g/100 kg BW/day) only in the pre-transport period for 42 days; NUTRA post: inclusion of NUTRA (10 g/100 kg BW/day) only in the post-transport period for 42 days; and NUTRA growth: inclusion of NUTRA (10 g/100 kg BW/day) during the whole experimental period (pre- and post-transport).

Every 42 days, hand-plucked samples were used to estimate the dietary nutritional value [22]. The samples were oven-dried at 55 °C for 72 h and then ground in a Wiley mill (Thomas Model 4, Thomas Scientific, Swedesboro, NJ, USA) to pass through a 1 mm mesh sieve. These samples were used for the determination of the CP levels (method 978.04; AOAC, 1995). The NDF and ADF contents were evaluated by a sequential analysis, as described by Van Soest et al. [23]. The cellulose was solubilized using 72% sulfuric acid, whereby the lignin content was obtained by the difference from the ADF. The true in vitro DM digestibility was determined, according to Van Soest and Robertson [24].

The quantitative and qualitative characteristics of the forage were subjected to statistical analyses, and there was no difference between the analyzed treatments ($p > 0.10$), with the averages being shown in Table 2.

2.10. Animal Performance

The animals were weighed after fasting for 16 h on d −42, d 42, d 84, d 126, d 168, and d 210 for the determination of their BWs and average daily gains (ADG). On d 0, the animals were weighed immediately before and after transport to evaluate if their BWs shrunk during transport (%). The shrunk BW from each period was also used to adjust the supplement amounts.

2.11. Blood Samples and Analyzes

The blood samples were collected via a jugular venipuncture into tubes (Vacuplast, 9 mL; Weihai Hongyu Medical Devices Co., Ltd., Weihai, China) with spray-dried sodium heparin. The samplings were performed on all the animals on d −42 and d −1 (pre-transport), d 0 (unloading), and d 3, d 7, d 14, d 42, and d 210 (post-transport).

The blood samples on d −42, d −1, d 3, d 7, d 14, d 42, and d 210 were taken at 08:00, before the supplementation. These samples were taken to evaluate the period in which the cattle were transported and arrived in a new place, facing stress-induced metabolic changes [7]. The blood samples were stored in ice boxes immediately after their collection and then centrifuged at 2500× *g* at 4 °C for 15 min. The blood samples were processed immediately after the blood collection and aliquots of the plasma were stored at −80 °C until further analysis.

All the samples were analyzed for cortisol by a radioimmunoassay (RIA) using a solid-phase commercial kit (Coat-a-count®, Diagnostic Products Corporation, Los Angeles, CA, USA); the urea, albumin, and total proteins were analyzed using commercial biochemical kits (Bioclin; Quibasa-Química Básica Ltda., Belo Horizonte, MG, Brazil) and a biochemical analyzer (Cobas Mira Plus; Roche Diagnostic Systems, Montreuil, France). The plasma samples (d −42, d −1, d 3, d 7, d 14, and d 210) were analyzed for glucose and aspartate aminotransferase (AST) (Bioclin; Quibasa-Química Basica Ltda., Belo Horizonte, MG, Brazil). The plasma samples (d −42, d −1, d 0, and d 210) were analyzed for creatinine, cholesterol, calcium, phosphorus, and magnesium (Bioclin; Quibasa-Química Basica Ltda., Belo Horizonte, MG, Brazil). The intra-and inter-assay variations of the plasma cortisol were 4.31% and 5.2%, respectively.

2.12. Statistical Analysis

All the data were analyzed as randomized complete block designs, using the MIXED procedures of SAS® University Edition software (SAS Institute, Cary, North Carolina, US). Each paddock was considered to be an experimental unit, where the bull (paddock) and paddock (treatment) were included as random effects in all the analyses. The variables, when evaluated over the experimental periods (ADG and blood parameters), were analyzed as repeated measures and tested for the fixed effects of treatment, time, and resulting interactions, using the paddock (treatment) as the subject. Different covariance structures were tested with the final choice, depending on the lowest value for the Akaike information criterion. The variables that were not evaluated by period were: the initial BW, final BW, and BW shrink (%), and were used in the model as fixed effects only for the effects of the treatments. All the results are reported as least squares means. The data were separated using PDIFF if a significant F-test was detected. The significance was set at $p \leq 0.05$, and tendencies were noted if $p > 0.05$ and ≤ 0.10.

3. Results

There was no interaction between the treatment and period ($p \geq 0.86$) in the post-transport ADG (d 0 to d 210) and total ADG (d −42 to d 210). There was no effect of the treatments ($p \geq 0.22$) on the ADGs and BWs during the experimental period (Table 3). There was no difference between the treatments ($p = 0.81$) for the BW shrink (Table 3). There was an effect ($p < 0.01$) of the periods on the ADG (Table 3). The ADG was lower in the pre-transport (mean of 0.100 kg/d) than in the post-transport period. The highest ADG was observed between d 168 and d 210 (mean of 1.08 kg/d).

Table 3. Performance of young Nellore bulls subjected to different immunomodulatory feed supplementation strategies in the pre- and post-transport periods during the growth phase.

Item	Treatments [1]				SEM	*p*-Value
	CON	Pre	Post	Growth		
Initial BW (d −42) (kg)	173	174	174	174	11.1	0.45
Post-transport BW (d 0; kg)	181	178	179	177	12.4	0.61
BW shrink (%)	6.98	7.32	6.54	7.04	0.61	0.81
Final BW (d 210; kg)	359	356	359	356	15.1	0.85
Pre-transport ADG (kg/day)	0.15	0.09	0.11	0.07	0.03	0.22
Post-transport ADG (kg/day)	0.87	0.87	0.88	0.87	0.03	0.96
Total ADG (d −42–d 210; kg/day)	0.75	0.74	0.76	0.74	0.02	0.93

ADG = average daily gain. [1] CON: no immunomodulatory supplementation; NUTRA pre: inclusion of NUTRA (10 g/100 kg BW/day) only in the pre-transport period for 42 days; NUTRA post: inclusion of NUTRA (10 g/100 kg BW/day) only in the post-transport period for 42 days; and NUTRA growth: inclusion of NUTRA (10 g/100 kg BW/day) during the whole experimental period (pre- and post-transport). Note: There was an effect of period ($p < 0.01$). There was no significant interaction between treatment and period for post-transport ADG and total ADG ($p \geq 0.86$).

There was a significant effect of the periods ($p < 0.01$) on the BWs (Figure 2). However, the initial BWs (d −42; 174 kg) and BWs on d 0 (179 kg) did not differ ($p = 0.16$) among the treatments. As expected, the BWs were higher ($p < 0.01$) in the following periods due to the greater ADGs.

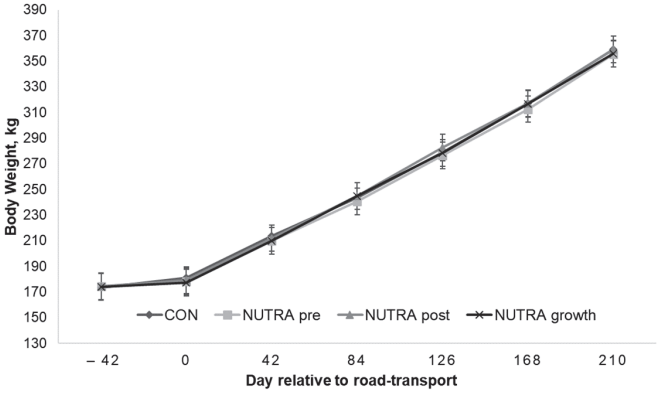

Figure 2. Body weight (BW) of young Nellore bulls subjected to different immunomodulator supplementation strategies in the pre- and post-transport during the growth phase. Additive: NutraGen added to the supplement at 10 g/100 kg BW/d. Treatments: ($p = 0.70$); period: $p < 0.01$; and treatment × period ($p = 0.93$). Animals were weighed before and after a 16 h shrink, except on d 0, in which BW was obtained after transport for 8 h.

There was no interaction between the treatment and period ($p \geq 0.17$), nor was there a treatment effect ($p \geq 0.14$) on any of the physiological variables that were measured in the plasma (Table 4). The sampling period influenced ($p < 0.01$) all the physiological variables in the plasma (Figures 3 and 4). The cortisol was higher on d 3 post-transport and reached its lowest concentration at the end of the growth phase period (d 210). From d 7 onwards, the cortisol concentration was similar to that on d −1. There was a trend towards a lower cortisol concentration during unloading than that on d 14 ($p = 0.058$). The glucose was greater during unloading (d 0), with lower concentrations on d 7 and d 14 post-transport. The total plasma proteins increased ($p < 0.01$) from d 0 to d 7 post-transport, and were lower ($p < 0.01$) in the afternoon blood samples (d 42 to d 210) than in the morning samples (d −42, d −1, d 3, d 7, and d 14). The plasma albumin concentration increased ($p < 0.01$) with the diet supplementation of the weaned calves (d −42 to d −1). At the same time, it responded negatively to the stress of road transport. There was a trend towards a lower albumin concentration ($p = 0.08$) on d 3 compared to d −1 (pre-transport), whereas it was lower ($p < 0.01$) on d 14. Moreover, the albumin concentration was lower ($p < 0.01$) in the afternoon (d 42 and d 210) than in the morning samples.

Table 4. Plasma concentrations of metabolites in young Nellore bulls subjected to different immunomodulatory feed supplementation strategies in the pre- and post-transport during the growth phase.

Item	Treatments [1]				SEM	*p*-Value [2]		
	Con	Pre	Post	Growth		T	P	T × P
Cortisol (ng/mL)	66.0	71.8	73.1	67.8	7.2	0.85	<0.01	0.93
Albumin (g/L)	19.3	19.2	18.6	19.5	0.37	0.30	<0.01	0.85
Total proteins (g/L)	54.4	54.1	54,1	56.4	1	0.36	<0.01	0.62
Urea (mmol/L)	4.73	4.42	4.73	4.93	0.19	0.39	<0.01	0.88
Creatinine (µmol/L)	102.5	108	111	103.2	2	0.14	<0.01	0.17
Glucose (mg/dL)	86.5	93.5	90.2	90.4	3.5	0.51	<0.01	0.81
Cholesterol (mmol/L)	3.01	2.86	2.95	2.92	0.06	0.52	<0.01	0.79
AST (U/L)	68.1	73.5	70.3	75.4	3.9	0.32	<0.01	0.5
Calcium (mmol/L)	1.74	1.73	1.67	1.76	0.03	0.26	<0.01	0.9
Phosphorus (mmol/L)	1.7	1.65	1.62	1.72	0.05	0.23	<0.01	0.78
Magnesium (mmol/L)	0.912	0.918	0.887	0.924	0.03	0.71	<0.01	0.55

AST = aspartate aminotransferase. [1] CON: no immunomodulatory supplementation; NUTRA pre: inclusion of NUTRA (10 g/100 kg BW/day) only in the pre-transport period for 42 days; NUTRA post: inclusion of NUTRA (10 g/100 kg BW/day) only in the post-transport period for 42 days; and NUTRA growth: inclusion of NUTRA (10 g/100 kg BW/day) during the whole experimental period (pre and post-transport). [2] T: treatment effect; P: sampling period effect; and T × P: effect of the treatment × period interaction. Note: blood samples were collected on days d −42, d −1, d 0, d 3, d 7, d 14, d 42, and d 210. Cortisol, albumin, total proteins, and urea were analyzed in all these periods. AST and glucose were analyzed on days d −42, d −1, d 0, d 3, d 7, d 14, and d 210. Creatinine, cholesterol, calcium, phosphorus, and magnesium were analyzed on days d −42, d −1, d 0, d 42, and d 210.

The plasma urea concentration increased ($p < 0.01$) during the experiment due to changes in the grazing and supplementation conditions. The urea concentration on d 0 (unloading) was lower than that on d −1 (one day before transport) and tended to be lower ($p = 0.06$) on d 0 than on d 14. The concentration of the enzyme aspartate aminotransferase was higher on d 0 (unloading; $p < 0.01$), with lower values on d −42, d −1, and d 210 ($p \leq 0.05$).

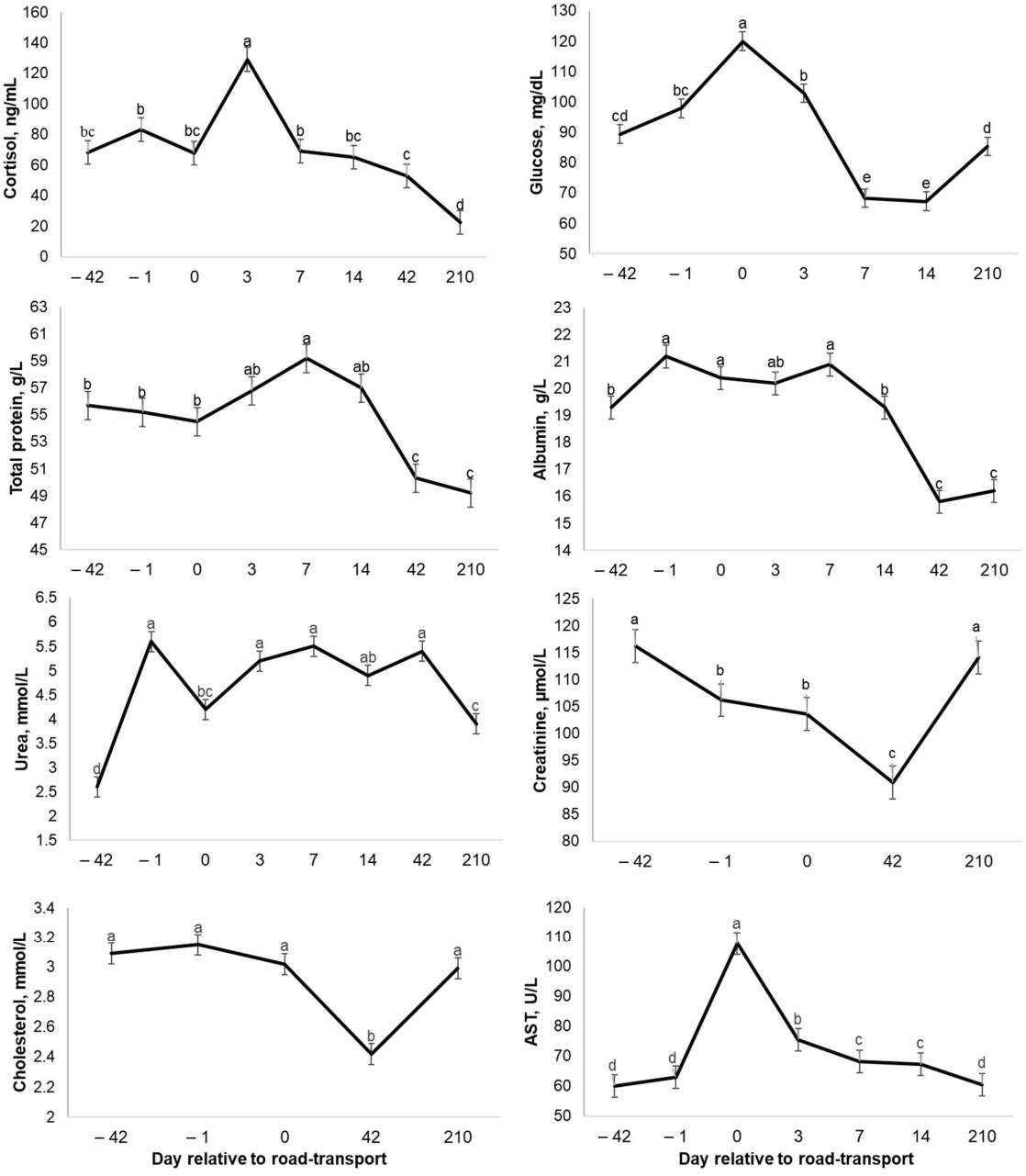

Figure 3. Plasma concentrations of cortisol, glucose, total proteins, albumin, urea, creatinine, and aspartate aminotransferase (AST) in young Nellore bulls subjected to different immunomodulatory feed supplementation strategies in the pre- and post-transport during the growth phase. Additive: NutraGen® added to the supplement at 10 g/100 kg BW/day. There was an effect of period on all variables ($p < 0.01$). [a,b,c,d,e] Values within the days followed by different letters differ at $p < 0.05$.

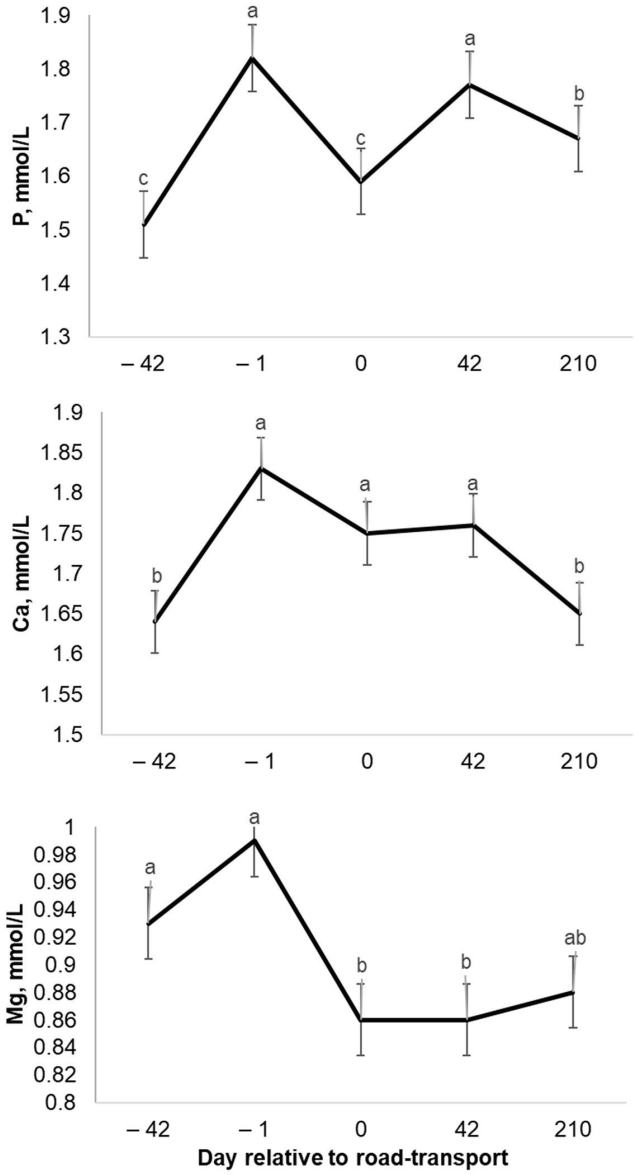

Figure 4. Plasma concentrations of phosphorus, calcium, and magnesium in young Nellore bulls subjected to different immunomodulatory feed supplementation strategies in the pre- and post-transport during the growth phase. Additive: NutraGen® added to the supplement at 10 g/100 kg BW/day. There was an effect of period on all variables ($p < 0.01$). [a,b,c] Values within the days followed by different letters differ at $p < 0.05$.

4. Discussion

This study shows the effects of NutraGen® supplementation on weaned Nellore cattle. In contrast to our hypothesis, we did not observe any additional benefits of NUTRA supplementation on the performances, metabolisms, and stress responses of Nellore calves that were transported in the post-weaning period. NUTRA supplementation benefits for animal performance were also not observed in Angus × Hereford calves that were purchased from

an auction market and transported for 12 h to a feedlot facility [11]. The authors also did not report any benefits of NUTRA on the feed efficiency, incidence, symptoms, and antibodies against bovine respiratory disease pathogens [11]. However, Lippolis et al. [11] started this NUTRA supplementation after the calves were exposed to immunological challenges caused by weaning, auction, transport, vaccination, mixing with animals from different sources, and feedlot entry [7]. In practical terms, a supplementation with NUTRA several weeks before a stress or immune challenge has been recommended [14].

In our study, for the NUTRA pre and NUTRA growth conditions, the animals received the feed additive supplementation for 42 days before being transported. These strategies allowed for the intake of NUTRA in advance of the transport (stressful event), although no effects on the animal performance and physiological parameters in plasma were observed. However, some particularities should be considered, such as the weaning method used (fence line weaning) and the period in which the calves were kept in the same paddock for weaning before being transported. These management practices are recommended to reduce stress, increasing the performance and health of beef calves compared to other methods of weaning and road transport immediately after weaning [15]. Moreover, the natural conditions of the grazing systems are usually less stressful for animals, which promotes an increased well-being compared to feedlot systems [25]. Studies in temperate regions with newly weaned animals upon feedlot entry report significant rates of morbidity and symptoms of bovine respiratory disease (BRD), resulting from the weakening of the immune system caused by stress [7]. It is worth mentioning that *Bos indicus* animals have a reduced acute-phase response to weaning and road transport stress compared to *Bos taurus* cattle, which indicates a greater resilience of Nellore cattle to stressful conditions [26]. According to Brandão et al. [27], NUTRA supplementation does not provide additional benefits for the innate immunocompetence of healthy beef cattle.

The lack of a treatment effect on the plasma parameters indicates that, under the experimental conditions of the present study, the NUTRA supplementation did not modulate the physiological responses of the cattle. However, the significant effects of the day and sampling intervals on the plasma-related variables indicate that the calves were exposed to stress associated with the transport procedures. It is noteworthy that the blood parameters should be interpreted based on similar sampling times (d −42, d −1, d 3, d 7, and d 14 at 800 h; d 42 and d 210 at 1500 h), given the circadian nature of cortisol secretion and its influence on metabolism [4].

The cortisol was higher on d 3 after the transport, unlike the expected peak during unloading. However, we believe that the cortisol concentration peaked during the transport, prior to the blood sampling, so that the rise in the cortisol during transport quickly returned to normal levels soon after the event [5,28]. The greater glucose concentration value during the unloading and on d 3 was probably because of the secretion of glucocorticoids such as cortisol, altering the glucose metabolism, consequently increasing its concentration in the blood [4,5]. This result corroborates with the hypothesis that the cortisol was higher before the sampling procedures during the unloading. It suggests that there was an increase in the cortisol during the transport, higher than that on d 3, and that this peak occurred before the blood collection.

The sharp increase in the cortisol levels elicited by the transport procedures and feedlot entry is accompanied by a transient response of acute-phase proteins in the cattle, particularly of haptoglobin and ceruloplasmin [7]. At the same time, this pattern correlates negatively with the DM intake and animal performance, leading to a subsequent reduction in immunocompetence. Therefore, this increases the BRD occurrence and morbidity of feedlot cattle [7]. Although we have not evaluated these acute-phase proteins, their transient responses can be deduced from the reduction in the plasma albumin concentration, which is negatively correlated with the acute-phase proteins [17,29]. The albumin concentration tended to decrease after the transport on d 3 and was lowest on d 14, even though there was an increase in the total protein concentration during the same period. Based on these responses, we can consider that, in case of the impossibility of dosing these acute-phase

proteins in the blood to identify an inflammatory response, the simultaneous dosage of the albumin–total protein pair may indicate an acute-phase inflammatory response triggered by cortisol, released as a result of the stress associated with the road transport. However, studies that also evaluate the acute-phase proteins during these procedures are necessary to validate this hypothesis.

Although there was evidence for the occurrence of acute stress in our study, the animals were supplemented on a pasture for 42 days after weaning and before transport, and were kept in a pre-established group. Preconditioning is a technique for reducing the stress of newly weaned animals that will be later transported [30]. After the transport, the animals were also kept in the same group and the same environment as before. On the other hand, management practices such as weaning followed by immediate transport, livestock auctions, the mixing of animals from different sources, extended periods of water and feed deprivation, vaccinations following these events, and feedlot entry are characterized as potentially more stressful situations for cattle. Therefore, these events are enough to deplete the body reserves to the point of impairing the biological functions and bringing an animal into a pre-pathological or pathological state, thus reducing the animal performance [5,7]. It is speculated, therefore, that there has not been sufficient stress in the studied management conditions to observe the benefits of NUTRA in Nellore animals, such as those obtained in experiments with dairy cows under conditions of thermal stress, transition periods, and sanitary challenges [12,13,31].

Aspartate aminotransferase is an indicator of muscle damage and may increase with trauma and muscle exercise, which can occur during transport [32]. In our study, there was probably muscle damage during the transport, since the AST had higher value during unloading. Contrary to what was expected, the plasma urea concentrations did not increase during the transportation, but were reduced relative to the pre-transport (d −1). This reduction was probably associated with a lower feed intake, and, consequently, the CP on the day of transport [33]. The animals were taken from the pasture to the corral at 06:00, and the transport and sampling procedures finished at approximately 17:00. Takemoto et al. [34] transported Holstein steers for 24 h and observed an increase in their plasma urea concentrations during unloading and up to 7 days after the transport, preserving similar feeding conditions. According to these authors, transport stimulates the deamination of amino acids and the use of carbon chains in energy metabolism, whereas nitrogen is metabolized as urea. Knowles et al. [35] also observed an increase in the plasma urea concentrations immediately after the transport, which suggests an increase in muscle protein breakdown. These results suggest that the challenge imposed on animals during transport is not enough to deplete the body reserves to the point of increasing the levels of urea in the blood via the deamination of amino acids from the muscle. It reinforces that the stress promoted by transport is not severe enough to cause a positive response associated with the use of an ingredient that modulates and stimulates the immune response of cattle under stressful conditions [6,31].

5. Conclusions

Under the conditions of this experiment, the supplementation with NutraGen® at 10 g/100 kg BW/day did not improve the physiological parameters and performances of grazing young Nellore bulls that were transported for 8 h. However, the short period in which the animals were subjected to transportation stress and the non-stressful grazing environment may have reduced the adverse effects of this stress on the animals, thus reducing the potential benefits of using the feed additive immunomodulator. New studies should be conducted based on more challenging situations to assess how the use of an immunomodulatory feed additive could benefit the performance of beef cattle.

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Institutional Review Board Statement: This study was carried out in accordance with the ethical principle established by the Brazilian Council for the Control of Animal Experimentation and approved by the Ethics Committee for the Use of Animals of the São Paulo State University, College of Agricultural and Veterinary Sciences, São Paulo, Brazil (process no. 006088/19).

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Bioactive Feed Additive for the Prevention of Clostridial Disease in High-Yielding Dairy Cattle

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Abstract: The purpose of this research is to develop and test a new approach to prevent clostridial disease in cattle, based on the use of a new compound biologically active feed additive (BFA). Some properties of the separate components of BFA are characterized. The research showed that a strain of the bacterium *Bacillus amyloliquefaciens*159 has an expressed antagonism to toxin-producing strains of *C. perfringens*. When using the test strains of *C. perfringens* from the ATCC collection (13,124 as type A, 10,543 as type C, 12,916 as type F), the anticlostridial activity of the tested strains varied, with size range of 14.0 ± 0.95–15.0 ± 1.28 mm of delayed growth zones. The bactericidal properties of lauric acid and the sorption properties of diatomaceous earth, included in BFA, were confirmed. The experiment was conducted on Holstein cows at the beginning of lactation (control, C (n = 15) vs. experimental E48 (n = 15), E80 (n = 15) and E112 (n = 15), 48, 80 and 112 g/head/day BFA, respectively. All cows were vaccinated with "Coglavax" (vaccine against bovine and sheep clostridial disease, Ceva-Phylaxia VeterinaryBiologicals, Hungary), reinjected two weeks before the experiment. At the end of the experiment (3.5 months after the vaccination and 3 months after the start of BFA feeding according to the scheme of the experiment), the immune response in the control and Group E48 to *C. perfringens* β-toxin remained at the initial level, while the response in Group E80 and Group E112 became higher under the influence of BFA feeding. Cows fed BFA saw a guaranteed improvement in non-specific resistance. The increase in serum lysozyme concentration in cows of Groups E was 1.01–2.91 mkg/mL vs. control ($p < 0.001$). TP, GLB, ALB/GLB vs. Groups C and E48 ($p < 0.001$); this stabilized and normalized while feeding Group E80 and E112 animals with BFA. They also had improved nitrogen, fat, mineral metabolism, as indicated by significant increase in ALB ($p < 0.05$), UREA ($p < 0.01$), CHOL ($p < 0.01$), and CHL ($p < 0.01$) vs. Groups C and E48. Consumption of BFA increased the amount of anti-oxidants in the blood (highest TAWSA values in Group E80 14.45 mg/g, $p = 0.002$). Serum TBA–AP/ CP ratio was directly related to TBA–AP (r = 0.87, $p < 0.001$), and decreased in Group E80. The milk productivity increased under the action of BFA; the average daily milk yield of the cows from the experimental groups for the period of the experiment (d0–d98) was 1.24–1.66 kg higher than that of the control. At the same time, Group E112 cows had a significant increase in milk yield (by 5.1%, $p = 0.03$ vs. Control). Thus, feeding BFA to dairy cows was found to improve resistance, prevent toxicoses and increase milk production of cattle, which can serve as an additional strategy for bioprotection of cattle against infection.

Keywords: dairy cows; *Clostridium*; *Bacillus*; probiotics; resistance; blood chemistry; antioxidants; detoxification; health benefits

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1. Introduction

Even if all feed production requirements are met, there are risks of xenobiotics of various etiologies entering the feed [1]. Once in animals, they cause serious health problems, can biotransform and accumulate in animal products, and thereby cause harm, not only to animals, but also to humans [2,3].

Meanwhile, animals consuming feeds containing various groups of xenobiotics suffer from damage to the limbs, liver, and digestive organs, metabolic disorders, postnatal complications, and reproductive disorders [4]. Often, contaminants of fungal (mycotoxins), bacterial (exo- and endotoxins), and chemical origin (pesticides and heavy metals) are present simultaneously in the feed, which has a synergistic negative effect on the animal organism [5–7].

The accumulation of xenobiotics determines changes in the intracellular content of dissolved substances, which can lead to volume changes, called isoosmotic volume changes, since the plasma membrane is easily permeable to water, and under normal conditions the body is able to conduct a rapid, multidirectional water flow to establish equilibrium, which is often disturbed by various pollutants [8,9]. This disturbance in the body can reach a state of oxidative stress when the balance between oxidants and antioxidants is disturbed. Stresses, including oxidative stress, are a special case of metabolic disorders, in which improper feeding and housing contribute to higher levels of endo- and exotoxins entering the bloodstream, causing inflammation in the animals, with a significant decrease in productivity [10].

For many years, it was believed that ruminants have absolute resistance to mycotoxins. However, the metabolism of mycotoxins in the rumen is not equivalent to their complete detoxification. Furthermore, their potential degradation depends to a large extent on the stability of the rumen microbial community and the pH, which varies depending on the feed composition [11]. It is known that high-yielding cows, transition cows, and calves are the most demanding in terms of optimal feeding and housing conditions. As a result of selection solely for milk productivity, low resistance, increased sensitivity to stresses, and pathological reaction to even insignificant changes in cattle maintenance and feeding are often observed in high-productivity cows. The presence of toxigens in feed is a factor leading to subclinical pathological conditions with a negative dynamic which, in the absence of a precise diagnosis and qualified help, is highly likely to lead to a fatal outcome. A typical example of this trend is the large number of high-yielding cattle that are culled immediately after calving or in the first months of lactation [12]. According to Hadley et al. [13], up to 80% of culled dairy cows have health problems.

The presence of toxins in feed primarily affects the rumen microflora, as many secondary metabolites are produced during digestion, negatively affecting numerous groups of microorganisms living in the rumen [14]. It is therefore a proven fact that ruminants are more affected by natural and natural pollutants than other species [15].

Many studies have been conducted on the effects of chemical toxins [16] and mycotoxins [17] on the ruminant organism. Mechanisms have also been developed to mitigate their effects on the animal organism, primarily related to measures to reduce emissions into the environment, to improve protocols for forage harvesting and storage, and to neutralize and remove them from the animal organism by feeding them targeted feed additives [18–20].

Bacterial toxins are no less dangerous [21]. It is well known that the secretion of proteins that damage host tissue is an integral part of the infectious processes of many bacterial pathogens [22,23]. In addition, toxins can be produced by many bacteria that live harmlessly in the animal's body—representatives of normal and opportunistic microflora: *Escherichia coli*, *Campylobacter* spp., *Shigella* spp., *Staphylococcus* spp., *Bacillus* spp., *Clostridium* spp., etc. Individual species of these can cause infection, either by reaching a specific organ or under the influence of certain conditions [24].

Much work has been undertaken to eliminate and/or reduce the incidence of anaerobic infections in veterinary medicine. Even today, despite the availability of specific

prophylactics, chemotherapeutic agents, and antibiotics against most infections, clostridia remains a serious problem and causes considerable economic damage [25,26].

The pathogenic anaerobic microorganisms that are the causative agents of clostridia are widespread in nature [27]. Their main reservoir is soil, and they also live in the intestines of animals and humans. Most clostridia are toxigenic infections. Exposure occurs through the formation of highly active toxins during growth, but other pathogenicity factors (hyaluronidase, lecithinase, collagenase, haemolysins, etc.) are also present. Clinically relevant clostridium species include the causative agents of independent diseases: tetanus, botulism, and emphysema, for example. However, in most cases the disease is caused by an association of species, as well as other anaerobes and aerobes. Although the clinical features of anaerobic diseases caused by different clostridial species differ greatly, there are several features that allow them to be grouped under the common name of Clostridiosis. These features include the ability to form spores, the anaerobic nature of the metabolic processes, the formation of highly active exotoxins, their habitation within animal intestines, and the fact that they often affect young animals with well-developed musculature [28].

In humans and livestock, *C. perfringens* causes intestinal infections, such as enteritis, enterocolitis, or enterotoxaemia. Enterotoxaemia in cattle is an acute or subacute syndrome with a mortality rate close to 100%, and is associated with the uncontrolled multiplication of *C. perfringens* in the small intestine, with excessive production of toxins [29]. These toxins act both locally and systemically, and can cause death within minutes to hours [30].

Often, the problem of clostridial disease, as well as the development of mycotoxicoses, is associated with a failure to follow the technology of canned feed production, which results in secondary fermentation in the feed and proliferation of proteolytic bacteria, mainly clostridia. Cattle are most at risk during the grazing season, which means that disease control and prevention should be particularly targeted at this time of year [31].

As all vaccines against clostridial disease are inactivated, two doses must be administered to heifers. In practice, however, the vaccinations are often administered too late, according to expert observations, and they are not always successful. In addition, spore-forming clostridia are resistant to broad-spectrum antibiotics, so their uncontrolled use in livestock further increases the risk of clostridial infections [32].

One way of combating clostridial disease is to develop an integrated animal bioprotection function. The bioprotection function is based on a scientifically proven combination of sorbents and toxin degraders, with carefully selected biologically active substances to improve the functioning of the digestive system and restore the function of the immune system in animals [33].

Anaerobic infections are a major problem for animal production worldwide. The damage consists of losses, not only from animal deaths, but also from unfavorable conditions in areas contaminated with pathogen spores and losses from quarantine measures and restrictions [21]. The economic damage caused by reduced productivity can only be estimated approximately. Therefore, in addition to proper vaccination, additional means are needed to provide full protection against and prevention of clostridia, including the use of feed additives that neutralize not only the toxins, but also the clostridia themselves, and their spores [34].

Research on the efficacy of feed additives for the prevention of clostridia in animals is still insufficient. It is of interest to study approaches to feeding animals complexes of substances that neutralize clostridia and/or their toxins. A new complex feed additive for dairy cows, which combines the functions of a sorbent, an immunomodulator, and a neutralizer, has been developed. Considering the specific character of the question under study, we have set an objective to investigate, on the one hand, the background values of clostridia presence in a modern Russian farm with a high level of fodder production and cattle breeding. On the other hand, we intend to reveal the possibility of preventing and increasing the immune status of cows with high productivity using a targeted bioactive feed additive, which can play an important role in the formation of new approaches in the integrated control of clostridia and its sequelae.

2. Materials and Methods

2.1. Study Site

The research was carried out on the grounds of Avangard LLC, located in the Ryazan region, Russia; in the laboratories of the feeding department, physiology and biochemistry department, and microbiology laboratory of farm animals at L.K. Ernst Federal Research Center for Animal Husbandry (FRCAH), located in Podolsk, Russia; in the laboratory of molecular and genetic studies of Biotroph Ltd., located in St. Petersburg, Russia.

2.2. Characteristics of the Compound Feed Additive

2.2.1. Composition of the Feed Additive

Bioactive feed additive (BFA) contains a toxin neutralizer, digestion modifiers, and natural substances of antibacterial nature (diatomite, enzyme–probiotic preparation, etc.).

For the experiment, experimental batches of multifunctional BFA were prepared. Composition of the additive, in 100 g:

(1) enzyme–probiotic component
- *B. amyloliquefaciens*159—not less than 6×10^7 CFU/g–0.1 g;

(2) antibacterial component
- lauric acid—4.2 g;
- pine bark extract (aqueous)—8.3 g;
- essential oil—0.064 g;

(3) sorbent constituent
- diatomite—40 g;
- wheat bran (as a filler)—55.8 g.;

The strain *B. amyloliquefaciens*159 was obtained from the collection of BIOTROF LLC (St. Petersburg, Russia). The original strain was extracted from the chyme of cow rumen. It is not a genetically modified organism.

The taxonomic identity of strain *B. amyloliquefaciens*159 was previously confirmed by whole-genome sequencing using the MiSeq platform (Illumina, Inc., San Diego, CA, USA). Application has been made (SUB12907853; Taxonomy ID: 3029762; BioProject ID: PRJNA937299) to deposit the genomic sequence in the BioProjects collection (NCBI, https://www.ncbi.nlm.nih.gov/bioproject/ (accessed on 21 Febuary 2023)).

According to the data obtained using conventional methods [35–37] strain *B. amyloliquefaciens*159 has no properties of toxigenicity and virulence, cell components of the strain are not toxic to laboratory animals.

Strain *B. amyloliquefaciens*159 was cultured at 37 °C on GRM nutrient broth medium of the following composition (g/L): enzymatic peptone, pancreatic hydrolysate of fish meal, sodium chloride, pH = 7.2 ± 0.2. (Siana LLC, Izhevsk, Russia).

2.2.2. Component Properties of the Feed Additive

In the laboratory of molecular genetic studies of Biotroph Ltd.:

- Anticlostridial activity of *B. amyloliquefaciens*159 strains was determined. A daily culture of *B. amyloliquefaciens*159 strain at a concentration of 1×10^8 CFU/mL, (grown on GRM nutrient broth at 37 °C for 24 h) was seeded with one linear stroke (strip) on the surface of dense RCB and blood agar media (BIOMEDIA LLC, Russia) along the diameter of a Petri dish, using a sterile swab. The cultures were incubated at 37 °C for 24 h. *C. perfringens* indicator strains from the American Type Culture Collection (ATCC) [38,39] (13124, 10543, 12916) were seeded in tubes with thioglyco-left medium and incubated at 37 °C for 24 h under anaerobic conditions using Gaz Pak Anaerobe Container System (BD) (Thermo Fisher Scientific Inc., Waltham, USA). The results were evaluated according to GPhM. 1.7.2.0009.15 (Determination of the specific activity of probiotics) [40]. The size of the no-growth zone of the test strain was considered. *C. perfringens* belonged to different toxinotypes [41]. Strain *C. perfringens* 13124 be-

longs to type A, because it produces alpha toxin (CPA), and perfringolysin O (PfoA), *C. perfringens* 12916 (type F) synthesizes CPA, and enterotoxin (CPE), *C. perfringens* 10543 (type C) simultaneously produces 4 toxins, CPA, CPE, beta2 toxin (CPB2), Pfoa. This suggested a high level of virulence [30,42]. In addition, the antimicrobial activity of *B. mucilaginsous*159 to other bacterial species, such as *E. coli* K-12 F + Str. R (KS-507), *Salmonella typhimurium* LT2, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* TO (obtained from the National Bioresource Center All-Russian Collection of Industrial Microorganisms of the Kurchatov Institute (VKPM)), may also be associated with the occurrence of intestinal enteritis in animals [43,44]. Petri dishes with Mueller–Hinton agar medium (BIOLITE SPB, Russia) were pre-seeded with test strains. Then, holes with a diameter of 8 mm were made with sterile metal cylinders. 100 μL of daily culture of strain *B. amyloliquefaciens*159 at a concentration of 1×10^8 CFU/mL, (grown on nutrient broth of GRM at 37 °C for 24 h) was placed in culture wells. Sterile GRM-bouillon nutrient medium was used as a control. Petri dishes were cultured in an aerostat at 38 °C. The level of antimicrobial activity was assessed after 24 h of growth.

- The bactericidal properties of lauric acid, a component of BFA, were determined. Lauric acid was introduced in concentrations (0.31; 0.16; 0.07 g/L, control without LA) into RCB agar culture medium (MERCK MILLIPORE, USA) to study growth zones of reference *C. perfringens* strains from ATCC collection (13124, 10543, 12916). Incubated under anaerobic conditions for 48 h.
- The true sorption capacity of diatomite with respect to aflatoxins (sum), ochratoxin A, T-2 toxin, zearalenone, and deoxynivalenol was determined [45]. «True» sorption capacity of this BFA component was determined by the difference between adsorption and desorption of mycotoxins from solutions. «True» sorption capacity of the sorbent was calculated by the following formula:

$$C = A - D$$

where: C—true sorption capacity, %; A—adsorption of toxins in an acid medium simulating the pH level in the stomach/choke of animals %; D—desorption of toxins in the conditions that simulate the alkaline medium in the intestines of animals, %.

Adsorption meant the total amount of mycotoxins bound by the sorbent during the time the feed was in an acidic environment (pH = 2.5) simulating the conditions of animal stomach/intestine. To determine mycotoxin adsorption, a standard solution of mycotoxin was prepared with a concentration of 1000 μg/cm^3. An exact weigh of 5.0 μg of dry mycotoxin standard (Romer Labs Inc., Getzersdorf, Austria) was dissolved in 5 cm^3 of methanol or distilled water (in the case of deoxynivalenol). Diatomite was placed into the solution at a concentration of 0.02 g/mL. The sorption process was carried out for 1 h at 37 °C in the thermostat with constant stirring using a V-1 plus personal vortex (BIOSAN Ltd., Riga, Latvia). The resulting suspension was precipitated by centrifugation for 5 min at 10,000 rpm.

The supernatant and the initial solution were analyzed for mycotoxin content by competitive enzyme immunoassay using Agra Quant test systems (Romer Labs Inc., Austria) according to the manufacturer's recommendations. Optical density was measured at λ = 450 nm using a Stat Fax 303+ microstrip photometer (Awareness Technology, Inc., Palm City, FL, USA).

Adsorption was calculated using the formula:

$$A = 100 \times (A_{initial} - A_{residual})/A_{initial}$$

where

A—adsorption, %;

$A_{initial}$—value of mycotoxin concentration in the initial working solution, μg/cm^3;

$A_{residual}$—value of mycotoxin concentration in the working solution after the sorption process, μg/cm^3.

To determine desorption, the total amount of mycotoxin removed from the adsorbent surface was measured in an alkaline environment close in pH to the conditions in the ruminant intestine. For this purpose, the obtained suspension containing the adsorbent particles with mycotoxin bound to them was alkalized to pH = 7.0. The suspension was incubated in the thermostat for 1 h at 37 °C with constant stirring using a V-1 plus vortex (Biosan Ltd., Riga, Latvia). The obtained suspension was centrifuged for 5 min at 10,000 rpm.

The supernatant and the initial solution were analyzed for mycotoxin content by ELISA.

2.3. Animals and Housing

The object of the study were Holstein cows (importing country: Hungary). The total number of cattle in Avangard LLC at the time of the study was 1968 milking cows, 274 dry cows and 1467 calves.

For the given experiment, we selected 60 lactating Holstein cows of the second lactation with the average milk yield of 34.16 ± 0.45 kg, days in milking (DIM) = 39.5 ± 1.4 days, fat content = 3.53 ± 0.01% and protein content in milk = 3.05 ± 0.01%. Cows were previously ranked in descending order by DIM, milk yield, fat and protein content in milk, and allocated to control (C, n = 15) and experimental (E48, n = 15; E80, n = 15; E112, n = 15) groups to obtain 48, 80, 112 g/head/day BFA, respectively. This allocation procedure was adopted to ensure that all experimental groups had the same DIM, milk yield and milk parameters at d 0. Feeding of the supplement (specially prepared capsules) was carried out with cows of groups E48–E80–E112 daily in the morning hours after milking with an individual food probe.

Cows were kept in group stalls with unlimited access to water and total mixed ration (TMR; 1.5 m linear cow space per cow) during the whole period of the experiment. Cows were milked twice a day using a carousel system (05-00 and 16-00 h).

2.4. Experimental Diets of Cows

The diet for dairy cows met the energy and nutritional value requirements for high-yielding animals (32–36 kg milk yield) [46,47]. Calculation of feeding rations was carried out by using the software complex KormOptimaExpert (Version 2016, KormoResurs LLC, Voronezh, Russia) (Table 1).

The chemical composition of feeds was determined at the Department of Animal Physiology and Biochemistry of the L.K. Ernst Federal Research Center for Animal Husbandry [48]. Feeds were grounded at a Laboratory mill (LMC-1M, NV-Lab, Moscow, Russia) and then analyzed for dry matter (DM) and crude ash (CA) (ISO 6496-83 and ISO 5984, respectively). Initial moisture content was determined by drying the samples at 65 ± 2 °C to constant weight, and drying at 103 ± 2 °C for 3 h to constant sample weight for two successive readings that determined the hygroscopic moisture content of the samples. In dry samples, the raw ash content was determined by burning the sample in a muffle furnace at 550 °C. The nitrogen content was analyzed with ISO 5983-2-2016, from which crude protein (CP) content was calculated with formula (Nitrogen × 6.25), crude fiber with ISO 6865-2015, crude fat ISO 6492:1999, calcium (Ca) ISO 6490-1:1985, phosphorus (P) ISO 6491-2016; ME—calculation method [48,49].

The total duration of the study was 98 days. Cows were fed 48, 80, and 112 g/head/day of BFA during the whole experiment period.

Table 1. Composition of the diet [1].

Parameter	Composition
Ingredient (kg DM/head/day)	
Corn silage	7.57
Alfalfa silage	2.87
Grass silage	0.56
Hay	0.66
Promatrix +	1.25
Beet molasses	0.53
Concentrate mixture [2]	10.36
Calculated nutrients	
Energy (MJ/kg DM)	11.36
Analyzed nutrients	
DM (kg)	23.80
Crude protein (% DM)	16.72
Crude fiber (% DM)	18.28
Starch (% DM)	22.47
Sugar, (% DM)	5.65
Crude fat, (% DM)	3.88
Calcium, (% DM)	0.72
Phosphorus, (% DM)	0.54

[1] DM, crude protein, crude fiber, starch, sugar, crude fat, calcium, phosphorus are results of chemical analysis of feed samples. [2] Concentrate mixture contained per 1 kg: wheat 150 g, barley 200 g, corn 200 g, soy meal 150 g, sunflower meal 250 g, monocalcium phosphate 20 g, premix 10 g, salt 10 g. Premix contained per 1 kg: vitamin A 2,500,000 IU; vitamin D_3 300,000 IU; vitamin E 1500 IU; vitamin B5 (pantothenic acid) 500 mg; choline chloride 40,000 mg; magnesium (Mg) 150,000 mg, sulfur (S) 100,000 mg, iron (Fe) 500 mg, manganese (Mn) 6000 mg; zinc (Zn) 7000 mg, copper (Cu) 1800 mg; iodine (I) 180 mg, cobalt (Co) 150 mg, selenium (Se) 30 mg.

2.5. Milk Performance and Analyses in Milk

Analysis of milk productivity during the experiment and during lactation was conducted according to Dairy Plan C21 (Version 5.285.093). The average daily milk yield was calculated from the results of control milkings before the experiment (d 0), on days 30, 60, 98 within the main period of the experiment (d 30; d 60; d 98). Average daily milk samples on these days were taken from each cow (n = 60) and in them, on an infraspectrometric analyzer (in the Department of Population Genetics and Genetic Bases of Animal Breeding at the L.K. Ernst Federal Research Center for Animal Husbandry) CombiFoss 7 DCC (FOSS, Denmark), fat and protein were determined by standard methods (ISO 9622/IDF 141:2013; AOAC 972. 16). Individual plastic flasks containing bronpol as a preservative were used for milk sampling. Based on the results obtained, the mean values for each indicator were calculated for the animal group before the start of the experiment and at subsequent sampling control points.

2.6. Vaccination and Level of Specific Immunity

All cows were vaccinated with "Coglavax" (vaccine against bovine and sheep clostridial disease, Ceva-Phylaxia VeterinaryBiologicals, Hungary), reinjected two weeks before the experiment. This induces the formation of immune responses in cattle and sheep against alpha, beta, and epsilon anatoxins of *C. perfringens* types A, B, C, D, and others, from 2–3 weeks after reintroduction, which lasts for 10–12 months. Blood was drawn from animals of the experimental groups at the beginning of the experiment (n = 60, d0) and at the end of the experiment (n = 55, 5 animals dropped out of the experiment, d98). Blood

samples from the subcostal vein were collected in vacuum containers (Vacuette, Greiner bioone, Kremsmünster, Austria), with blood clotting activator. Blood samples were stored on ice immediately after extraction and centrifuged 2 h after delivery. All samples were centrifuged at 4 °C at 4500 rpm for 10 min (Tagler centrifuge machine, table model SM-12 with rotor RU-06, NV-LAB, Moscow, Russia). Thereafter, serum samples were stored at −20 °C until analysis. In the Department of Feeding of Farm Animals L.K. Ernst Federal Research Center for Animal Husbandry, the content of antibodies to *C. perfringens* beta toxin in the blood serum were studied. The studies were performed on an automatic microplate photometer Immunochem-2100 (High Technology Inc, North Attleboro, MA, USA) by solid-phase enzyme immunoassay, using a Monoscreen AbELISA *C. perfringens* beta toxin reagent kit (BIO K 317/2, Bio-X Diagnostics, Rochefort, Belgique). Blood samples without hemolysis were collected (n = 40 at the beginning of the experiment, n = 50 at the end of the experiment). We also used 2 additional serum samples from animals diagnosed with *C. difficile* disease as positive controls.

The degree of positivity of each sample was evaluated using a scale (Figure 1).

Calculated value	Degree of positivity
<20	0
20≤ % inh <40	X
40≤ % inh <60	XX
60≤ % inh <80	XXX
80≤ % inh	XXXX

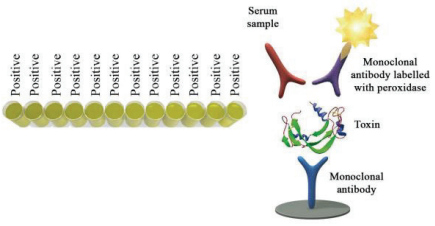


Figure 1. Levels for assessing serum sample positivity.

2.7. Molecular Genetic Studies to Detect Some Species of C. perfringens and Their Toxins in the Rumen Contents

Molecular and genetic studies were carried out by the laboratory of Biotroph Ltd. Samples of rumen contents sampled at the end of the experiment (n = 12, 3 animals from each group) and dietary feed (soybean meal, corn grains, DDGS, straw, corn silage; alfalfa haylage) were tested for the presence of toxin genes produced by microorganism *C. perfringens*: alpha-toxins 1 and 2 (*Cpa*); beta-toxin (*cpb*) and epsilon-toxin (*ext*). The analysis was performed using the PCR method. DNA was isolated using standard methods using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., Waltham, USA) according to the attached instructions. The following specific primers (5′→3′) were chosen for analysis: for the alpha-toxin *Cpa* gene, F: GCTAATGTTACTGCCGCCGTTGA, R: CCTCTGATACATCGTGTAAGAATC, for the *cpb* beta-toxin gene—F: GCGAATGCTGAATCATCTA, R: GCAGGAACATTAGTATATCTTC, for the *etx* epsilon-toxin gene—F: GCGGTGATATCCATCTATTC, R: CCACTTACTTGTCCTACTAAC.

Amplification reactions were performed using a DTLight amplifier (DNA-Technology, Moscow, Russia) and the Tersus Plus PCR kit (Eurogen, Moscow, Russia) according to the manufacturer's protocol. Mode and conditions of amplification were as follows: 3 min at 94 °C (prewarming); 40 s at 94 °C, 60 s at 55 °C, 90 s at 72 °C (34 cycles).

2.8. Bacterial Strains and Culture Conditions

In the Laboratory of Microbiology of L.K. Ernst Federal Research Center for Animal Husbandry, in selected blood samples, by conventional methods, the indices of nonspecific resistance of experimental animals were determined: bactericidal, lysozyme activity of serum, and phagocytic activity of blood cells.

Strains *E. coli* ATCC 25922 and *M. luteus* (*lysodeicticus*) ATCC 4698 were obtained from Federal Budget Institution of Science «State Research Center for Applied Microbiology & Biotechnology». These bacteria were cultured in Tryptic Soy Agar (TSA) (Merck, Rahway,

Germany) at 37 °C for 24 h. The cultures were suspended in phosphate-buffered saline and adjusted by Densi-La-Meret (PLIVA-Lachema Diagnostika, Brno, Czech Republic) for phagocytosis assay to 4.5 McF (*E. coli*), for bactericidal activity assay to 1.9 McF (*E. coli*), for lysozyme activity assay to 0.6 McF (*M. luteus*), and used within 15 min.

2.8.1. Phagocytosis Assay

E. coli culture (0.5 mL) was added to 0.5 mL of blood and incubated on shaker at 37 °C for 30 min. The sediment of mixture was smeared, fixed with 96% methanol, stained with Romanowsky–Giemsa method, and viewed under microscope (90×). *E. coli*-engulfed neutrophils were counted as positive cells. We analyzed 100 neutrophils per slide. The following parameters were determined:

Phagocytic activity (PA) = (Number of neutrophils involved in phagocytosis/all neutrophils) × 100%;

Phagocytic index (PI) = Number of *E. coli* cells ingested/100 active neutrophils;

Phagocytic amount (PAM) = Number of phagocytosed bacteria cells/all neutrophils.

2.8.2. Lysozyme Activity Assay

Lysozyme was measured by turbidimetric method in a spectrophotometer USAMultiskan FC (ThermoFisher Scientific Inc., Vantaa, Finland). Tubes of blood serum (0.1 mL) were heated (56 °C) in a water bath for 30 min, then 1.4 mL of standard *M. luteus* culture was added and incubated at 37 °C for 3 h. The following parameters were determined: lysozyme activity of blood serum (LA), concentration of serum lysozyme (lysozyme, μg/mL), activity unit (AU) per 1 mg protein (AU/TP).

Lysozyme activity (LA) of a blood serum is calculated using the following formula:

$$\%LA = ((\Delta Do) \times 100/Do1) - ((\Delta Dk) \times 100/Dk1)$$

Do is the difference in the optical density of the prototype,
Dk is the difference in the optical density of the control,
Do1 is the optical density of the prototype immediately,
Dk1 is the optical density of the control.

The concentration of lysozyme in serum was calculated based on calibration with dilutions of chicken egg-white lysozyme (L6876, Sigma-Aldrich, St. Louis, MO, USA) ranging from 0.1 to 51.2 μg/mL.

Due to variations in protein content in the blood serum of animals, the level of lysozyme activity was converted and expressed in arbitrary units of activity per 1 mg of protein (activity units per 1 mg of TP or AU/TP).

2.8.3. Bactericidal Activity of Blood Serum

Bactericidal activity (BA) of blood serum was measured by turbidimetric method in a spectrophotometer UNICO-2100 (United products & instruments, Ins., Suite E Dayton, NJ, USA) at OD540. *E. coli* culture (0.005 mL) was mixed with 4.5 mL of Tryptic Soy Broth (TSB) (Merck, Rahway, Germany) and 0.5 mL of blood serum in sterile tubes. Control was 0.5 mL of physiological saline with phosphate buffer instead of serum. All tubes were cultured at 37 °C for 5 h.

Percentage of BA was calculated from the following formula:

$$\%BA = ((Dk - Do)/Dk)) \times 100$$

Dk is optical density of control;
Do is optical density of experimental sample.

2.9. Blood Sampling and Analyses

Blood samples from the jugular vein were collected at the start (d 0) and at the end (d 98) of the main experimental BFA-feeding period at 09-00 h. Blood was collected in vacuum containers (Vacuette, Greiner bio-one, Kremsmünster, Austria), with clotting activator. At the same time, blood was drawn from each cow into vacuum containers (Vacuette, Greiner bio-one, Kremsmünster, Austria) with K3 EDTA. Blood samples were delivered to the Department of Physiology and Biochemistry of Farm Animals of L.K. Ernst Federal Research Center for Animal Husbandry within 2 h. To determine erythrocytes (RDC), leukocytes (WBC), hemoglobin (HGB), and hematocrit (HCT), a hematological analyzer ABC VET analyzer (Horiba ABZ, France) with Uni-Gem reagent kits (ReaMed, Russia) was used. To obtain serum, the samples were centrifuged (3000 rpm) for 20 min, with further storage of the separated serum at −20 °C until analysis. An automatic biochemical analyzer Erba Mannheim automatic XL-640 (Lachema s.r.o., Brno, Czech Republic) was used for analysis with determination of: AST, ALT by UV kinetic method without pyridoxal phosphate, IFCC; ALP—IFCC AM buffer; TP—biuret method; ALB—BKZ method; CREA—Jaffe kinetic method; UREA—Uricase GLDG, kin. method; TBIL—quantification by Walters and Gerarde method; electrolytes: Ca—ARSENAZO III method, P—Ammonium molybdate, Mg—Xylidine blue.

To assess the antioxidant status (AOS), the total amount of water-soluble antioxidants (TAWSA) in blood serum of experimental animals was determined by amperometric method (chromatograph, TsvetYauza 01-AA, SPA Himavtomatika PLC, Moscow, Russia) in samples. The concentrations of TBK-active products were determined using "TBK-Agat" kits (Agat-Med, Russia), and ceruloplasmin (CP) activity was determined using the Revin method. To characterize the prooxidant–antioxidant status of cows, the ratio of TBK-AP to ceruloplasmin (CP) levels was calculated.

2.10. Statistical Analyses

Statistical analyses of the data were performed with STATISTICA software (version 13RU, StatSoft, Inc., 2011; www.statsoft.com (accessed on 27 December 2022)) by using a general linear model. Each group was considered as an experimental unit in measuring milk performance. For analyzing blood characteristic, the experimental units were individual animals. The relationship between the factor and the parameters was revealed using animal sampling, single-factor and two-factor analysis of variance (ANOVA), and Dunnett's test, with testing of individual measures by Tukey's multiple comparison method. Statistical differences were considered highly significant at $p < 0.01$, significant at $p < 0.05$ and values between $p \geq 0.05$ and $p \leq 0.1$.

2.11. Ethical Approval

Ethical approval for the study was provided by the bioethical commission of the L.K. Ernst Federal Research Center for Animal Husbandry (protocol #2022-02/1, dated 14 February 2022).

3. Results

3.1. Characteristics and Properties of the Components of the Bioactive Feed Additive (BFA)

The bacterial strain *B. amyloliquefaciens*159 was found to have pronounced antagonism to toxin-producing strains of *C. perfringens*. In a study of indicator strains of *C. perfringens* from the ATCC collection (13124, 10543, 12916), the anti-clostridial activity of strain *B. amyloliquefaciens*159 varied, with growth retention zones ranging in size from to 14.0 ± 0.95 to 15.0 ± 1.28 mm, at an incubation temperature of 37 °C and exposure for 48 h. These data characterize the strain as a "strain with high antagonistic activity" (Figure 2, Table 2).

(**A**) (**B**) (**C**)

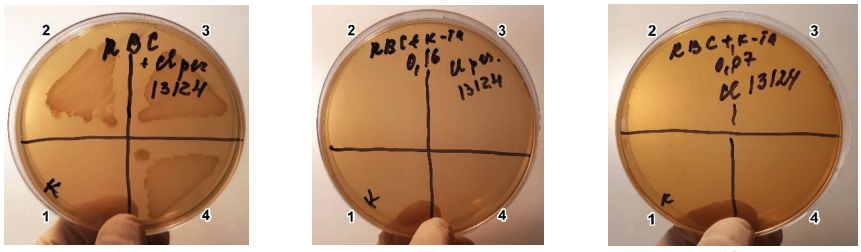
Figure 2. Antagonism of *B. amyloliquefaciens*159 strain against toxin-producing strains in in vitro experiments (**A**) *C. perfringens* 10543, (**B**) *C. perfringens* 12916, (**C**) *C. perfringens* 13124, 1—growth zone of test microorganism, 2—growth inhibition zone of test microorganism, 3—growth zone of *B. amyloliquefaciens*159.

Table 2. Inhibition zones of test cultures influenced by strain *B. amyloliquefaciens*159 in in vitro experiments.

Name of Test Culture	Length of Oppression Zone of Test Cultures, mm (n = 5, M ± SEM)
C. perfringens® ATCC 13124	14.0 ± 0.95
C. perfringens® ATCC 10543	15.0 ± 1.10
C. perfringens® ATCC 12916	15.0 ± 1.28
E. coli K-12 F + Str.R (KS-507)	15.5 ± 1.56
Salmonella typhimurium LT2	8.3 ± 0.61
Staphylococcus aureus ATCC 29213	22.0 ± 2.29
Pseudomonas aeruginosa TO	24.1 ± 1.64

The sensitivity of the bacterial strains *E. coli* K-12 F + Str.R (KS-507), *Salmonella typhimurium* LT2, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* TO against *B. mucilaginsous* 159. The growth inhibition zones of these strains ranged from 8.3 ± 0.61 to 24.1 ± 1.64 mm.

The bactericidal properties of lauric acid in BFA were determined. The addition of lauric acid at concentrations (0.31; 0.16; 0.07 g/L) to RCB media completely suppressed the growth of three *C. perfringens* reference strains from the ATCC collection (13124, 10543, 12916) (Figure 3, Table 3).



(**A**) (**B**) (**C**)

Figure 3. Bactericidal properties of lauric acid against the toxin-producing strain *C. perfringens* 13124 in in vitro experiments ((**A**)—control without LA: 1—sector without test-strain (for control of medium sterility), 2, 3, 4—sectors inoculated with test-strain; (**B**)—test with addition of LA at 0.16 g/L; (**C**)—test with addition of LA at 0.07 g/L: 1—sector not seeded with the test strain, 2, 3, 4—sectors seeded with the test strain (no growth of the test strain)).

Table 3. Growth suppression of *C. perfringens* strains by lauric acid at different concentrations.

Daily Culture	Concentration of Lauric Acid, g/L			Control [a]
	0.31	0.16	0.07	
C. perfringens® ATCC 13124	No growth	No growth	No growth	No growth
C. perfringens® ATCC 10543	No growth	No growth	No growth	No growth
C. perfringens® ATCC 12916	No growth	No growth	No growth	No growth

[a] As a control for the sterility of the nutrient medium, one sector on each Petri dish was left unseeded.

The study of in vitro sorption properties of the studied sorbent—diatomite, as one of the important components of BFA—showed that the true sorption capacity when added to the medium in a concentration of 0.14 g/L was: aflatoxins (sum)—95%, ochratoxin A—30.0%, T-2 toxin—86.3%, zearalenone—36.1%, deoxynivalenol—25.3%.

3.2. Molecular Genetic Studies of Feed and Rumen Contents of Cows

A PCR study detected the presence of the alpha-toxin gene (*Cpa*) in rumen fluid samples of cows of Group C (No. 1509) and Group E48 (No. 1547; 1118), as well as in feed samples: maize, barley, soya and draff. The presence of the Beta-toxin gene (*cpb*) was also detected in rumen fluid sample No. 1465 (Group E80) and maize. The epsilon-toxin gene (*etx*) was also present in the maize sample (Figure 4).

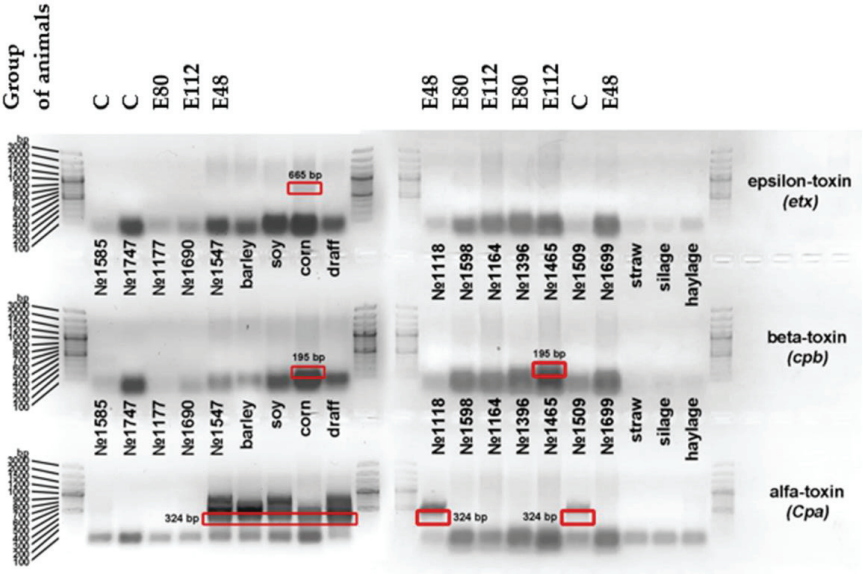
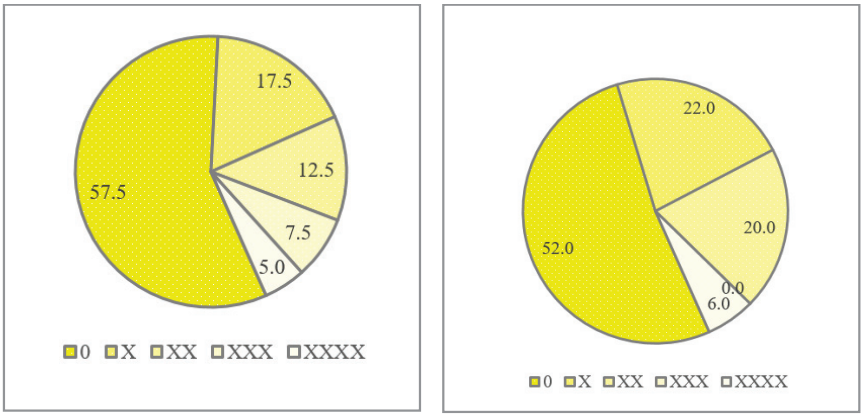


Figure 4. Agarose gel electrophoresis of alpha, beta and epsilon amplicons of *C. perfringens* toxin genes detected by polymerase chain reaction in rumen contents and feed ingredients (barley, corn, draff) of lactating cows (Avangard LLC, Russia): C—control group of cows, E48, E80, E112—experimental groups of cows, the symbol "№" and a four-digit number indicates the No.

3.3. Level of Serological Response

The level of serological response increased slightly after three months of experiment in the studied animals as a whole. At the beginning of the experiment, a positive serological response was observed in 42.5% of animals (categories X, XX, XXX, XXXX), at the end of the experiment the level was 48% of the examined samples (Figure 5).



(**A**) (**B**)

Figure 5. Level of serological response of animals to *C. perfringens* β-toxin at the beginning ((**A**), *n* = 40) and at the end ((**B**), *n* = 50) of the experiment (% inh by *C. perfringens* β-toxin).

It was noted that, in the animals (2 animals) diagnosed with clostridiosis, an immune response was identified at the level of <20 (positivity level "0").

Analysis of the data by animal group at the beginning of the test showed that the response distribution was almost equal, except for serum samples from Group E112 animals, where the response was the lowest (80% of the samples at the positive "0" level) Figure 6).

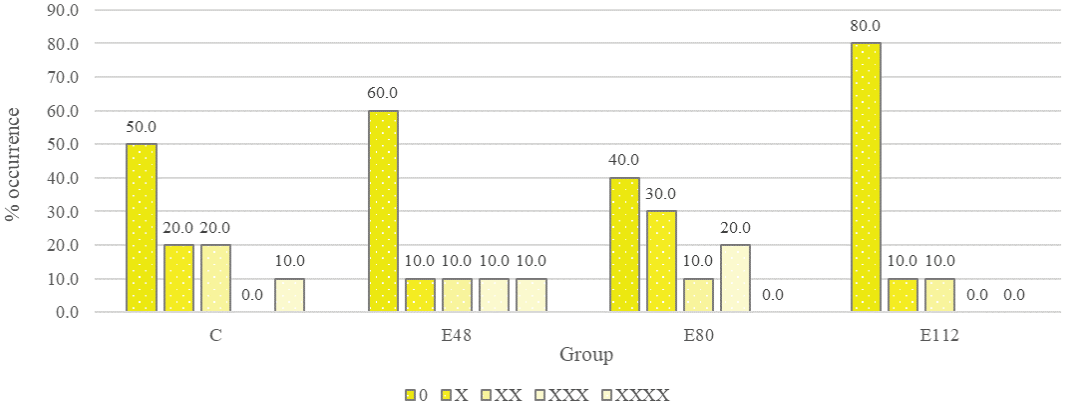


Figure 6. Distribution of samples by response rate and level of occurrence at the beginning of the experiment (*n* = 10).

At the end of the experiment (3.5 months after vaccination and 3 months after the start of BFA feeding according to the scheme of the experiment), the immune response in the control and Group E48 remained at the same level as at the beginning of the experiment, while the response in Group E80 and E112 was improved by feeding BFA (Figure 7).

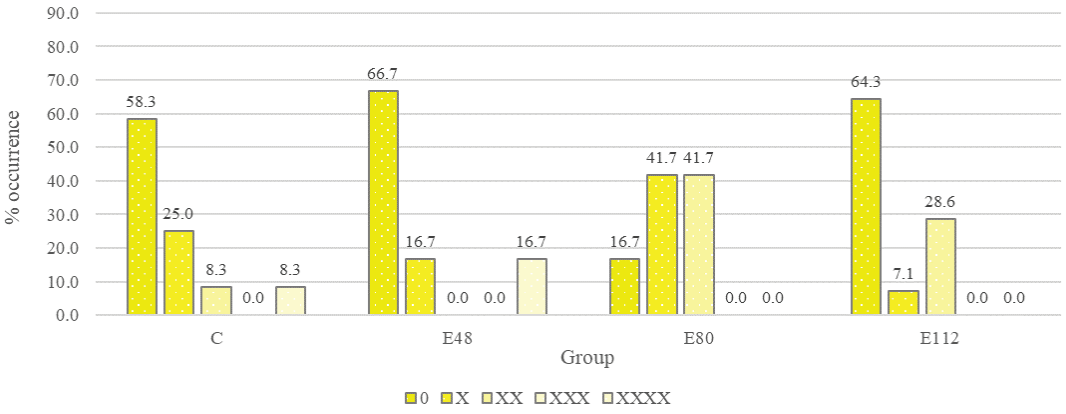


Figure 7. Distribution of levels of positive samples by level of occurrence at the end of the experiment (n = 10–14).

3.4. Level of Non-Specific Resistance

The study of nonspecific resistance of experimental animals in the control and experimental groups at the end of the experiment revealed significant inter-group differences (at the beginning of the experiment, the differences in the studied parameters between the groups were not significant) (Table 4). Feeding on BFA increased the serum lysozyme concentration of experimental cows by 1.01–2.91 mkg/mL compared to the control ($p < 0.001$).

Table 4. Indicators of nonspecific resistance of experimental animals [1].

Parameter	Group [2]				SEM	*p*-Value
	C	E48	E80	E112		
at the start of the experiment (d0)						
LA, %	33.47 ± 9.00	25.70 ± 6.95	42.65 ± 11.40	29.47 ± 10.94	379.03	0.57
Lysozyme, mkg/mL	0.93 ± 0.21	0.69 ± 0.20	0.75 ± 0.28	0.76 ± 0.07	0.16	0.80
AU/TP	1.33 ± 0.74	0.79 ± 0.21	2.53 ± 1.50	1.34 ± 0.98	3.80	0.56
BA, %	54.29 ± 4.41	52.17 ± 2.21	37.41 ± 11.94	38.81 ± 12.02	311.29	0.33
PA, %	36.40 ± 0.57	40.80 ± 2.79	40.20 ± 2.16	43.00 ± 3.82	27.43	0.29
PI	3.71 ± 0.12	4.07 ± 0.40	3.43 ± 0.17	3.60 ± 0.41	0.37	0.42
PAM	1.35 ± 0.06	1.65 ± 0.14	1.38 ± 0.11	1.51 ± 0.08	0.04	0.13
at the end of the experiment (d98)						
LA, %	40.49 ± 4.55	38.62 ± 7.89	41.85 ± 8.41	35.85 ± 2.92	162.24	0.89
Lysozyme, mkg/mL	1.11 ± 0.42 ^{c}	2.12 ± 0.57 ^{c}	2.62 ± 0.39	4.02 ± 0.36 ^{ab}	0.77	0.001
AU/TP	1.85 ± 0.45	1.25 ± 0.33	1.51 ± 0.42	0.93 ± 0.11	0.51	0.25
BA, %	26.95 ± 3.27	31.62 ± 5.29	33.80 ± 3.41	37.61 ± 5.23	77.65	0.32
PA, %	45.20 ± 1.19	50.40 ± 2.02	49.40 ± 1.96	48.80 ± 1.52	8.88	0.07
PI	3.45 ± 0.37 ^{c}	2.30 ± 0.53 ^{ac}	3.15 ± 0.26	3.43 ± 0.09 ^{b}	0.29	0.01
PAM	1.55 ± 0.14	1.15 ± 0.08 ^{c}	1.56 ± 0.17	1.68 ± 0.09 ^{b}	0.07	0.03

[1] Means (±standard error) within a row (overall) followed by different superscript are significantly different, general linear model (GLM), $p < 0.05$, least significant difference test. [2] C = 0 g, E48 = 48 g, E80 = 80 g, E112 = 112 g, levels of supplement BFA. Significant to Group: C—^{a}, to E48—^{b}, to E112—^{c}, at $p < 0.05$, according to Tukey's test. For each group, n = 5.

3.5. Blood Parameters of Experimental Animals

At the beginning of the experiment, some blood values were outside the physiologically adequate reference limits (Table 5). Specifically, TP, GLB, AST, and WBC were above the upper limits, while A/G, ALB, Ca/P, and HGB were below the optimum.

Table 5. Biochemical and morphological parameters of the blood at the start of the experiment (d0) [1].

Parameter	Group [2]				Physiological Value [3]	*p*-Value
	C	E48	E80	E112		
TP (g/L)	85.28 ± 2.42	93.66 ± 5.00	90.34 ± 5.29	87.94 ± 3.25	72–86	0.46
ALB (g/L)	21.76 ± 1.21	19.42 ± 2.36	20.56 ± 0.93	20.00 ± 1.55	25–36	0.70
GLB (g/L)	63.52 ± 2.36	74.24 ± 4.58	69.78 ± 5.60	67.94 ± 3.75	40–64	0.29
ALB/GLB	0.34 ± 0.02	0.27 ± 0.11	0.30 ± 0.03	0.30 ± 0.04	0.6–1.0	0.39
UREA (mmol/L)	4.29 ± 0.38	3.19 ± 0.21	3.08 ± 0.40	2.92 ± 0.31	2.35–7.06	0.02
CREA (mmol/L)	83.93 ± 4.47	77.15 ± 4.33	82.87 ± 6.63	74.18 ± 6.44	63–162	0.48
ALT (IE/L)	19.98 ± 0.72	29.64 ± 10.25	15.20 ± 1.12	15.16 ± 2.94	12–35	0.15
AST (IE/L)	115.28 ± 15.92	144.56 ± 15.42	85.06 ± 14.28	81.44 ± 13.05	46–108	0.012
ALP (mmol/L)	62.0 ± 4.57	63.00 ± 12.13	74.20 ± 12.17	57.20 ± 4.20	41–187	0.53
CHOL (mmol/L)	3.63 ± 0.62	2.99 ± 1.08	2.94 ± 0.69	3.25 ± 1.03	2.35–8.30	0.92
TBIL (µmol/L)	6.25 ± 2.71	4.42 ± 0.89	3.20 ± 0.84	2.05 ± 0.42	1.2–8.2	0.19
GLU (mmol/L)	3.53 ± 0.58	4.29 ± 0.28	3.95 ± 0.23	3.69 ± 0.23	1.65–4.19	0.40
CHL (mmol/L)	98.20 ± 3.53	101.32 ± 1.31	103.14 ± 0.74	101.68 ± 0.40	90–110	0.27
Ca (mmol/L)	2.46 ± 0.06	2.44 ± 0.04	2.54 ± 0.06	2.49 ± 0.06	2.03–3.14	0.53
P (mmol/L)	1.99 ± 0.22	2.09 ± 0.17	2.15 ± 0.13	1.99 ± 0.08	1.13–2.90	0.81
Ca/P	1.08 ± 0.13	1.14 ± 0.08	1.13 ± 0.09	1.06 ± 0.04	1.4–2.32	0.87
Mg (mmol/L)	0.93 ± 0.12	0.63 ± 0.06	0.90 ± 0.06	0.99 ± 0.16	0.79–1.35	0.08
Fe (µmol/L)	20.92 ± 4.23	18.04 ± 4.57	18.51 ± 2.79	21.35 ± 2.75	12.96–34.14	0.85
WBC (10^9/L)	13.79 ± 1.27	19.83 ± 3.54	12.17 ± 0.80	22.76 ± 1.59	5.3–16.6	0.003
RBC (10^{12}/L)	8.86 ± 0.91	8.15 ± 0.56	7.70 ± 0.46	8.10 ± 0.46	5.2–8.2	0.46
HGB (g/L)	81.38 ± 7.00	77.72 ± 3.43	70.24 ± 2.75	74.22 ± 4.93	84–122	0.26
HCT (%)	41.43 ± 3.69	39.83 ± 1.75	35.98 ± 1.34	38.09 ± 2.31	23.2–34.2	0.28

[1] Means (±standard error) within a row (overall) followed by different superscript are significantly different, general linear model (GLM), $p < 0.05$, least significant difference test. [2] C = 0 g, E48 = 48 g, E80 = 80 g, E112 = 112 g, levels of supplement BFA. For each group, $n = 5$. Marked: —below normal, —above normal. [3] According to [50].

Blood sampling after 3 months showed that there were some changes under the influence of lactation (Table 6). ALB, AST, Ca/P, and WBC returned to normal, which can be considered positive, but not related to the factor we studied (BFA).

Blood parameters such as TP, GLB, ALB/GLB stabilized and normalized in Groups E80 and E112 animals under the influence of BFA feeding vs. Groups C and E48 ($p < 0.001$). By the end of the experiment, the animals of Groups E80 and E112 had improved nitrogen, fat, and mineral metabolism, as evidenced by the significant increases in ALB ($p < 0.05$), UREA ($p < 0.01$), CHOL ($p < 0.01$), CHL ($p < 0.01$) vs. Groups C and E48.

Using the amperometric detection method, the serum antioxidant content of the experimental animals was determined at the end of the experiment (Table 7). The lowest value for total water-soluble antioxidants (TAWSA) was found in the serum of control cows, 10.19 mg/g; the highest TAWSA values were in Group E80, 14.45 mg/g ($p = 0.002$).

The TBA–AP/CP ratio in cow serum was directly related to TBA–AP content (r = 0.87, $p < 0.001$) and decreased in Group E80 animals.

Table 6. Biochemical and morphological parameters of the blood at the end of the experiment (d98) [1].

Parameter	Group [2]				Physiological Value [3]	*p*-Value
	C	E48	E80	E112		
TP (g/L)	98.40 ± 3.45	97.00 ± 2.56	86.42 ± 1.37	82.96 ± 1.68	72–86	0.0002
ALB (g/L)	32.12 ± 0.99	30.46 ± 1.67	33.62 ± 0.62	34.36 ± 0.55	25–36	0.045
GLB (g/L)	66.28 ± 3.02	66.54 ± 3.79	52.80 ± 1.04	48.60 ± 2.14	40–64	0.00008
ALB/GLB	0.49 ± 0.02	0.47 ± 0.05	0.64 ± 0.01	0.71 ± 0.04	0.6–1.0	0.0002
UREA (mmol/L)	2.58 ± 0.50	2.30 ± 0.16	4.21 ± 0.66	4.94 ± 0.59	2.35–7.06	0.002
CREA (mmol/L)	75.07 ± 3.37	67.81 ± 4.62	68.85 ± 1.45	66.99 ± 1.82	63–162	0.19
ALT (IE/L)	18.94 ± 4.18	13.60 ± 2.97	29.92 ± 2.27	29.84 ± 2.85	12–35	0.0014
AST (IE/L)	80.10 ± 3.58	70.80 ± 9.18	86.84 ± 9.78	85.36 ± 4.22	46–108	0.32
ALP (mmol/L)	68.80 ± 4.77	73.60 ± 9.09	56.00 ± 6.35	66.20 ± 4.51	41–187	0.22
CHOL (mmol/L)	3.23 ± 0.54	2.48 ± 0.56	5.18 ± 1.15	6.12 ± 0.74	2.35–8.30	0.007
TBIL (µmol/L)	1.49 ± 0.46	1.16 ± 0.03	0.91 ± 0.09	1.12 ± 0.03	1.2–8.2	0.31
GLU (mmol/L)	1.23 ± 0.18	1.87 ± 0.22	1.68 ± 0.21	1.76 ± 0.29	1.65–4.19	0.18
CHL (mmol/L)	101.66 ± 0.65	103.28 ± 0.77	104.78 ± 0.80	105.76 ± 0.93	90–110	0.005
Ca (mmol/L)	2.66 ± 0.14	2.44 ± 0.04	2.66 ± 0.04	2.58 ± 0.02	2.03–3.14	0.12
P (mmol/L)	2.36 ± 0.14	2.59 ± 0.24	2.44 ± 0.06	2.26 ± 0.09	1.13–2.90	0.37
Ca/P	1.51 ± 0.13	1.28 ± 0.12	1.45 ± 0.05	1.52 ± 0.05	1.4–2.32	0.21
Mg (mmol/L)	1.10 ± 0.18	0.89 ± 0.08	1.09 ± 0.06	1.19 ± 0.07	0.79–1.35	0.22
Fe (µmol/L)	20.18 ± 5.79	13.83 ± 4.39	22.79 ± 2.57	20.84 ± 5.19	12.96–34.14	0.48
WBC (10^9/L)	15.65 ± 2.49	11.51 ± 1.50	11.09 ± 1.20	10.42 ± 0.60	5.3–16.6	0.10
RBC (10^{12}/L)	4.00 ± 0.25	7.63 ± 0.43	8.13 ± 0.42	8.66 ± 0.19	5.2–8.2	0.32
HGB (g/L)	75.22 ± 3.91	66.88 ± 1.89	73.46 ± 3.69	79.43 ± 3.12	84–122	0.07
HCT (%)	18.61 ± 1.31	35.61 ± 1.16	38.89 ± 2.07	41.59 ± 1.45	23.2–34.2	0.09

[1] Means (±standard error) within a row (overall) followed by different superscript are significantly different, general linear model (GLM), $p < 0.05$, least significant difference test. [2] C = 0 g, E48 = 48 g, E80 = 80 g, E112 = 112 g, levels of supplement BFA. For each group, $n = 5$. Marked: —below normal, —above normal. [3] According to [50].

Table 7. Antioxidant blood parameters at the end of the experiment (d98) [1].

Parameter	Group [2]				*p*-Value
	C	E48	E80	E112	
TAWSA (mg/g)	10.19 ± 0.78 ^{c}	10.73 ± 0.77 ^{c}	14.45 ± 0.78 ^{abd}	11.38 ± 0.72 ^{c}	0.002
CP (mg/L)	280.60 ± 24.43	293.80 ± 25.22	265.60 ± 3.62	283.60 ± 10.87	0.69
TBA–AP (µmol/L)	2.11 ± 0.19	1.48 ± 0.12 †	1.81 ± 0.13	1.89 ± 0.29	0.05
TBA–AP/CP	0.008 ± 0.0004 ^{b}	0.005 ± 0.00 ^{acd}	0.007 ± 0.001 ^{b}	0.007 ± 0.001 ^{b}	0.011

[1] Means (± standard error) within a row (overall) followed by different superscript are significantly different, general linear model (GLM), $p < 0.05$, least significant difference test. [2] C = 0 g, E48 = 48 g, E80 = 80 g, E112 = 112 g, levels of supplement BFA. Significant to Group: C—^{a}, to E48—^{b}, to E80—^{c}, to E112—^{d}, at $p < 0.05$, according to Tukey's test. † is a trend towards significance. For each group, $n = 5$.

3.6. Milk Productivity and Milk Content of Dairy Cows

The average daily milk yield of the cows in the experimental groups of cows (d0–d98) was on average 1.24–1.66 kg higher than that of the control. At the same time, Group E112 cows had a significant increase in milk yield (by 5.1%, $p = 0.03$ vs. Group C) (Figure 8).

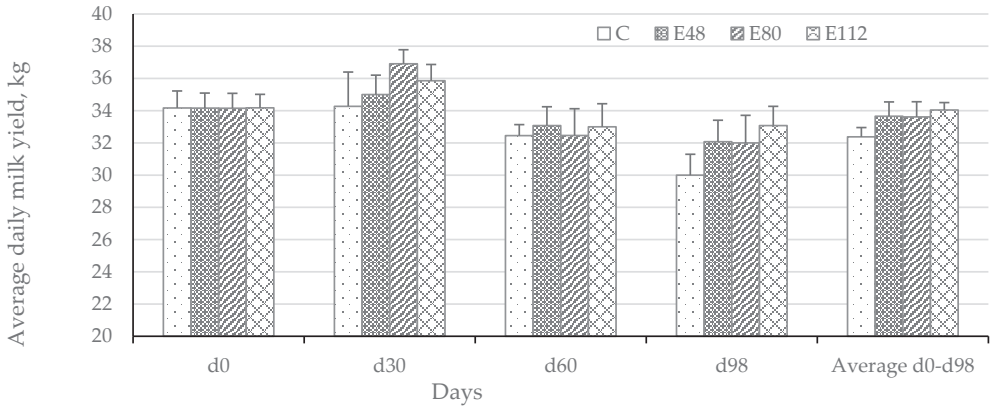


Figure 8. Milk yield of cows (n = 12) of natural fat, kg.

No significant differences in fat and protein content were found in studies of milk composition and quality (Table 8).

Table 8. Dairy productivity and milk quality parameters during the experiment (average per head).

Parameter [1]	Group [2]				*p*-Value
	C	E48	E80	E112	
Total milk yield of natural fat for the period, kg	3172.8 ± 56.7	3297.9 ± 87.4	3294.2 ± 92.2	3335.8 ± 45.4 *	0.41
% fat	3.58 ± 0.02	3.57 ± 0.02	3.54 ± 0.02	3.56 ± 0.01	0.46
% protein	3.06 ± 0.01	3.06 ± 0.01	3.05 ± 0.01	3.05 ± 0.01	0.41
Total milk yield of 3.4% of milk, kg	3334.5 ± 51.8	3459.1 ± 90.5	3428.2 ± 85.8	3488.4 ± 38.1 *	0.41
Average daily yield of 3.4% milk, kg	34.04 ± 0.53	35.30 ± 0.92	34.98 ± 0.88	35.60 ± 0.39 *	0.4
% of control	100	103.74	102.81	104.62	-
Total fat yield, kg	113.4 ± 1.8	117.6 ± 3.1	116.6 ± 2.9	118.6 ± 1.3 *	0.42
Total protein yield, %	97.1 ± 1.7	100.8 ± 2.7	100.4 ± 2.7	101.9 ± 1.3 *	0.98

[1] Means (± standard error) within a row (overall) followed by different superscript are significantly different, general linear model (GLM), $p < 0.05$, least significant difference test. [2] C = 0 g, E48 = 48 g, E80 = 80 g, E112 = 112 g, levels of supplement BFA. *—$p < 0.05$ vs. control, according to Dunnett's test. For each diet, n = 45 (3 taking × 15 head).

The effect of BFA feeding was already evident after a 4-week feeding period. Converted to 3.4% fat milk showed a difference in milk yield of 2.8–4.6% (p = 0.02 Group E112 vs. Group C), while fat content in milk of cows from experimental groups was 3.54–3.57 vs. 3.58% ($p > 0.05$) in control. The slight decrease in milk fat content is explained by the increase in gross milk production.

4. Discussion

In today's livestock farming conditions, vaccination is a reliable way to control clostridium disease [51]. A vaccine helps the body's immune system identify and eradicate life-threatening pathogens such as *Clostridium*. However, due to the widespread distribution of pathogens in the environment, the acute or hyperacute course of the disease, and the severity of tissue damage in the body, treatment of animals is almost 100% ineffective compared to specific prophylaxis [26]. Earlier research [26] had shown that clostridiosis in productive cattle mainly occurs in the form of malignant oedema, with the following prevalence: *C. septicum*—found in 34.5% of cases, *C. perfringens* type A—23.25%, *C. perfringens* type C—14.25%, *C. perfringens* type D—6.5%, *C. oedematiens*—2.5%, *C. sordellii*—6.5%. According

to [52] *C. perfringens* type A (56.66%), type D (26.66%) and type B (16.66%) were, respectively, the most common bacteria in gut samples from 30 cattle and calves with enterotoxemia. On the other hand, there is evidence that dairy cows are not a major source of the potentially pathogenic enterotoxin gene for humans [53]. The type diversity of *C. perfringens* was low because primarily type A was cultivated. The enterotoxin gene was present in only 0.8% of all isolates. Nevertheless, it poses a serious risk and causes considerable economic damage to the industry [34].

C. perfringens causes several diseases in animals and humans, including histotoxic and intestinal infections. *C. perfringens* toxins can be divided into major, minor and enterotoxins. Strains of *C. perfringens* are classified into five toxinotypes (A, B, C, D and E) based on the production of four major toxins. Chromosome-encoded CP toxins type A (CPA) are causative agents of histotoxic infection in humans and animals, and are the leading pathogenetic factors in the development of gas gangrene. The structural genes encoding these toxins, *cpa* (or *plc*), are located on the chromosome. CPA activates the extracellular signal-regulated kinase pathway, and thereby induces oxidative stress in the affected cells and the production of interleukin-8 by stimulating the action of kinase and mitogene-activated protein kinase. Beta-toxin SR (CPB), a pore-forming toxin, has a similarity to the amino acid sequence of the pore-forming toxins of *Staphylococcus aureus* in 20–28% of strains. This toxin is extremely sensitive to trypsin. *In vivo*, CPB causes necrotizing enteritis in sheep, cattle and horses [28]. Epsilon-toxin CP (ETX) is considered to be the most potent clostridial pore-forming toxin after botulinum and tetanus toxins, and is the cause of necrotizing enteritis and enterotoxemia in sheep, cattle and horses. Thus, the role of each *C. perfringens* toxin has been established in animal disease, and the mechanism of action of these toxins in vivo has been studied, which makes it possible to develop more effective vaccines against diseases caused by these microorganisms [30].

It is now generally accepted that the unjustified use of antibiotics to prevent and treat infectious diseases causes selection pressure on bacteria, leading to increased antimicrobial resistance and loss of microbial diversity in the environment [54]. Feed additives should be an additional prevention strategy. Therefore, in developing an effective natural feed additive for the prevention of clostridial diseases, we relied on modern approaches to improve the specific and non-specific resistance of livestock. Antimicrobial peptides, immune regulators, probiotics and bacterial toxin binding mannan oligosaccharides [55–57] are known to be used as preventive agents. The frequent lack of significant treatment effects, as well as the low reproducibility of the results obtained, leads to the conclusion that, although various feed additives may contribute to the health and immune status of animals, no conclusions can be drawn from these results regarding their effect on the prevention of clostridiosis. According to some researchers [58], high efficacy of treatment and prevention of bovine diseases such as abomasitis, and enteritis caused by *C. perfringens*, can be achieved by using a comprehensive approach. This should include prevention of *C. perfringens* proliferation, reduction of exotoxin production, restoration of normal gastrointestinal microflora and gut architecture. The use of probiotic strains selected for the biodegradation of toxic compounds in feed additives is promising [59]. The use of probiotic strains and immunostimulants has great potential as therapy, but requires further development [56,60]. Such bacteria, in addition to their powerful antimicrobial effect, are capable of exerting versatile effects on the host organism. Probiotics, used for prevention or treatment of clostridia, act through multiple possible mechanisms, including the restoration of normobiotic resistance, antagonistic activity, and synthesis of a wide range of metabolites, which have a protective effect on the main targets of *Clostridia* [61–63]. They protect cells from damage and reduce the level of expression (activity) of apoptosis (cell death) and inflammation genes [64,65].

The choice of *B. amyloliquefaciens*159 as the basis of BFA was based on numerous data [66,67] that suggest that *Bacillus* bacteria can effectively restore the composition of the host microbiota, increase its resistance to infectious diseases, and contribute to improved animal performance. Currently, various commercial probiotics contain bacilli as active

ingredients, due to their ability to colonize the digestive system, their high stability in relation to endosporogenesis, which allows them to survive in stressful situations, such as the aggressive conditions of the internal environment of the body, and their longer shelf life [68]. Representatives of this taxon are known to be capable of synthesis of a wide range of metabolites with antimicrobial properties, including bacteriocins (subtilin, bacillocin 22, sublancin 168 and others) that have an antagonistic effect against a wide range of pathogens [69,70]. In an in vitro experiment, we observed pronounced antimicrobial, particularly anticlostridial, activity of *B. amyloliquefaciens*159, which was probably due to the diffusion of antimicrobial metabolites into agar. Neutralizing toxins, antioxidants, and increasing the nonspecific resistance of animals can serve as important additions for the complex prophylaxis of clostridiosis. Lauric acid has strong antimicrobial activity due to its function of disordering lipid membranes of microorganisms [66,71]. Pine extract (pycnogenol) has antioxidant activity due to its high content of procyanidides, catechins, polyphenols, as well as antimicrobial and anti-inflammatory properties [72]. We used diatomite as an enterosorbent in BFA. The unique firing technology, at temperatures of 800 °C, significantly increases the specific surface area of the sorbent (up to 40 ha/kg), which makes it the most effective among other sorbents [46,73]. More research is needed to study the use of neutralizers of clostridial toxins, as well as the role of individual substances in the formation of additional animal bioprotection.

In a healthy animal or poultry organism, immunity must perform the task of resisting clostridia. However, antibiotics, often used in the treatment of *Clostridia*, not only do not increase it, but also reduce it, making the organism more helpless and unable to defend itself [74]. Our study was based on previous findings, that certain strains of the bacteria *Bacillus* spp. Are biostimulants of non-specific immunity [75]. Some strains are known to reduce the effects of oxidative stress, apoptosis and inflammatory gene expression levels [76]. In addition, pine bark extract, chosen as an ingredient of BFA, is also known for its immunomodulatory properties [77]. The combined action of these components of the feed additive resulted in a significant increase in the level of humoral immunity of cows, which is especially important at the beginning of lactation. The main integral indicators of natural resistance of cattle are the bactericidal (BA) and lysozyme activity (LA) of blood serum. Using the feed additive increased the concentration of lysozyme in the blood serum of cows of group E112 by 3,6 times compared to the control ($p < 0.001$). BA levels increased by 10.7% (Table 3). It is important to note that vaccines against clostridia are not always effective. In some cases, experts attribute their lack of effectiveness against clostridia to the low level of production in animals of specific antibodies in response to the introduction of the antigen [78]. Prior to the experiment, the animals of the experimental groups were vaccinated with a preparation against bovine clostridiosis. The drug should cause formation of immune response in animals against α-, β- and ε-anatoxins of *C. perfringens* types A, B, C, D and others, 2–3 weeks after repeated administration, and immunity in this case should be maintained for 10–12 months. The use of BFA in the feeding of dairy cows contributed to a better serological response after immunization with the vaccine and natural contact with *C. perfringens*. The level of serological response in the control group decreased slightly after three months of experience. At the beginning of the experiment, a positive serological response was observed in 50% of the animals, and at the end of the experiment the serological response rate was 41.6% of the number of animals examined (Figure 5). In contrast, the serological response rate in the experimental groups increased at the end of the experiment. The positive effect of the drug on the effectiveness of vaccination is explained by the fact that the intestinal tract of mammals is the largest immune organ and consists of cells of non-hematopoietic (epithelium, Paneth cells, goblet cells) and hematopoietic (macrophages, dendritic cells, T cells) origin, while simultaneously being a home to symbiotic microbiota, which largely modulate immune cell activity, including through regulation of gene expression [79]. A disturbed microbiome, which is observed in most animals in intensive livestock farming conditions [80], is capable of causing a reduction in vaccination efficacy. Intestinal dysbiosis contributes to systemic inflammation, immunosuppression

and a lesser response to vaccines. It has previously been demonstrated in model organisms that dysbiosis reduced specific IgG responses to various vaccines, and the transplantation of fecal commensal microbiota helped to stimulate vaccine responses [81]. Due to the healing of the microbiome and the manifestation of immunomodulatory activity in our experiment, the biopreparation had a positive effect on postvaccination immunity. It is known that the microbiome can influence the efficacy of vaccination through various mechanisms, including direct effects (e.g., through the structural component of the flagellin protein), as well as the synthesis of lipopolysaccharides, volatile fatty acids and other metabolites that can activate Toll-like receptors, and, in turn, stimulate the response of dendritic cells [82]. Thus, Bron et al. [83], after analyzing the available data on the adjuvant activity of three strains of lactobacilli, noted a role in improving vaccination efficacy in compounds such as lipoteichoic acid in *Lactiplantibacillus plantarum*, the GG (also known as LGG) proteins p40 and p75 secreted by *Lacticaseibacillus rhamnosus*, and surface layer protein A (SlpA) in *L. acidophilus* strain NCFM. Earlier results in poultry have also shown that a number of probiotic bacteria increase efficacy against Newcastle disease [84] and avian influenza [85].

Active oxygen species (AOS) are produced during mitochondrial oxidative metabolism as well as in the cellular response to xenobiotics [86] and changes in the rumen microbiome. When AOS suppress the cellular antioxidant defense system, whether by increasing AOS levels or decreasing cellular antioxidant capacity, oxidative stress occurs, affecting the rumen microbiome among others [87]. In turn, it is known that the microbiota of the digestive system can affect the levels of enzymes that metabolize xenobiotics, both in the gut and in the liver [88]. Glutathione (GSH) is present in all mammalian tissues, as the most abundant non-protein thiol that protects the body against oxidative stress [89]. GSH is also a key determinant of redox signaling. Being vital for xenobiotic detoxification, it regulates cell proliferation, apoptosis, immune function and fibrogenesis. GSH biosynthesis occurs in the cytosol in a highly regulated manner. The interrelation of antioxidants and glutathione is caused by their overproduction of indirect biological activity, allowing them to realize their action through signal pathways, mediated by NF-κB and LPS/TLR-4 (proinflammatory cascades), STAT3 and MAR kinases, PI3K/Akt/mTOR (autophagy), and G-protein coupled receptors [90–92]. Comparative analysis of the total amount of water-soluble antioxidants is directly related to the induction of ARE-dependent phase II xenobiotic detoxification enzymes and antioxidant enzymes directly involved in the induction of total GST activity in U937 myeloid cells, catalyzing reactions of chemical modification of xenobiotics with GSH participation (conjugation and nucleophilic substitution reactions of xenobiotics and GSH, and reduction of organic peroxides to alcohols with the formation of GSSG). Thus, the accumulation of antioxidants indicates a better level of stress resistance in productive animals, adaptive qualities, and mobility of biochemical pathways in the body under the conditions of intensive milk production. Our data indicate that the feeding of BFA led not only to an increase in TAWSA, but could also improve the antioxidant properties of the milk. The conjugacy of lipid peroxidation and the antioxidant system can be assessed by calculating the correlation between a number of components of these systems and the correlations between them (Table 6). The TBA–AP / CP ratios of blood serum of cows we determined were in direct relation to the TBA–AP content, and decreased in the blood of Group E48 animals ($p < 0.01$). The highest TBA–AP content was observed in the blood serum of the control group, which probably indicates the activation of free radical processes in cows against the background of high productivity at the beginning of lactation. Thus, the use of BFA significantly improved the antioxidant protection of the body of the experimental animals. We attribute some differences in blood values with physiological norms at the beginning of lactation (Table 4), firstly to the physiological period (cows average 34th day of lactation), secondly to high productivity, and thirdly to feeding. There were intergroup differences (AST, UREA, $p < 0.05$; WBC, $p < 0.01$) but they were not systematic. Under the influence of the factor under study, important parameters, such as TP, GLB, A/G, and RDC, stabilized and normalized in the animals of groups E80 and E112, compared to the animals of groups C and E48. Therewith, the following intergroup differences were observed: under

the influence of BFA feeding, the TP content in blood serum decreased to normal ($p < 0.001$), ALB level increased ($p < 0.05$), and GLB decreased to normal ($p < 0.001$). This stabilized the A/G-ratio from 0.47–0.49 to 0.64–0.71 ($p < 0.001$) in the E80 and E112 groups, against the background of urea and ALT increase ($p < 0.01$). This indicates an optimization of protein metabolism when using BFA, and is also consistent with the higher milk productivity of the experimental groups. Better productivity and milk fat content were connected with the improvement in carbohydrate-lipid metabolism: the blood serum cholesterol concentration in the cows of the experimental groups increased ($p < 0.01$). GLU in blood of group C animals was below the norm, while in animals of experimental groups its concentration corresponded to the norm ($p > 0.05$). It is also necessary to note the tendency ($p < 0.10$) of WBC to decrease in the blood serum of cows in group E112, which reflects the positive effect and characterizes the decrease in inflammatory processes in animals of experimental groups under the influence of BFA. It should be noted that during the three months of the experiment, the cows in the experimental groups maintained their productivity at a high level, unlike the animals in the control group. At the end of the experiment, the average daily milk yield was higher in the experimental groups (Table 7), which indicates not only the ability of the chosen strategy to prevent clostridiosis and improve cattle health and resistance, but also shows the short-term effect expressed in additional milk production.

5. Conclusions

To date, the problem of Clostridial disease remains unresolved. A wide range of clostridial anatoxin, bacteria-anatoxin, or whole culture vaccines are used worldwide to protect animals against the disease [50]. The use of probiotics and holistic supplements for the prevention of clostridiosis is becoming relevant [59,93,94]. The synergy of complementary microorganisms and biologically active substances resulted in the improvement of animal organism resistance, including an increase in organism reactions to the specific β-toxin of *C. perfringens*. There was a significant improvement of metabolism and increased antioxidant status in cows. When future research in this direction is conducted, it should study the influence of the factor under study on the specific immunity of cows against other kinds of toxins; on rumen metabolism and the microbiological background of the rumen and intestine; on the hormonal background of the animals; and on reproductive ability. This will give an opportunity to assess in a new way the developed method of bovine clostridia prophylaxis. Thus, the use of BFA can serve as a functional technique for the implementation of an animal's biosecurity strategy against toxins, providing a practically valid technique for future use in farm animal diets. Based on our research, we consider the dosage of 80 g/head/day of BFA to be most appropriate. Studies will continue with this dosage in the future.

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Informed Consent Statement: This study did not involve humans.

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Bioactive Compounds of Barbatimão (*Stryphnodendron* sp.) as Dietary Additive in Lamb Diets

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Abstract: This study aimed to evaluate barbatimão bark extracts as a feed additive and substitute for lasalocid sodium (LAS) for feedlot lambs. Lambs were distributed into three treatments: LAS (0.018 g of lasalocid sodium), DBB (1.500 g of dried and milled barbatimão bark), and BHE (0.300 g of barbatimão hydroalcoholic extract). There was no effect ($p = 0.32$) of the inclusion of DBB and BHE extracts on the average daily gain. Inclusion of BHE in lamb diets reduced ($p < 0.05$) the fatness score compared to LAS, which was similar to DBB. The BHE decreased the yellowness intensity and hue angle ($p < 0.05$) of meat compared to the LAS. Animals that consumed DBB and BHE had a reduced ($p = 0.04$) total cholesterol level. Thus, the use of barbatimão bark extracts can replace lasalocid sodium in the diet of feedlot lambs, with no detrimental effects on performance or metabolic parameters.

Keywords: ruminants; tannins; bioactive compounds; saponins; feed additives

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1. Introduction

Lasalocid sodium (LAS) is a synthetic product used as an additive in animal feed, related to its potential to mitigate methane (CH4) emissions, improve animal performance [1], and increase production profitability. However, the market is increasingly demanding, resulting in the rejection of chemicals in animal protein production, due to the evolution of antibiotic-resistant pathogens [2]. In the search for substitutes for synthetic products, the animal feed additive industry has intensified its investments in biocompounds [3]. These biocompounds modify ruminal subtract availability and microbial ecosystem, thus reducing CH4 emissions [4]. The natural additive effects are related to the type of diet (high or low concentrate), concentration and amount of ingested additive, mode of action in the gastrointestinal tract, and physiological state of the animal [3,5]. In addition, the composition of biocompounds in plants can be affected in several ways, from the plant development to the final extraction [6].

Barbatimão (*Stryphnodendron* sp.) is a plant native to the Brazilian savanna and produces several chemical metabolites in its secondary metabolism, such as tannins and saponins [7]. These biocompounds have antimicrobial, healing, anti-inflammatory, and antioxidant activities; thus, it is a plant used by communities in traditional medicine [8,9]. Tannins are phenolic compounds with properties that precipitate proteins [10]. High doses of tannins (>5% in dry matter; DM) in a ruminant diet may lead to a reduction in DM intake, digestibility, and performance [11]. However, at moderate doses, tannins potentiate nutrient use efficiency, due to the greater availability of these nutrients in the small intestine [3]. Saponins are active photochemical components of plants, which are part of the plant defense system, and that have an antimicrobial and antioxidant potential [8,12] that can affect ruminal microorganisms. In the literature, several studies have shown

that the inclusion of sources of tannins and saponins in ruminant diets can reduce enteric methane production, improving feed efficiency and animal performance [11,13,14].

In this context, barbatimão extract has advantages because of its various active components and modes of action, being a natural and safe option as an additive [15]. Thus, the objective of this study was to evaluate the effects of the use of barbatimão bark extracts, as a food additive in place of LAS, on the metabolic parameters, performance, and carcass characteristics of feedlot lambs.

2. Materials and Methods

2.1. Location and Experimental Facilities

The experiment was carried out at the Federal University of Grande Dourados. The Animal Use Ethical Committee of the Federal University of Grande Dourados, Brazil, approved the experimental animal procedures (Protocol 032.2020).

2.2. Collection, Production, and Phytochemical Analysis of Extracts

Barbatimão barks (*Stryphnodendron rotundifolium*) were manually collected from several trees during the summer season in the morning and dried in a forced ventilation oven at 55 °C for 72 h. Then, the material was milled in a Willey mill with a 2 mm sieve, to obtain the dry barbatimão bark (DBB). Barbatimão hydroalcoholic extract (BHE) was obtained by submersion of 250 g of DBB in a water–ethanol solution 50:50 [16] and incubated in a water bath at 60 °C for 60 min. Hot filtration was performed in a funnel with four layers of cheesecloth and dried (40 °C) to a constant weight. Then, the material was macerated until a powder granulometry was obtained.

The extracts were submitted to phytochemical evaluation [17], to confirm the secondary metabolite classes [18]. The presence of triterpenes and steroids was confirmed through hydrolysis of the dry methanolic extract. This procedure was performed with potassium hydroxide (0.5 mol/L) and with reflux for 1 h. Then, the compounds were extracted with ethyl ether and then submitted to a Liebermann–Burchard reaction. To determine the presence of secondary metabolite classes, the reactions of characterization intensities were classified as follows: negative reaction (− = 0%), partial intensity (±/+ = 10%), low intensity (+ = 50%), medium intensity (++ = 75%), and high intensity (+++ = 100%) [18]. The extracts were solubilized at a concentration of 1 mg/mL in methanol, to analyze phenolic compounds and flavonoids [19]. The content of phenolic compounds was determined based on the Folin–Ciocalteau colorimetric method [19]. The tannin content was determined using the Folin–Denis spectrophotometric method, with tannic acid as a reference [20].

2.3. Animals, Diets, and Experimental Design

Twenty-four lambs, non-castrated males, with 150 ± 4.59 days of age and 21.2 ± 3.63 kg of body weight (BW) were used. At the beginning of the experiment, the lambs were weighed, identified, and dewormed (Baycox, 1 mL/3.5 kg BW). The lambs were allocated to individual covered stalls with an area of 2.2 m^2 (1.5 m × 1.5 m), with a cement floor lined with rice husk. They had free access to water and had access to ground oat hay (*A. sativa*) and concentrate ad libitum for 14 days for adaptation.

After the adaptation phase, the experimental period began, consisting of three periods of 14 days, counting 42 days of performance evaluation plus six days up to the slaughter, totaling 48 days for the experiment. The experimental diet (Table 1) was formulated based on soybean meal, ground corn, oat hay, and specific commercial mineral supplement for sheep, according to the National Research Council (NRC) [21], to meet the requirement of lamb weight gain of 300 g/animal/day, with the dry matter intake estimated at 3.5% of BW. The forage:concentrate ratio was 20:80. The total diet was offered at 07:00 and 12:00, adjusting the amount provided every three days, considering 5% of leftovers.

Table 1. Ingredients and chemical composition of the experimental diet.

Ingredients	Quantity (g/kg DM)
Hay	200.0
Ground corn	632.0
Soybean meal	152.0
Salt	8.0
Calcium carbonate	8.0
Composition	
Dry matter (g/kg as feed)	895.0
Organic matter (g/kg of DM)	918.0
Crude protein (g/kg of DM)	210.0
Ether extract (g/kg of DM)	35.0
Neutral detergent fiber (g/kg of DM)	247.0
Acid detergent fiber (g/kg of DM)	138.0

The experimental designed used randomized blocks, with three treatments and eight repetitions. The lambs were blocked based on BW, and the treatments were randomly distributed within each block. The treatments tested were addition of DBB, 1.50 g/animal/day; addition of BHE, 0.300 g/animal/day; and addition of LAS, 0.018 g/animal/day. DBB and BHE were mixed with 30 g of concentrate and supplied as a top-dress at morning meals, to ensure total intake. For LAS, 0.150 g/kg of the ionophore (Taurotec-Zoetis, Campinas, São Paulo, Brazil; 15% of lasalocid sodium) was mixed into the diet.

2.4. Animal Performance

The dry matter intake was determined daily from the difference between the offered feed and the leftovers. The leftovers were weighed before the morning meal. A composite sample of diet ingredients and leftovers was collected per period per animal and stored in a freezer at −20 °C. At the end of the collection period, the samples were pre-dried in a forced ventilation oven at 55 °C for 72 h. Lastly, the samples were milled (1 mm) in a Willey mill for further analysis.

The lambs were weighed on the first day (initial body weight) and at the end of each experimental period, with fasting of solids and liquid in the previous 12 h. The average daily gain was measured from the difference in BW between the beginning and the end of the experimental period, divided by the number of days. The relation between dry matter intake and average daily gain was used to determine the feed conversion ratio. The body condition at slaughter was evaluated by two specialists, based on a scale from 1 to 5, with a 0.5 variation.

2.5. Chemical Composition

Diet and extract samples were analyzed for dry matter content (ID 934.01), ash (ID 930.05), crude protein (CP, ID 981.10), and ether extract (ID 920.39) [22]. Neutral detergent fiber and acid detergent fiber contents were evaluated according to Van Soest [23].

2.6. Slaughter and Carcass Evaluation

All slaughter procedures were performed according to the Regulation of Industrial and Sanitary Inspection of Products of Animal Origin and the rules of Technical Regulation of Methods of Insensitization for the Humanitarian Slaughter of Butcher Animals [24]. The lambs were slaughtered at the end of the experiment, after 62 days in the feedlot (14 of adaptation + 48 of the experiment). The lambs were submitted to solid fasting for 16 h and weighed to determine the body weight at slaughter. The lambs were desensitized by electronarcosis, suspended by the hind legs, and the carotid arteries and jugular vein were sectioned for bleeding, then they were skinned and eviscerated. The full and empty gastrointestinal tract, bladder, and gallbladder were weighed, and the weight of the abiotic components was obtained from the difference between the full and empty weights. The empty body weight was determined from the subtraction of the abiotic components from

body weight at slaughter. Carcass conformation and fatness indexes were determined by two specialists, based on a scale from 1 to 5, with a 0.5 variation, where: 1—Very poor; 1.5—Poor; 2—Acceptable; 2.5—Average; 3—Good; 3.5—Very Good; 4—Superior; 4.5—Very Superior, and 5—Excellent [25].

The carcasses were weighed to obtain the hot carcass weight. The carcasses were suspended by the leg tendons and stored in a cold room at 4 °C for 24 h, and were posteriorly weighed to obtain the cold carcass weight. Then, the yields of the hot carcass and cold carcass and the loss by cooling were determined [26].

The meat color in the Longissimus thoracis et lumborum muscle was determined [27] using a digital colorimeter (Minolta CR-400, Minolta Co., Osaka, Japan), calibrated in the CIELAB system. The luminosity (L*), red intensity color (a*), and yellow intensity color (b*) [28] were measured. The saturation index (chroma; C*) was determined according to the equation:

$$C* = \sqrt{(a*^2) + \left(b*^2\right)} \quad (1)$$

The definition of metric hue angle (HUE) was determined according to the equation:

$$\text{HUE} = \text{arctangent } (b*/a*) \quad (2)$$

2.7. Metabolic Parameters

Blood samples were taken by jugular vein puncture, four hours after morning feeding, on the 11th day of each experimental period with a vacutainer with heparin. The samples were centrifuged immediately after collection at 3000 rpm for 15 min, and the plasma was frozen for further analysis. The plasma glucose concentration was determined by the enzymatic-colorimetric method of glucose-oxidase, using a commercial kit (Sigma C.C.). Total cholesterol was evaluated using a Cholesterol Labtest Diagnóstica commercial kit. The concentration of urea, aspartate aminotransferase, and alanine aminotransferase were evaluated using Diagnóstica commercial kits.

Urine was sampled after slaughter. Creatinine and urea were evaluated using Gold Diagnóstica commercial kits. The colorimetric method was used for allantoin determination [29]. Blood and urine tests were performed at the Laboratory of the Veterinary Hospital of the University Center of Grande Dourados. Creatinin was used to estimate the total urine excretion.

2.8. Statistical Analysis

The data were analyzed using MIXED PROC from the Statistical Package of SAS (SAS University Edition), except for the data from carcass conformation and fatness, which were analyzed by proc npar1way (SAS University Edition). The means were compared using the Tukey test. Significance was declared when $p < 0.05$. The statistical model included treatment as a fixed effect and the block as a random effect.

3. Results

The high tannin contents of barbatimão bark extracts (Table 2) did not interfere in acceptability because it did not reduce the lamb dry matter intake ($p = 0.56$; Table 3). Barbatimão bark extract's effects were similar to (LAS), and there was no change in the performance variables average daily gain, feed conversion ratio, initial body weight, body weight at slaughter, empty body weight, and body condition at slaughter ($p > 0.05$) with the addition of DBB and BHE (Table 3). The inclusion of DBB and BHE did not influence the variables related to body weight at slaughter and yield ($p > 0.05$), such as hot carcass weight, cold carcass weight, loss by cooling, hot carcass yield, cold carcass yield, and carcass conformation of lambs. The BHE showed a lower carcass fatness index ($p = 0.04$).

Table 2. Chemical composition and secondary metabolic compounds of dry barbatimão bark (DBB) and barbatimão hydroalcoholic extract (BHE).

Chemical Composition	DBB	BHE
Dry matter (g/kg as feed)	362.0	765.2
Organic matter (g/kg of DM)	980.2	975.1
Crude protein (g/kg of DM)	109.0	29.0
Ether extract (g/kg of DM)	6.0	9.0
Neutral detergent fiber (g/kg of DM)	473.0	9.0
Acid detergent fiber (g/kg of DM)	446.0	6.0
Phenolic compounds (mg/g of DM)	89.8	93.2
Flavonoids (mg/g of DM)	35.0	39.1
Tannins (mg/g of DM)	453.70	479.1
Secondary metabolic compounds *		
Phenolic compounds	++	++
Flavonoids	+	+
Tannins	+++	+++
Naphtoquinone	−	−
Coumarin	+	+
Triterpenes and Steroids	+	+
Cyanogenic heterosides	+	+
Cardioactive heterosides	+	+
Reducing sugars	+	+
Saponins	+	+
Alkaloids	−	−

* The presence of secondary metabolic compounds was classified as follows: negative reaction (− = 0%), partial intensity (±/+ = 10%), low intensity (+ = 50%), medium intensity (++ = 75%), and high intensity (+++ = 100%).

Table 3. Productive performance characteristics and carcass evaluation of lambs fed with diets containing lasalocid sodium (LAS), dried barbatimão bark (DBB), or dry barbatimão hydroalcoholic extract (BHE).

Item	LAS	DBB	BHE	[1] SEM	*p*-Value
Dry matter intake (g/day)	880.2	866.8	828.3	51.20	0.56
Average daily gain (g)	221.8	189.2	198.4	0.02	0.32
Feed conversion ratio	3.9	5.1	4.2	0.55	0.09
Initial body weight (kg)	21.6	20.6	21.1	0.97	0.31
Body weight at slaughter (kg)	31.0	29.5	30.5	1.47	0.61
Empty body weight (kg)	27.6	25.9	26.5	1.20	0.40
Body condition at slaughter	2.7	2.4	2.7	0.19	0.22
Total weight gain (kg)	9.1	8.2	8.6	1.04	0.69
Weight hot carcass (kg)	15.6	14.4	14.7	0.68	0.25
Weight cold carcass (kg)	15.0	14.0	14.2	0.65	0.27
Hot carcass yield (%)	50.2	49.0	48.6	0.94	0.23
Cold carcass yield (%)	48.4	47.4	47.0	0.96	0.31
Loss by cooling (%)	4.2	3.3	3.3	0.57	0.23
Conformation [2]	2.3	2.2	2.1	0.11	0.40
Fatness [3]	3.1 a	2.7 ab	2.5 b	0.17	0.04

[1] Standard error mean; [2] Conformation estimated by a scale of 1 (no fat) to 5 (excess fat); [3] Estimated fatness by a scale of 1 (no fat) to 5 (excess fat); a, b Averages followed by different letters on the same line differ ($p < 0.05$) from each other.

The parameters of luminosity, red intensity, and chroma were not influenced ($p > 0.05$) by the DBB and BHE (Table 4). However, the BHE lambs had a lower intensity of yellow and hue angle compared to the other treatments. The barbatimão bark extracts in the diets did not influence ($p > 0.05$) the levels of glucose and urea in the blood and urea and allantoin in urine of the lambs (Table 5). The barbatimão bark extracts reduced ($p = 0.01$) the total blood cholesterol levels compared to the LAS treatment. The barbatimão bark extracts did not influence ($p > 0.05$) the aspartate aminotransferase and alanine aminotransferase levels.

Table 4. Meat color of lambs fed with diets containing lasalocid sodium (LAS), dried barbatimão bark (DBB), or dry barbatimão hydroalcoholic extract (BHE).

Item	LAS	DBB	BHE	[1] SEM	*p*-Value
Luminosity, L*	39.9	39.8	38.9	0.58	0.38
Intensity of red, a*	16.7	16.7	15.7	0.58	0.17
Intensity of yellow, b*	4.6 a	4.6 a	3.7 b	0.27	0.01
Chroma, C*	17.3	17.4	16.2	0.32	0.27
Hue angle, HUE	15.6 a	15.5 a	13.2 b	0.93	0.04

[1] Standard error mean; a, b Averages followed by different letters on the same line differ ($p < 0.05$) from each other.

Table 5. Blood and urinary parameters of lambs fed with diets containing lasalocid sodium (LAS), dried barbatimão bark (DBB), or dry barbatimão hydroalcoholic extract (BHE).

Item	LAS	DBB	BHE	[1] SEM	*p*-Value
Blood					
Glucose (mg/dL)	75.8	66.4	66.0	5.21	0.33
Total cholesterol (mg/dL)	56.4 a	39.7 b	41.5 b	5.01	0.01
Urea (mg/dL)	55.2	43.4	40.5	5.15	0.11
Aspartate aminotransferase (mg/dL)	119.4	119.7	127.2	15.69	0.91
Alanine aminotransferase (mg/dL)	19.2	20.0	16.8	1.99	0.45
Urine					
Urea (mg/day)	3189.0	2336.2	2612.4	357.85	0.23
Alantoin (mg/day)	835.0	624.5	563.6	108.02	0.15

[1] Standard error mean; a, b Averages followed by different letters on the same line differ ($p < 0.05$) from each other.

4. Discussion

Normally, a high tannin content is associated with harmful effects, such as reduced feed intake [30]. However, if the supply is moderate, there are no harmful impacts of intake [5], as occurred in this study. Barbatimão bark extract's effects were similar to LAS, by reducing ruminal proteolysis and promoting the flow of dietary protein into the duodenum. This was inferred to be because the flow of dietary protein with microbial protein increased the availability of nitrogen compounds for protein synthesis in the animal [31]. In addition, the presence of tannins and saponins in the barbatimão bark extracts was associated with reduced energy expenditure, justified by the ability of these phenolic compounds to reduce the process of methanogenesis [32]. Consequently, there is reduction in energy losses by decreasing ruminal methane production [4]. Studies with sheep fed a diet containing less than 50 g of tannins/kg of dry matter showed no effect on daily dry matter intake, presenting a higher feed efficiency and daily weight gain than a treatment without tannins [30,33]. Furthermore, carcass characteristics and meat quality were not affected by tannins [34].

Regardless of the treatment, body weight at slaughter and carcass yields were similar, and this may indicate a positive relation of body weight at slaughter with carcass yield. All treatments had an average within the appropriate variation range, from 40 to 50% for sheep [35]. Loss by cooling was not influenced by treatments, with established indices for sheep ranging from 1 to 7% [36]. Loss by cooling is related to the carcass fatness classification, because it is related to age, nutritional management, live weight, and carcass conformation [37].

The lower carcass fatness index on BHE may be related to the level of extract used in the diet, resulting in a lower influence of barbatimão biocompounds on lambs. The BHE carcasses had an acceptable fatness index (2 to 2.4), with an average of 2.4, followed by DBB, with a medium fatness index (2.5 to 3.0) and LAS with a fatness index considered good (3.0 to 3.4) [25]. Carcass fatness index is directly connected to adiposity, which predicts the tissue composition of the carcass [38]. In addition, it reduces fluid loss and shortening of muscle fibers and increases meat darkening during the cooling process [39].

The BHE may have influenced the meat color through the activity of barbatimão biocompounds, probably due to the level of extract used, since this additive has an antioxidant

action, which can interfere in the meat color [40]. Meat values of b* (9.44), C* (25.29), and HUE (21.92) of feedlot Pantaneiros lambs reported in the literature [41] differ from the values of this study, which were lower.

To verify the metabolic alteration, the blood and urinary parameters were evaluated. Barbatimão bark extracts reduced the total blood cholesterol levels compared to the LAS treatment. Cholesterol levels are indicative of energy balance [42]. The reduction in blood cholesterol concentration can be explained by a possible decrease in rumen acetate production, since acetate is a precursor to cholesterol synthesis in ruminants [43]. The barbatimão tannins can form complexes with fibers, inhibiting the cellulolytic bacteria action and causing lower acetic acid production [44]. Similarly, the antimicrobial activity of saponins present in barbatimão is more evident for Gram-positive bacteria [45]. Inhibition of Gram-positive bacteria in ruminants reduces the proportion of acetate produced in the rumen [46].

The aspartate aminotransferase and alanine aminotransferase enzymes are markers of liver damage and can be identified in the cytoplasm, liver cell mitochondria, cardiac system, and skeletal muscle [42]. Our study's results corroborate the literature reports [47]. This literature indicates that appropriate doses (<5% in dry matter) do not cause adverse effects, even with extensive biotransformation of liver metabolism in mammals and intestinal microbiota. Thus, the extracts used in the present study did not cause liver injury in the lambs.

5. Conclusions

The use of barbatimão bark extracts may replace lasalocid sodium in the diet of feedlot lambs, with no detrimental effects on performance and metabolic parameters. Therefore, it is suggested that further studies are conducted to evaluate the bioavailability of the biocompounds and the inclusion of higher doses of milled bark and hydroalcoholic extract of dried barbatimão in the diet of feedlot lambs.

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Meat Quality of Male Layer-Type Chickens Slaughtered at Different Ages

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Abstract: An experiment with male layer-type chickens of the Lohmann Brown Classic breed was carried out at the Institute of Animal Science-Kostinbrod, Bulgaria, aiming to investigate the effect of age at slaughter on the meat quality. The birds were reared in a controlled microclimate, with an initial stocking density of 22 birds/m^2. At five weeks of age, fragmentation of the stocking density was applied, decreasing the number to seven birds/m^2. Chickens were slaughtered at five and nine weeks of age at an average live weight of 329 g and 1096 g, respectively. After slaughter, 10 chickens from each age group were subjected to analysis to determine the quality of breast and thigh meat. The results of the study showed that the age affected the meat quality parameters of the male layer-type chickens and its effect differed between the breast and thigh. The chickens slaughtered at nine weeks of age displayed a lower pH but darker meat color ($p < 0.001$) than those slaughtered at five weeks. Furthermore, the older birds showed a significant decrease in the intramuscular fat content in thigh meat ($p < 0.01$) and a tendency for diminishing in breast meat. This decrease corresponded to the lower percentage of monounsaturated fatty acids (MUFA) in the meat of the nine-week-old chickens ($p < 0.01$). On the other hand, the meat of the older chickens displayed a higher content ($p < 0.01$) of polyunsaturated fatty acids (PUFA), especially n-6, leading to a considerably higher n-6/n-3 ratio.

Keywords: male layer-type chickens; age; meat quality; fatty acids

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1. Introduction

The increasing demands of consumers regarding the quality of poultry meat produced globally require intensive selection of broilers for rapid growth and low feed intake. The selection according to these indicators has an economic effect but leads to negative changes in the quality of the harvested meat [1]. According to Baldi et al. [2] the major meat quality concerns are associated with abnormalities in breast meat, such as wooden breast, white striping, and spaghetti meat, that affect alone or in combination the meat of fast-growing broilers. These growth-related abnormalities not only impair the appearance of the meat, but also have a detrimental effect on the technological qualities [3]. Hence, the interest in the meat of slow-growing chickens has considerably increased. It has gradually grown in popularity in the market as a product with excellent taste and dietary values [4,5], and in some EU countries its production has increased significantly in the recent years [6].

Previous studies [7,8] found that the growth rate and the feed conversion of male layer-type chickens meet the minimum criteria for slow-growing chickens; even in the first four weeks of rearing their feed intake resembles slow-growing broilers. This reveals possibilities for their utilization and the conversion of a waste product from the production of female layer chickens into a secondary product for an innovative, independent, and economically sustainable niche for producing high-quality poultry meat products. After

hatching, the male layer-type chickens are often used in the manufacturing of pet foods or are culled due to their low live weight. This poses both animal welfare and ethical issues [9] provoking sharp criticism in society [10] and made it necessary to explore other ways to use layer-type cockerels. Three methods for utilization have emerged to avoid culling of this type of chicken: in ovo sex determination, use of dual-purpose breeds, and rearing male cockerels for meat. In Bulgaria, research on this topic commenced in 2010; however, studies on the meat quality of these birds are scarce. Our previous studies with male layer-type chickens showed that despite the certain disadvantages of the performance traits of these birds compared to the fast-growing broilers, they also have more positive traits, such as low deposition of abdominal fat and lower intramuscular and subcutaneous fat [8]. We found that the meat of layer-type cockerels slaughtered at 12 weeks of age is not inferior in quality to that obtained from slow-growing chickens; on the contrary, they have a higher protein content, a higher WHC, and lower intramuscular fat [8]. The quality of meat is determined by various factors, among which is the age at slaughter [11]. Dal Bosco et al. [12] compared chickens from different genotypes slaughtered at different ages and showed that the meat of younger chickens was more tender, while the older birds had lighter meat. Li et al. [13] confirmed higher tenderness of the meat in younger chickens and found improved water-holding capacity with increasing age. Age can affect the nutritional profile and healthy value of the meat, which is also determined by its fatty acid profile. In slow-growing chickens slaughtered at different ages [14], a significantly higher content of saturated fatty acids in thigh meat and a higher content of polyunsaturated fatty acids in both the breast and thighs of older chicks was reported. Furthermore, the content of cholesterol in meat was significantly lower in chickens slaughtered at an older age. Determining the optimal age for slaughtering animals and poultry is crucial to obtain the best characteristics in the carcass and meat and the highest nutritional and health values [13,15–17].

With a current scarcity of scientific information on the male layer-type chickens, this research was conducted to provide a better insight into the differences in the meat quality of this type of chickens slaughtered at different age. The data obtained in this study will complement the current scientific knowledge on slow-growing chickens and can also be used as a basis for further research on the processing quality of this type of meat.

2. Materials and Methods

2.1. Ethical Procedure

The experimental protocol used in this study, including the animal management and housing, was designed in compliance with the guidelines of the European and Bulgarian legislation regarding the protection of animals used for experimental and other scientific purposes [18]. The protocol was based on the permit for use of animals in experiments No. 227 of the Bulgarian Food Safety Agency (Statement No. 193 of the Bulgarian Animal Ethics Committee, prot.No.18/02.07.2020).

2.2. Experimental Birds and Housing

The trial involved 800 male layer-type chickens of the Lohmann Brown Classic breed hybrid and was carried out in the experimental poultry farm at the Institute of Animal Science-Kostinbrod, Bulgaria. The 1-day-old chickens were supplied by Bulagro 97 AD. The birds were housed in 5 pens each containing 160 chickens and were reared conventionally until slaughter. The initial stocking density was 22 chickens/m^2 and at 5 weeks of age, this was decreased to 7 birds/m^2. The fragmentation of the stocking density of the chickens was undertaken through preliminary weighing of each chicken and differentiation by the live weight. Thus, all the male layer-type chickens with a final body weight of $\leq$360 g were slaughtered at 5 weeks of age, and the remaining chickens were reared until 9 weeks old. The chickens were reared in deep litter and a controlled microclimate. The lighting regime was 3 h light and 3 h dark which repeated during the 24 h cycle. Feeding was ad libitum with standard broiler feed (Tables 1 and 2) according to the instructions for Ross

308 broilers [19]. Water was provided through gravity drinkers. During the trial period, the live weight of the birds was controlled weekly.

Table 1. Composition of the diet of the male layer-type chickens.

Component	Content, %
Maize	47
Wheat	15.12
Sunflower meal	8
Soybean meal	24.3
Sunflower oil	2
Limestone	1.26
Monocalcium phosphate	1.25
Salt	0.19
Met	0.2
Lys	0.195
Vitamin premix [1]	0.2
Mineral premix [1]	0.2
Choline chloride	0.085
Calculated composition	
Crude protein, %	20.00
Fat, %	4.60
Ash, %	5.70
Crude fibers	5.50
Ca	0.90
P	0.65
Metabolizable energy, kcal/kg	3000

[1] Vitamin and mineral premix provided the following per kg of diet: Fe,185 mg; Cu, 25 mg; Zn, 120 mg; Mn, 145 mg; I, 1.70 mg; Se, 0.45, vit. A 10500 IU; vit. D, 3750 IU; vit. E, 45 mg; BHT, 0.10 mg; endo-1,4 beta-xylanase 1500EPU.

Table 2. Fatty acid composition of the feed.

Fatty Acid	C14:0	C16:0	C16:1	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-3	C20:0
%	0.33	8.89	0.37	2.77	30.74	55.31	1.33	0.26

2.3. Slaughtering Procedure and Sampling

The chickens were slaughtered at 5 and 9 weeks of age in a commercial poultry abattoir at an average live weight of 329 g and 1096 g, respectively. The birds were stunned, decapitated, and bled. The carcasses were then plucked and eviscerated. Feet and edible viscera (heart, liver, gizzard) were removed in order to obtain the ready-to-cook carcass. The carcasses were then chilled and stored at 4 °C for 24 h. After 24 h of chilling, 10 chickens from each age group were randomly selected for meat quality analysis. The breast (pectoralis profundus et superficialis) and thighs of each carcass were collected, skinned, and deboned. One part of the samples was immediately used for analysis of meat technological quality while the other was vacuum-packed and stored frozen for further analysis of the proximate composition.

2.4. Analysis of the Meat Quality

2.4.1. Measurement of pH and Color

Muscle pH and color were measured at the time of deboning of the breast and thigh cuts. The pH measurements were undertaken using a portable pH meter equipped with a glass electrode. Calibration prior to use at pH 4.0 and 7.0 was performed. The surface color of the breast and thigh muscles was measured by Croma meter CR-410 (Konica Minolta Inc., Osaka, Japan) using CIE values expressed as lightness (L*), redness (a*), and yellowness

(b*). A measuring area of 50 mm, illuminant D65, and 2° standard observer were used. The instrument was calibrated using a standard white plate. The measurements of the pH and color were undertaken at 3 locations in the muscles and the results were averaged.

2.4.2. Determination of Water Holding Capacity (WHC)

Water holding capacity measured as free water content (%) was determined according to the filter press method as described by Honikel and Hamm [20].

2.4.3. Texture Analysis

The tenderness of the meat was measured by Warner–Bratzler shear force (WBSF) using a Belle texture analyzer (Agrosta, Serqueux, France). The measurements were undertaken on cooked meat from breast and thigh at the day of carcass analysis. The breast and thigh meat pieces were weighed, placed into plastic bags, sealed, and cooked in a water bath at 80 °C until the internal temperature of the meat reached 70 °C. The bags were then removed and the meat was left to cool at room temperature for approximately 30 min. The meat pieces were dried to eliminate any water left on the surface. Shear force was evaluated on cores cut from the thickest part of the cooked samples by cutting them perpendicularly to the direction of the fibers [21].

2.4.4. Proximate Analysis

The content of moisture, protein, fat, and ash in the breast and thigh meat was assessed according to the methods of AOAC [22].

2.5. Fatty Acid Profile

The fatty acid composition of the feed and meat was determined according to the method of Bligh and Dyer [23] with slight modifications [24]. Lipids were extracted from 10 g of the muscle/feed sample and homogenized using a HG-15D homogenizer (Witeg Labortechnik GmbH, Wertheim, Germany) with 10 mL of chloroform and 20 mL of methanol for 30 s. Following this, 10 mL of chloroform and 10 mL of NaCl (1% in distilled water) were added to the mixture and homogenized for 30 s. The samples were centrifuged (4000 rpm for 10 min) and finally the chloroform layer was evaporated. The fatty acids were trans esterified following the procedure described by Domínguez et al. [25] with some modifications: 20 mg of extracted fat dissolved in 1 mL of toluene was mixed with 2 mL of a sodium methoxide (0.5 N) solution, vortexed for 10 s, and allowed to stand for 15 min at room temperature. Then, 4 mL of a H_2 SO_4 solution (10% of H_2 SO_4 in methanol) was added, vortexed for 10 s, and left for 5 min before adding 2 mL of saturated sodium bicarbonate solution. Fatty acid methyl esters were extracted as 1 mL of hexane was added to the samples, vortexed for 10 s, and the organic phase was transferred to an appropriate GC vial. Separation and quantification of FAMEs were carried out using a gas chromatograph (CSi 200 series, Cambridge Scientific Instruments Ltd., Ely, UK) equipped with a capillary column (DM-2330:30 m × 0.25 mm × 0.20μm) and hydrogen as a carrier gas. The oven temperature was first set to 160 °C for 0.2 min, then raised to 220 °C at a rate of 5 °C/min and then held for 5 min. The temperatures of the detector and injector were 230 °C. Methyl esters were identified through comparison of the retention times of the standards. Fatty acids are presented as percentages of the total amount of the methyl esters (FAME) identified. The amount of each fatty acid was used to calculate the atherogenic (AI) and thrombogenic (TI) indices [26]:

$$AI = (4 \times C14{:}0 + C16{:}0)/[MUFA + \Sigma(n-6) + \Sigma(n-3)];$$
$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/[0.5 \times MUFA + 0.5 \times (n-6) + 3 \times (n-3) + (n-3)/(n-6)]$$

2.6. Statistical Evaluation

Results are presented as mean ± SD. Comparisons between the 2 age groups in regard to the meat quality traits and the fatty acid profiles were performed through *t*-test (JMP v.7, SAS Institute Inc. Cary, NC, USA).

3. Results

3.1. Effect of Age on the Technological Quality of Meat

The pH values were significantly lower in the breast ($p < 0.05$) and thighs ($p < 0.001$) of the older chickens (Table 3). In breast the pH ranged from 5.67–5.73, while for the thighs this trait varied from 6.08 to 6.25.

Table 3. Quality traits of the breast and thigh meat.

Item	5 Weeks	9 Weeks	Sig.
Breast			
pH 24	5.73 ± 0.05	5.67 ± 0.074	*
L*	59.46 ± 2.65	54.14 ± 1.27	***
a*	16.04 ± 1.18	14.16 ± 1.18	**
b*	11.22 ± 1.23	8.76± 1.28	***
WHC, %	39.87 ± 2.06	35.58± 2.45	***
WBSF, kg	2.45 ± 0.81	2.60 ± 0.72	ns
Thigh			
pH 24	6.25± 0.09	6.08 ± 0.11	***
L*	52.47± 2.89	44.72 ± 1.47	***
a*	20.38 ± 1.30	19.22 ± 0.68	ns
b*	8.87 ± 0.61	6.79 ± 0.46	***
WHC, %	33.08 ± 3.20	33.93 ± 1.22	ns
WBSF, kg	1.95 ± 0.75	1.86 ± 0.31	ns

WHC: Water holding capacity; WBSF: Warner–Bratzler shear force. Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns—non-significant.

The color parameters of breast and thigh meat differed between the chickens slaughtered at five and nine weeks of age. The older birds had darker meat with lower L* ($p < 0.001$) and b* values ($p < 0.001$). On the other hand, the redness was significantly higher in the breast of the chickens slaughtered at five weeks of age ($p < 0.01$) and tended to be higher in the thighs of the younger birds but without significant difference.

The water holding capacity (Table 3) as expressed by lower percentage of free water was better in the breast of the chickens that were slaughtered at nine weeks of age ($p < 0.001$), while no such difference was observed regarding this trait in the thigh meat. No difference between the age groups was found in regard to the shear force values.

3.2. Proximate Composition

Difference between the age groups regarding the proximate composition was observed in thighs (Table 4). The intramuscular fat content decreased significantly in the older cockerels (2.72% vs. 3.80%, respectively, for the nine- and five-week-old male layer-type chickens, $p < 0.01$). The chickens slaughtered at nine weeks old also displayed higher ash ($p < 0.001$) and moisture ($p < 0.05$) contents.

Table 4. Proximate composition of the breast and thigh meat.

Item	5 Weeks	9 Weeks	Sig.
Breast			
Protein, %	21.10 ± 0.85	21.65 ± 0.66	ns
Intramuscular fat, %	0.54 ± 0.30	0.41 ± 0.17	ns
Moisture, %	75.40± 0.73	75.748 ± 0.61	ns
Ash, %	0.96 ± 0.06	0.99 ± 0.05	ns
Thigh			
Protein, %	17.46 ± 0.65	17.56 ± 0.61	ns
Intramuscular fat, %	3.80 ± 0.75	2.72 ± 0.57	**
Moisture, %	75.95 ± 1.01	76.81 ± 0.33	*
Ash, %	0.87 ± 0.06	0.96 ± 0.034	***

Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns—non-significant.

3.3. Fatty Acid Composition

The fatty acid composition of the meat of the male layer-type chickens differed between the two groups (Table 5).

Table 5. Fatty acid (% FAME) profile of the breast and thigh meat.

Fatty Acid	5 Weeks	9 Weeks	Sig.
Breast			
C14:0	0.61 ± 0.51	0.47 ± 0.10	ns
C14:1	0.10 ± 0.07	0.09 ± 0.08	ns
C15:0	0.26 ± 0.31	0.16 ± 0.04	ns
C16:0	20.43 ± 0.51	20.69 ± 1.18	ns
C16:1n-7	3.34 ± 0.95	2.10 ± 0.86	**
C17:0	0.15 ± 0.07	0.08 ± 0.02	*
C17:1	0.23 ± 0.14	0.63 ± 0.51	*
C18:0	8.84 ± 0.99	9.89 ± 0.99	*
C18:1n-9	30.99 ± 3.98	26.75 ± 2.53	**
C18:2n-6	23.38 ± 1.99	23.20 ± 1.68	ns
C18:3n-6	0.12 ± 0.01	0.12 ± 0.02	ns
C18:3n-3	0.69 ± 0.17	0.41 ± 0.13	***
C20:0	0.29 ± 0.08	0.26 ± 0.08	ns
C20:2n-6	0.36 ± 0.07	0.48 ± 0.17	ns
C20:3n-6	0.93 ± 0.21	1.00 ± 0.27	ns
C20:4n-6	6.25 ± 1.89	9.36 ± 1.88	**
C20:5n-3	0.19 ± 0.04	0.11 ± 0.04	***
C22:4n-6	1.46 ± 0.42	2.49 ± 0.55	***
C22:5n-3	0.67 ± 0.16	0.85 ± 0.19	*
C22:6n-3	0.71 ± 0.26	0.86 ± 0.21	ns
Thigh			
C14:0	0.43 ± 0.15	0.54 ± 0.06	*
C14:1	0.10 ± 0.05	0.16 ± 0.04	*
C15:0	0.10 ± 0.03	0.17 ± 0.04	***
C16:0	19.65 ± 1.27	19.22 ± 1.55	ns
C16:1n-7	4.39 ± 1.04	4.02 ± 1.18	ns
C17:0	0.12 ± 0.03	0.19 ± 0.03	***
C17:1	0.18 ± 0.07	0.21 ± 0.06	ns
C18:0	8.55 ± 1.01	8.43 ± 0.89	ns
C18:1n-9	36.92 ± 3.42	32.18 ± 1.88	***
C18:2n-6	23.44 ± 2.41	26.90 ± 2.21	**
C18:3n-6	0.14 ± 0.02	0.18 ± 0.02	***
C18:3n-3	0.93 ± 0.03	0.72 ± 0.08	***
C20:0	0.37 ± 0.09	0.31 ± 0.12	ns
C20:2n-6	0.19 ± 0.08	0.30 ± 0.11	*

Table 5. *Cont.*

Fatty Acid	5 Weeks	9 Weeks	Sig.
C20:3n-6	0.32 ± 0.06	0.36 ± 0.07	ns
C20:4n-6	2.84 ± 0.51	4.33 ± 0.90	***
C20:5n-3	0.06 ± 0.02	0.02 ± 0.01	**
C22:4n-6	0.68 ± 0.13	1.16 ± 0.35	***
C22:5n-3	0.32 ± 0.08	0.31 ± 0.07	ns
C22:6n-3	0.27 ± 0.08	0.29 ± 0.07	ns

Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns—non-significant.

The changes in the percentage of the individual fatty acids affected by age did not follow the same pattern in breast and thigh. The breast meat showed significantly higher contents of C17:1, C18:0, C20:4n-6, C22:4n-6, and C22:5n-3 in the older chickens. On the other hand, the breast meat of these birds displayed a lower percentage of C16:1n-7, C17:0, C18:1n-9, C18:3n-3, and C20:5n-3. More fatty acids were affected by the age in the thigh meat. The male layer-type chickens, slaughtered at nine weeks of age had a significantly higher content of C14:0, C14:1, C15:0, C17:0, C18:2n-6, C18:3n-6, C20:2n-6, C20:4n-6, and C22:4n-6, while the percentages of C18:1n-9, C18:3n-3, and C20:5n-3 were lower. In regard to the total amounts of the fatty acids, generally, the meat from older chickens had lower MUFA ($p < 0.01$) and higher PUFA ($p < 0.01$), while the total percentage of SFA remained unaffected (Table 6). The values of n-6/n-3 ratio increased with age in both breast and thighs, while the P/S ratio was higher only in the thigh meat of the older chickens. The values of AI and TI were similar in both age groups.

Table 6. Lipid nutritional indices of the breast and thigh meat.

Item	5 Weeks	9 Weeks	Sig.
Breast			
SFA	30.58 ± 1.48	31.55 ± 1.58	ns
MUFA	34.66 ± 4.74	29.57 ± 2.45	**
PUFA	34.76 ± 4.26	38.88 ± 2.30	**
n-6/n-3	14.38 ± 1.09	16.30 ± 2.13	**
P/S	1.13 ± 0.13	1.23 ± 0.11	ns
AI	0.33 ± 0.03	0.33 ± 0.03	ns
TI	0.74 ± 0.04	0.78 ± 0.04	ns
Thigh			
SFA	29.22 ± 1.31	28.86 ± 1.11	ns
MUFA	41.59 ± 3.61	36.57 ± 2.85	**
PUFA	29.19 ± 3.01	34.57 ± 33.43	**
n-6/n-3	17.47 ± 1.72	24.79 ± 2.25	***
P/S	1.00 ± 0.10	1.20 ± 0.11	**
AI	0.30 ± 0.03	0.30 ± 0.02	ns
TI	0.73 ± 0.04	0.72 ± 0.04	ns

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; P/S: poly-/saturated fatty acids; AI: atherogenic index; TI: thrombogenic index. Significance: ** $p < 0.01$; *** $p < 0.001$, ns—non-significant.

4. Discussion

Meat quality, including poultry meat, is very complex and might be affected by many factors [27]. This study illustrates the effect of age on the physical traits, chemical composition, and fatty acid profile with related lipid nutritional indices in the meat of male layer-type chickens. One of the physical traits that is known to be crucial for meat technological quality and safety is the ultimate pH [28]. The rapid pH decline postmortem and low values of pH are associated with PSE-like conditions, poor WHC, and functionality [29]. On the other hand, higher pH values can decrease the shelf life of the meat making it more susceptible to bacterial spoilage [30]. The pH also depends on the muscle type,

which could explain the difference in this trait between the breast and the thigh meat. The values of pH that were observed in this study are within the normal range according to the limits set by Zhang and Barbut [31] for the five-week-old chickens. The effect of age on pH was reported in broilers [32,33] and also in spent hens [34]. In line with our results, these studies showed lower pH in the birds slaughtered at an older age. However, in a previous experiment [35], significantly increased pH values were reported in the breast and thigh meat of male layer-type chickens slaughtered at 12 weeks of age when compared to 5-week-old chicks. Contrary to us, Lichovníková et al. [36] did not find a difference in pH in the breast meat of male layer-type chickens, reporting values similar to ours (5.73–5.77); however, they were significantly higher than the pH in broiler meat.

Water holding capacity is very important for meat functional properties and determines the quality for further meat processing [37]. It was demonstrated that both pH and WHC correlate with meat color [38,39]. The results of the trial revealed a darker color of the breast and thigh meat in the older chickens, despite the lower pH in this age group, which is usually correlated with a lighter meat color [38]. On the other hand, the higher WHC in the older birds in breast meat corresponds to the lower L*, since the lower percentage of free water decreases the light reflected from meat surface [40]. In addition to the lower L* values determined in the male chickens slaughtered at the age of nine weeks, this age group also showed lower yellowness (b*) in the breast and thigh meat. This is in line with the results in broilers [41] and in Da-Heng meat-type chickens [13].

Usually, the ageing of animals is associated with lower tenderness of meat, mainly due to a decrease in the collagen solubility [42]. In poultry, the results of the effect of age on the tenderness of meat are contradictory. In heavy lines of broilers aged 35–63d there was no effect of age at slaughter on the shear force [41]. Recently, it was found that the tenderness of breast and thigh meat of broilers slaughtered at 28 days of age was significantly higher when compared to those at 30, 32, and 34 days of age [43]. However, the authors did not observe differences between age 30 and 34 days. In slow growing chickens, Wang et al. [44], found a significant increase in the shear force of breast and thigh meat between age 63 d and 105 d. On the other hand, they found no effect of age on the shear force in native chickens reared under a semi-free-range system [45]. The values of the shear force reported in our study are similar to those of Choo et al. [46], when comparing egg-type males, white mini broilers, and commercial broilers (Ross 308). As shown in Table 3, the shear force values were higher in the breast, which can be attributed to the higher content of intramuscular fat [47]. Nevertheless, the values of the shear force that were measured in this experiment classify the meat as "very tender" (<3.62 kg) [48].

The proximate composition differs between the groups only for thigh meat. While the intramuscular fat decreased in the older chickens, the moisture and the ash content increased in these birds compared to the five-week-old birds. Decreased intramuscular fat and increased moisture with aging in chickens have been reported in previous studies with slow-growing [14] and male layer-type chickens [35]; however, results on the effect of the slaughtering age on this parameter in poultry have been rather inconsistent. Dal Bosco et al. [12] observed increased lipid content at an older age in the breast meat of various commercial chicken genotypes reared organically and little to no effect on the moisture. When comparing dual-purpose chickens with layer hybrids, Mueller et al. [49] observed decreased intramuscular fat in Lohmann Brown chickens with prolonged age, but not in the other studied hybrids. The intramuscular fat and its composition are important for organoleptic characteristics but also in the health value of meat and meat products [50]. In the present study, despite the different number of individual fatty acids affected by age in breast and thigh meat, in both meat cuts the MUFA was lower in the older birds. This was determined mostly by the decreased percentage of C18:1n-9 in the breast and thigh, and also corresponds with the decreased intramuscular fat in the meat, particularly in thighs. In a previous study on two slow-growing lines slaughtered at 9 and 18 weeks, a similar decrease in MUFA in the chickens slaughtered at an older age was observed [51]. An extensive review [52] has well outlined the useful properties of C18:1n-9 for immunomodulation,

treatment, and prevention of cardiovascular and autoimmune diseases, metabolic disorders, skin injuries, and even certain types of cancer. Hence, the decrease in the percentage of C18:1n-9 and MUFA can be a disadvantage for the male layer-type chickens slaughtered at the age of nine weeks, compared to those slaughtered at five weeks old.

On the other hand, this was compensated by the lack of changes in the SFA but a significant increase in the PUFA. It should be noted that the increase in PUFA is mainly at the expense of n-6 PUFA. The major n-6 fatty acid in the poultry meat is C18:2n-6. A significant increase in this fatty acid was found in the thigh meat of the nine-week-old chickens. Since it is essential and derived exclusively from feed, its increase in the meat of older chickens might be explained by the higher feed consumption at this age compared to those at five weeks old [48]. The content of C18:2n-6 in the feed as presented in Table 2 is 55.31%. The increase in this fatty acid was also accompanied by increase in C20:4n-6, both in breast and thigh of the older chickens. In a recent study [53], it was recommended that for normal physiological function, the body requirement for optimal n-6/n-3 ratio is approximately 1–2:1. In this study, the n-6/n-3 ratio is much higher. This indicates a certain imbalance of the fatty acid profile of the male layer-type chickens in regard to PUFA that can be improved through feeding strategies or housing systems with access to pasture [54]. Meat has often been implicated in imbalanced fatty acid intake by consumers due to some meats naturally having a low PUFA to SFA ratio (P/S). Thus a P/S ratio of no less than 0.1 is recommended [55]. In the current study, in the older chickens the P/S ratio was higher than the set limit, ranging from 1.13–1.23 in breast muscle versus 1.00 to 1.20 in thighs.

5. Conclusions

The results of the study showed a significant effect of age in meat quality parameters of male layer-type chickens that was different in the different meat portions (breast and thigh). Generally, the chickens slaughtered at nine weeks of age displayed a lower pH and darker color. In regard to the nutrient components, older age was associated with a significant decrease in intramuscular fat in thighs and tended to diminish in breast meat. Its reduced content corresponded to the lower MUFA in the nine-week-old layer-type cockerels. On the other hand, the meat of older chickens was richer in PUFA, especially n-6, significantly increasing the n-6/n-3 ratio. As a whole, the meat of nine-week-old male layer-type chickens showed certain disadvantages in regard to the fatty acid profile which opens the possibilities for further studies on different feeding strategies or housing systems to improve this trait.

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Effects of a Blend of Live Yeast and Organic Minerals as an Alternative to Monensin on Intake, Digestibility, Performance and Beef Quality of Nellore Bulls Finished on Pasture with High Concentrate Supplementation

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Abstract: Effects of a blend of live yeast and organic minerals as an alternative to monensin and inorganic trace minerals for beef cattle finished on pasture with high concentrate supplementation, on growth performance, intake, digestibility, liver trace mineral and carcass characteristics were evaluated. Forty-eight Nellore bulls were blocked by initial body weight and randomly assigned to one of the two experimental diets. The animals were raised in an experimental pasture divided into 12 paddocks equipped with an electronic system for monitoring individual feeding behavior and feed intake. Treatments were: (1) Monensin (MON), 30 mg/kg supplement dry matter of sodium monensin and trace minerals supplementation from inorganic sources; (2) AdvantageTM (ADV), 1.6 g/kg supplement DM of a blend of live yeast (*Saccharomyces cerevisiae* strains) and organic trace minerals. The use of ADV instead of MON led to greater supplement intake and greater intake of dietary components. Bulls fed ADV also had higher digestibility of dry matter, organic matter, crude protein and non-fiber carbohydrates. Bulls fed MON had a greater number of visits to the feeder, however with a shorter time spent feeding per visit. The use of ADV resulted in higher average daily weight gain, and feed efficiency was similar between treatments. In the carcass, ADV tended toward greater Longissimus muscle area. Liver Zn concentration tended to be lower in the ADV treatment. The use of ADV generated higher meat lightness and redness. In summary, the blend of live yeast and organic minerals can be an alternative to monensin and inorganic sources of trace minerals for beef cattle finished on pasture with high concentrate supplementation, without negative effects on supplement feed efficiency and with benefits to animal growth.

Keywords: carcass; chromium supplementation; ionophore; nutrition; yeast-based blend

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1. Introduction

Technologies in feed additives are of interest in ruminant nutrition, to promote health, growth, feed efficiency and improvement on carcass characteristics, thus bringing greater productivity to livestock activity. Sodium monensin is an ionophore that has been used to improve feed efficiency and decrease the risk of acidosis in finishing beef cattle [1,2]. However, there is a growing demand from consumers for meat produced free from the use of antibiotics, due to the public concern of increasing antimicrobial resistance in people.

Several non-antimicrobial products have been evaluated as feed additives, especially for cattle fed high-concentrate diets, in order to replace the use of monensin without impairing feed efficiency and rumen health [3,4]. Yeast-based products have been studied for

this purpose, however with variable results [3,5]. Batista et al. [6], in a meta-analysis study considering several yeast-based feed additives, reported that the main benefits of yeast products for beef cattle were in improving digestibility and rumen health, and generating greater average daily gain (ADG). However, the authors emphasize that the benefits in animal performance may be present in low magnitudes [6], requiring investments in new technologies in yeast products, such as research on specific strains and combination with other technologies, in order to achieve greater benefits in animal performance.

A topic that has been of increasing interest in cattle supplementation is the use of organic complexed sources of Co, Cu, Mn, Se and Zn in place of inorganic sources [7]. This is in addition to the supplementary use of Cr, which was established as essential by NASEM [8], however without established requirements. In this context, organic sources of trace minerals have been recognized for showing greater absorption, retention and biological activity compared to inorganic minerals [9–11], thus enabling the reduction of supplementary levels without harming the mineral status during a finishing period. Vellini et al. [12] observed greater feed efficiency and Longissimus muscle area (LM area) in feedlot-finished Nellore bulls with supplementation of zinc amino acid complex in association with chromium methionine, compared to mineral supplementation from inorganic sources including zinc, but without chromium. In addition, the use of a blend of live yeast and organic minerals instead of monensin or a diet without feed additives increased milk and solids yield of dairy cows during the hot season [13].

Because of the possible improvement on digestibility, rumen health and performance, with the use of live yeast and the positive impacts of organic sources of trace minerals, including chromium, on feed efficiency and carcass characteristics, we hypothesized that the combination of technologies, such as specific strains of live yeast + organic minerals, could replace the traditional use of monensin and inorganic sources of trace minerals, during the finishing phase of beef cattle. Therefore, the objective of this experiment was to evaluate the effects of a blend of live yeast and organic minerals as an alternative to monensin and inorganic trace minerals on intake, digestibility, performance and beef quality of Nellore bulls finished on pasture with high concentrate supplementation.

2. Materials and Methods

All experimental procedures in this study were carried out in accordance with the ethical principle established by the Brazilian Council for the Control of Animal Experimentation and approved by the Ethics Committee for the Use of Animals of the Sao Paulo State University, College of Agricultural and Veterinary Sciences, Sao Paulo, Brazil (process no. 014782/19).

2.1. Experimental Area, Animals, Treatments

This experiment was conducted from June to October 2018 at the Research Center of APTA (Agência Paulista de Tecnologia dos Agronegócios) in Colina, São Paulo, Brazil. The experimental period comprised the dry season in central Brazil (20°43′5″ S, 48°32′38″ W), which is characterized by low rainfall and low pasture quantity and nutritional quality. The area was an experimental pasture, consisting of Marandu grass (*Brachiaria brizantha* cv. Marandu) implanted at the beginning of the rainy season of 2015, and divided into 12 paddocks of 1 ha each. The paddocks were equipped with an electronic system for monitoring individual feeding behavior and feed intake (Intergado Ltd., Contagem, Minas Gerais, Brazil; [14]). Each paddock was grazed by four animals (Two/treatment), with individual access to the electronic feeder for each animal. The electronic feeders allow the intake of concentrate by only one animal per specific feeder, allowing the application of treatments with different supplementation strategies and/or different feed additives, with the animals being in the same pasture, therefore the same grazing conditions.

In total, 52 Nellore bulls (BW = 519 ± 25 kg, 33 ± 3 months) distributed in a randomized complete block design were used. Animals were stratified by initial body weight (initial BW) and distributed to the grazing area. Four animals, representative of different

BW blocks, were slaughtered at the beginning as the baseline group to represent the initial carcass weight of the remaining animals (n = 48). All animals originated from the same research center and had the same feed and health management throughout the growth phase, until the beginning of the experiment, being raised in a pasture area with free access to water and mineral supplement from inorganic sources.

The experimental period lasted 112 days, divided into four periods of 28 days. This period comprised the adaptation phase to concentrate intake and the finishing phase of the bulls on pasture, where high concentrate supplementation was provided (Table 1). This system of finishing cattle on pasture has been practiced in Brazil with satisfactory results in terms of productivity and meat quality [15,16]. Adaptation to concentrate intake occurred in the first 28 days (first experimental period). It started with the offer of concentrate at 1% of the initial BW, and every three days the offer was increased by 0.25% of the BW, until reaching the equivalent of 2% of the BW, after which concentrate was offered ad libitum.

Table 1. Ingredient and nutrient composition of supplements.

Item (g/kg of DM)	MON [1]	ADV [2]
Cracked corn	802	802
Citrus pulp	100	100
Peanut meal	60	60
Urea	10	10
Optigen®	5.0	5.0
Macromineral mix [3]	22.3	22.3
Inorganic trace mix [4]	0.126	-
Advantage™ [5]	-	1.60
Rumensin 200	0.153	-
Chemical Analysis, g/kg of DM		
Crude protein	127	127
Neutral detergent fiber	158	158
Ash	25.7	25.7
Ether extract	34.9	34.9
Total carbohydrate	775	775
Non-fibrous carbohydrate	616	616

[1] Supplement with monensin (30 mg/kg of DM) and trace minerals from inorganic sources. [2] Monensin-free supplement, with a blend of live yeast and organic minerals. [3] Containing (Supplement DM basis) 6.5 g/kg limestone, 5.0 g/kg dicalcium phosphate, 1.1 g/kg sulfur, 1.1 g/kg magnesium oxide, 4.3 g/kg potassium chloride, 4.3 g/kg salt. [4] Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. Sources of trace mineral included copper sulfate, zinc oxide, manganese monoxide, calcium iodate, sodium selenite, and cobalt sulfate. [5] Feed additive with live yeast (*Saccharomyces cerevisiae* strains 1026 + 8417; amount of 3.2 × 108 cfu/g in the final supplement) and organic sources of trace minerals. Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr. Sources of trace mineral included copper proteinate, manganese proteinate, zinc proteinate, iron proteinate, selenium-enriched yeast, and chromium yeast.

The experimental treatments were (1) Monensin (MON), 30 mg/kg supplement DM of sodium monensin (Rumensin; Elanco) and trace minerals supplementation from inorganic sources at the following rates (mg/kg) in concentrate: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. Inorganic trace mineral sources included copper sulfate, zinc oxide, manganese monoxide, calcium iodate, sodium selenite, and cobalt sulfate; it was formulated to meet or exceed NASEM [8] recommendations. (2) Advantage™ (ADV), 1.6 g/kg supplement DM of Advantage (Alltech®). Advantage™ is a blend of live yeast (*Saccharomyces cerevisiae* strains 1026 + 8417; 3.2×10^8 cfu/g) and organic minerals supplemented at the following rates (mg/kg) of concentrate: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr. Organic trace mineral sources included copper proteinate, manganese proteinate, zinc proteinate, iron proteinate, selenium-enriched yeast, and chromium yeast.

Monensin inclusion level was in accordance with Lemos et al. [17]. Advantage™ was provided following manufacturer's recommendations (Expected daily intake of 3 g/100 kg

of BW). Both additives were provided mixed with the concentrate. Concentrate supplement were formulated to meet or exceed NASEM requirements [8] for protein and energy, to an average daily gain of 1.5 kg (Table 1). The concentrate supplement was provided once daily (08:00 h) in quantity for ad libitum intake, with an expected intake of approximately 2% of BW. The amount of concentrate offered was adjusted when the leftovers were greater than 10% for four consecutive days; this criterion was established to avoid variations in the daily dry matter intake.

2.2. Pasture Characteristics

Every paddock received all the treatments; therefore, the grazing conditions were similar across treatments. Quantitative and qualitative assessments of the pasture conditions, measuring forage mass, structural components, and chemical composition, were performed (Table 2) at the beginning of the experiment and every 28 days. The forage mass was estimated using the double sampling method [18]. The quantitative and structural components of the forage sward were evaluated at medium heights. Forage samples were separated into green leaf, dead leaf, green stem, and dead stem. After separation, forage components were weighed and oven-dried at 55 °C for 72 h to obtain the partial DM and the proportion of each component in the forage sward (Table 2). Hand-plucked samples were used to estimate the dietary nutritional value [19]. Samples were oven-dried at 55 °C for 72 h and then ground in a Wiley mill (Thomas Model 4, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1 and 2-mm mesh sieve, for further analysis. Samples were analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF), organic matter (OM), lignin, ether extract (EE), non-fibrous carbohydrate (NFC) and nitrogen (N).

Table 2. Characteristics of the forage sward, grazed by Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Periods					SEM
	d 0	d 28	d 56	d 84	d 112	
Quantitative and structural characteristics						
Sward height, cm	38.4	30.7	28.5	26.5	29.0	2.30
Forage mass, kg DM/ha	5946	4331	3961	3271	3802	508
Senescent leaf, g/kg DM	208	210	190	270	80.0	35.0
Senescent stem, g/kg DM	694	790	810	730	750	23.0
Green leaf, g/kg DM	4.03	0.0	0.0	0.0	120	27.0
Green stem, g/kg DM	94.0	0.0	0.0	0.0	50.0	21.0
Chemical composition, g/kg DM						
Organic matter	943	934	956	955	947	4.80
Neutral detergent fiber	830	839	840	841	717	2.51
Acid detergent fiber	499	505	502	505	384	1.40
Lignin	90.8	92.9	91.1	97.0	52.7	1.41
Crude protein	34.2	32.8	32.9	27.9	79.6	1.46
Ether extract	9.92	7.55	6.02	6.41	11.83	0.82
Non-fibrous carbohydrate	68.4	54.7	77.0	77.5	139	5.25

In vitro dry matter digestibility (IVDMD).

2.3. Feed Intake and Digestibility

Individual concentrate supplement intake was monitored daily throughout the experimental period, by calculating the difference between the DM of concentrate offered and refusals. The behavior of concentrate supplement intake in terms of time spent feeding per day, number of visits per day to the feeder, and time spent feeding per visit were monitored daily by the electronic system for monitoring individual feeding behavior and feed intake. The mean per animal per period was considered later for statistical analysis.

Forage intake was determined in 24 animals randomly selected from the 48 experimental animals. To determine fecal excretion, the external marker, titanium dioxide (TiO_2) and the internal marker, indigestible NDF (iNDF) were used [20]. Estimates of forage intake and total tract digestibility were evaluated in the second and fourth experimental periods (d 28 to 56 and d 84 to 112). The TiO_2 was offered during the last 10 days of the respective experimental period at 10 g/animal/d. Six days of supply aimed at establishing the marker's fecal flow, and the last four days to collect fecal samples, respectively, at: 1700, 1300, 1000, and 0700 h, to represent fecal excretion during the day [21]. Feces were pooled by animals within period, dried to a constant weight at 55 °C, ground as described above, and stored for analysis of chemical composition and TiO_2. On the same days of fecal collections, a hand-plucked forage sample was used to quantify the iNDF of forage.

Forage DMI was estimated as previously described by Miorin et al. [22], where the fecal output of the internal marker (iNDF) was corrected for the contribution of the concentrated supplement as follows:

$$\text{Forage DMI} = \text{FE} \times [\text{iMF}] - \text{DMIS} \times [\text{iMS}]/[\text{iMH}]$$

where FE is the fecal excretion, DMIS is the DMI of supplement, [iMF], [iMS] and [iMH] are the concentrations of the internal marker in feces, supplement, and forage, respectively. Total DMI was obtained by addition of forage and supplement DMI. The total tract apparent digestibility was estimated using the following model:

$$\text{DM digestibility} = (\text{DMI} - \text{FE})/\text{DMI} \times 100$$

Digestibility of DM, OM, CP, NDF, EE and NFC were calculated based on the intake and fecal excretion of individual components.

2.4. Animal Performance and Carcass Assessments

Bulls were weighed at the start (0 d) of the experiment, and at the end of each experimental period, after fasting for 16 h (feed and water). Average daily gain (ADG) was calculated for each experimental period. Concentrate supplement feed efficiency (supplement G:F) was calculated by dividing the ADG by the supplement intake.

As mentioned above, four animals were slaughtered at the beginning of the experiment (randomly selected within the BW blocks, to estimate the initial carcass weight of the remaining animals. Animals were transported to the slaughterhouse (Minerva Foods, Barretos, SP, Brazil) located 20 km from the research facility. After arrival at the slaughterhouse, animals were kept in resting pens for 18 h (free access to water) and then submitted to humanitarian slaughter under Brazilian Federal Inspection, and the hot carcass weight (HCW) was obtained. The hot carcass weight (HCW) was measured after evisceration without kidneys, pelvic, and heart fat. Carcass gain was calculated using a linear equation to predict the initial HCW. The equation was applied to the initial BW (kg) to determine a predicted initial HCW (kg), as follows:

$$\text{HCW}_{\text{initial}} = -22.866 + 0.6038 \times \text{BW}_{\text{initial}} \ (R^2 = 0.99)$$

At the end of the evaluation period, all remaining animals were slaughtered following the same procedure as that of the baseline groups, and the final HCW was obtained. Subsequently, the carcasses were placed in a cold chamber at 2 °C for 24 h. Carcass-adjusted gain was determined by subtracting the final HCW from the initial HCW dividing the results by the days on evaluation period. Twenty-four hours after slaughter, ribeye area and backfat thickness were measured on the left side of the carcass, between the 12th and 13th ribs [23]. Twenty-four hours after slaughter and cooling, four 2.54 cm-thick steaks from *longissimus thoracis* muscle were collected from the left side of the carcass from the 13th rib toward the head for chemical composition, color, Shear Force and cooking loss analyses. Each steak was identified, and vacuum packed in polyethylene bags (water vapor

permeability < 10 g/m^2/24 h at 38 °C and oxygen permeability < 40 mL/m^2/24 h at 25 °C) and stored at −20 °C until further analysis.

2.5. Liver Samples

Liver samples were collected on the day of slaughter from the left lobe of each liver after being inspected by the Brazilian Federal Inspection personnel. Each sample was placed in numbered cryotubes corresponding to the carcass order, placed on ice and transported to the Research Center Laboratory and stored at −80 °C until analyzed for trace mineral concentrations. Liver samples were collected only at slaughter: (1) because our goal was to evaluate the final liver trace mineral concentrations of bulls after receiving the supplementation during the finishing period, and (2) to avoid a surgery-induced inflammatory response in the beginning of the study that could interferes with growth performance and physiological parameters [24]. It is worth mentioning that before the beginning of the experiment the animals were raised in the same group, grazing and mineral supplementation conditions, therefore the possibility of variation between animals in the initial liver trace mineral concentrations, were randomly distributed among the treatments at the beginning of the experiment.

2.6. Laboratory Analyzes

Samples of concentrates, forage and feces previously ground to 1 mm were analyzed for contents of DM (method 930.15; [25]), CP (N × 6.25; Kjeldahl method 984.13; [25]), ether extract (method 920.39; [25]), ADF and lignin (method 973.18; [25]), ash (method 942.05; [25]), and NDF using α-amylase [26]. The NFC content was calculated according to Hall et al. [27] as NFC = 1000 − [(NDF − NDIP) + CP + ether extract + ash], with values expressed as grams per kilogram of DM. The indigestible NDF of forage, concentrates, and feces was determined by an in-situ incubation procedure for 288 h [20]. After incubation, bags were removed from the rumen, washed in running tap water until bleaching, and subjected to NDF analysis as previously described. The determination of TiO_2 concentrations in feces was performed through spectrophotometry read at 410 nm as described by Myers et al. [28]. Liver samples were sent to a commercial laboratory (Bio Minerais Análises Químicas Ltda, Campinas, São Paulo, Brazil) for analysis of the concentrations of Cu, Mn, Se and Zn using inductively coupled plasma atomic emission spectrometry–ICAP 6300–Thermo scientific.

For the proximate analysis, steaks were thawed overnight at room temperature (4 °C), ground and used to analyze composition (protein, ether extract, ash and moisture) using near infrared analyses (AOAC method: 2007–04) using a Foodscan™ (FOSS, Hillerod, Denmark). The cooking loss was performed according to Aroeira et al. [29]. The steaks were weighed and grilled at 160–180 °C until their center reached an internal temperature of 71 °C [30]. After temperature stabilization, the steaks were weighed, and cooking loss was calculated as a percentage of the weight of the steaks before the cooking process. Shear force was analyzed using the Warner–Bratzler square shear force method. Six rectangular core samples (1.0 × 1.0 × 2.5 cm) were manually removed from each steak parallel to the muscle fibers. The core was completely sheared perpendicular to the muscle fibers in the Texture Analyzer (Brookfield, model CT325K, Middleboro, MA, EUA) with Warner–Bratzler blade of a 1.016 mm and 200 mm min–1 of speed. The maximum force (kg) was measured, and the average value was calculated for each steak.

The instrumental color analysis was performed on the surface of the steaks using a CM–700 spectrophotometric colorimeter and the CIELAB system with an 8 mm aperture, illuminant A, and 10° observer angle. Before the color readings, each steak was removed from the vacuum package and exposed to atmospheric air for 30 min for blooming and oxygenation of myoglobin. The lightness (L*), redness (a*), and yellowness (b*) components were recorded using an average of five readings per steak. The polar coordinates chroma (C*) and hue angle (h*) were also determined as: C* = [(a*)2 + (b*)2] × 0.5 and h* = tan − 1 (b*/a*).

2.7. Statistical Analysis

All data were analyzed as randomized complete block design using the MIXED procedures of SAS® University Edition software. Two bulls per treatment per paddock were the experimental unit, where bull(paddock) was included as random effect in all analyses. The variables when evaluated over the experimental periods (Supplement Intake, Supplement intake behavior, forage intake, digestibility, ADG and supplement G:F) were analyzed as repeated measures, and tested for fixed effects of treatment, time, and resulting interaction, using paddock(treatment) as the subject. Different covariance structures were tested with the final choice depending on lowest value for the Akaike information criterion. The variables that were not evaluated by period: Carcass and meat parameters, and liver trace minerals, were used in the model as a fixed effect only the effect of treatments. All results are reported as least squares means. Data were separated using PDIFF if a significant F-test was detected. Significance was set at $p \leq 0.05$, and tendencies were determined if $p > 0.05$ and ≤ 0.10.

3. Results

Concentrate supplement intake, when measured during the periods of simultaneous evaluation of forage intake and apparent digestibility (d 28 to 56 and d 84 to 112), was 18.9% higher in animals receiving ADV ($p < 0.01$) compared to animals supplemented with monensin and inorganic sources of trace minerals (MON; Table 3). However, the forage intake was not different between treatments ($p = 0.73$), also there was no Treat (treatment) × Per (period) interaction ($p = 0.75$). There was an effect of the experimental period ($p < 0.01$) on the estimate of forage intake, where a lower intake was observed between d 28 to 56 (1.88 kg of DM/d) and higher intake observed in the final period (d 84 to 112; 2.37 kg of DM/d). The total DM intake was 14.6% higher in animals receiving ADV ($p < 0.01$), consequently there was a higher intake of OM, CP, EE, and NFC ($p \leq 0.01$), and a trend towards higher intake of NDF ($p = 0.10$). The use of ADV instead of MON also resulted in greater digestibility of DM, OM, CP ($p \leq 0.04$), and tended toward greater digestibility of NFC ($p = 0.06$), however there was no effect of treatments or Treat × Per interaction on digestibility of NDF ($p \geq 0.53$).

Table 3. Intake and digestibility of Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Treatment [1]		SEM	*p*-Value		
	MON	ADV		Treat	Per	Treat × Per
			Intake, kg/d			
Supplement	8.50	10.49	0.41	<0.01	0.27	0.90
Forage	2.15	2.10	0.10	0.73	<0.01	0.75
Total DM	10.81	12.66	0.41	<0.01	0.48	0.79
OM	10.45	12.26	0.40	<0.01	0.52	0.78
CP	1.16	1.41	0.05	<0.01	0.50	0.87
NDF	3.16	3.45	0.12	0.10	0.18	0.98
EE	0.370	0.415	0.01	0.01	<0.01	0.40
NFC	5.84	7.17	0.28	<0.01	0.28	0.88
			Digestibility, g/kg DM			
DM	625.6	672.9	16.3	0.03	0.03	0.80
OM	660.0	701.1	15.3	0.04	0.03	0.77
CP	525.0	607.4	25.6	0.02	0.01	0.45
NDF	527.6	528.2	14.8	0.97	< 0.01	0.53
EE	764.7	769.0	20.9	0.87	< 0.01	0.11
NFC	776.1	813.7	14.5	0.06	0.48	0.76

[1] Treatment, MON: Supplement with monensin (30 mg/kg of DM) and supplemental trace minerals from inorganic sources. Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. ADV: Monensin-free supplement, with a blend of live yeast and organic minerals (Advantage; Alltech), live yeast (*S. cerevisiae* strains 1026 + 8417; 3.2 × 108 cfu/g). Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr.

Concentrate supplement intake, when evaluated throughout the experimental period (d 0–112) was 9.5% higher in animals receiving ADV instead of MON (Table 4). However, the intake was similar (p = 0.27) during the first experimental period (d 0–28), which involved the adaptation of the animals to a high concentrate diet. In this context, post-adaptation intake (d 29–112) was 12.2% higher in animals receiving ADV. The behavior of supplement intake in terms of time spent feeding per day, number of visits per day to the feeder, and time spent feeding per visit are shown in Figure 1. There was effect of the experimental period for all responses ($p < 0.01$). However, there was no Treat × Per interaction ($p \geq 0.19$). Regarding the treatment effects, it was observed that the animals supplemented with ADV had a lower number of visits to the feeder and a longer time spent feeding per visit ($p < 0.01$), with no significant change in the total time spent feeding per day (p = 0.38).

Table 4. Supplement intake and animal performance of Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Treatment [1]		SEM	*p*-Value
	MON	ADV		
Supplement Intake, kg/d				
Adaptation (d 0–28)	7.51	7.77	0.19	0.27
Post-adaptation (d 29–112) [2]	9.20	10.48	0.39	<0.01
Total (d 0–112)[2]	8.80	9.73	0.27	<0.01
Performance, live measures				
Initial BW, kg	518.6	518.4	7.1	0.97
Final BW, kg	635.0	647.7	8.9	0.22
ADG adaptation, kg	0.41	0.46	0.11	0.73
ADG post-adaptation, kg [2]	1.30	1.42	0.05	0.02
ADG (d 0–112), kg [2]	1.07	1.18	0.05	0.06
G:F adaptation	0.053	0.056	0.015	0.88
G:F post-adaptation [2]	0.148	0.142	0.006	0.47
G:F d 0–112 [2]	0.126	0.121	0.006	0.61

[1] Treatment, MON: Supplement with monensin (30 mg/kg of DM) and supplemental trace minerals from inorganic sources. Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. ADV: Monensin-free supplement, with a blend of live yeast and organic minerals (Advantage; Alltech), live yeast (*S. cerevisiae* strains 1026 + 8417; 3.2 × 108 cfu/g). Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr. [2] Least-squared means are based on repeated measures.

Supplementation with ADV tended (p = 0.06) toward greater ADG (9.3% or 0.110 kg/d) considering the entire experimental period (Table 4). Although no Treat × Per interaction was observed (p = 0.61), ADG measured only in the first period (d 0–28) was a similar between the treatments (p = 0.73), while the post-adaptation ADG was higher (p = 0.02) in animals fed ADV (8.4% or 0.120 kg/d). However, the final BW was similar between treatments (p = 0.22). Additionally, there were no differences between treatment on measures of feed efficiency of concentrate intake (Supplement G:F; $p \geq 0.47$).

In the evaluation of animal performance based on carcass gain (Table 5), there were no differences in carcass ADG or final HCW ($p \geq 0.16$). Nevertheless, ADV fed bulls tended toward greater LM area (5.8%; p = 0.07), without a difference in subcutaneous fat thickness (p = 0.92).

Liver Cu, Mn, Se and Zn data are shown in Table 6. There were no treatment differences in liver concentrations of Cu, Mn and Se ($p \geq 0.27$). However, ADV supplementation tended (p = 0.06) to result in a lower concentration of Zn.

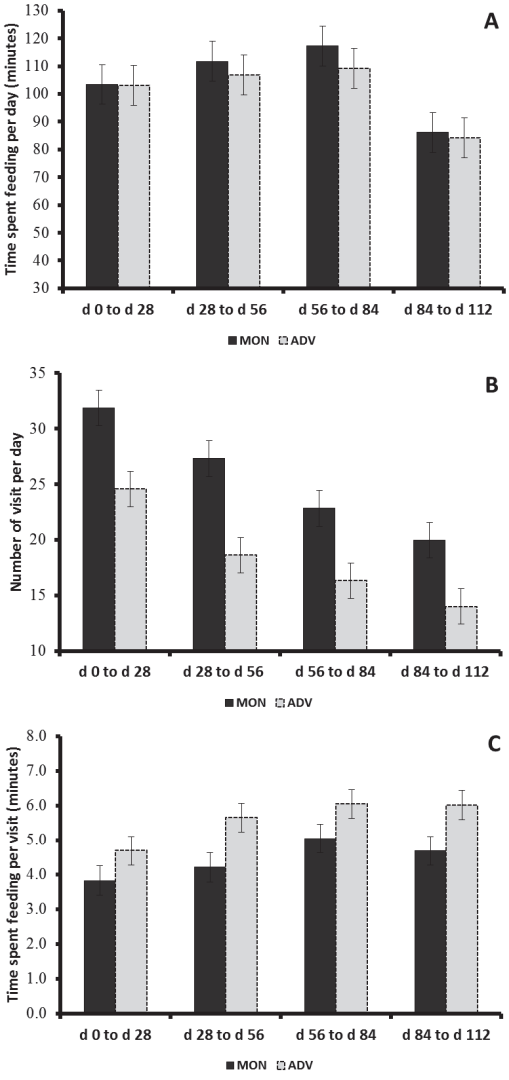

Figure 1. Supplement intake behavior of Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV). Time spent feeding per day (**A**), statistical effects: Period ($p < 0.01$), Treatment ($p = 0.38$), interaction Treat × Per ($p = 0.94$); Number of visits per day (**B**), statistical effects: Period ($p < 0.01$), Treat ($p < 0.01$), interaction Treat × Per ($p = 0.74$); Time spent feeding per visit (**C**), statistical effects: Period ($p < 0.01$), Treat ($p < 0.01$), interaction Treat × Per ($p = 0.19$).

In the evaluations of meat quality characteristics, there were no differences in meat proximate analysis ($p \geq 0.19$; Table 7), cooking loss ($p = 0.28$) or Warner–Bratzler Shear Force ($p = 0.69$). However, there was a tendency for ADV fed animals to have a greater carcass pH ($p = 0.08$). On meat colorimetric parameters, bulls fed ADV had higher L* and b* than those fed MON ($p \leq 0.03$).

Table 5. Carcass measures of Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Treatment [1]		SEM	*p*-Value
	MON	ADV		
Initial HCW, kg	290.3	290.1	4.2	0.95
Final HCW, kg	376.1	385.5	5.6	0.20
Carcass ADG, kg	0.77	0.84	0.03	0.16
Supplement G:F	0.090	0.088	0.004	0.70
Dressing, %	59.2	59.2	0.30	0.98
Fat thickness, mm	4.08	4.12	0.39	0.92
LM area, cm^2	74.9	79.5	1.79	0.07

[1] Treatment, MON: Supplement with monensin (30 mg/kg of DM) and supplemental trace minerals from inorganic sources. Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. ADV: Monensin-free supplement, with a blend of live yeast and organic minerals (Advantage; Alltech), live yeast (*S. cerevisiae* strains 1026 + 8417; 3.2 × 108 cfu/g). Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr.

Table 6. Liver concentrations of Cu, Mn, Se and Zn in Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Treatment [1]		SEM	*p*-Value
	MON	ADV		
		Minerals		
Cu, mg/kg	493.0	532.7	50.8	0.78
Mn, mg/kg	9.70	9.69	0.349	0.98
Se, mg/kg	1.32	1.23	0.064	0.27
Zn, mg/kg	174.2	157.6	5.18	0.06

[1] Treatment, MON: Supplement with monensin (30 mg/kg of DM) and supplemental trace minerals from inorganic sources. Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. ADV: Monensin-free supplement, with a blend of live yeast and organic minerals (Advantage; Alltech), live yeast (*S. cerevisiae* strains 1026 + 8417; 3.2 × 108 cfu/g). Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr.

Table 7. Meat characteristics of Nellore bulls finished on pasture with high concentrate supplementation, with monensin (MON) or a blend of live yeast and organic minerals (ADV).

Item	Treatment [1]		SEM	*p*-Value
	MON	ADV		
pH	5.58	5.69	0.04	0.08
		Proximate analysis		
Moisture, g/kg	744	741	2.10	0.34
CP, g/kg	229	229	1.02	0.73
EE, g/kg	16.8	19.4	1.60	0.19
Ash, g/kg	10.3	10.4	0.11	0.54
		Colorimetric parameters		
Ligthness (L*)	35.7	36.9	0.43	0.03
Redness (a*)	17.0	17.5	0.33	0.25
Yellowness (b*)	12.5	13.8	0.25	<0.01
Chroma (C*)	21.2	22.0	0.44	0.21
Hue angle (h*)	36.6	37.2	0.29	0.11
Cooking loss, %	31.2	32.5	0.85	0.28
WBSF, (N) [2]	54.0	52.4	2.94	0.69

[1] Treatment, MON: Supplement with monensin (30 mg/kg of DM) and supplemental trace minerals from inorganic sources. Supplemental trace minerals per kg of supplement: 0.68 mg Co, 12 mg Cu, 1 mg I, 15.01 mg Mn, 45.6 mg Zn, 0.18 mg Se. ADV: Monensin-free supplement, with a blend of live yeast and organic minerals (Advantage; Alltech), live yeast (*S. cerevisiae* strains 1026 + 8417; 3.2 × 108 cfu/g). Supplemental trace minerals per kg of supplement: 0.14 mg Co, 5.34 mg Cu, 0.67 mg I, 10.62 mg Mn, 6.40 mg Fe, 16.0 mg Zn, 0.12 mg Se, 0.53 mg Cr.
[2] WBSF = Warner–Bratzler Shear Force.

4. Discussion

Sodium monensin is the feed additive that has been widely used for beef cattle finished in feedlots in Brazil [2]. Using a meta-analytic approach, Duffield et al. [1] demonstrated that the use of monensin in growing and finishing beef cattle diets can improve animal performance by reducing DMI (3%) and increasing both ADG (2.5%) and feed efficiency (6.4%). According to Tedeschi et al. [31], the main effects of feeding monensin to ruminants include reduction of feed intake, inhibition of ruminal protein degradation, thus increasing protein escape from the rumen, and an increase in propionate and decrease in methane production in the rumen. Our hypothesis was that a new feed additive, combining live yeast and organic trace minerals, would support performance in finishing beef cattle receiving high concentrate supplementation, without the use of monensin. In fact, a recent meta-analysis demonstrated the benefits of using yeast-based products for beef cattle fed high-grain diets, in terms of nutrient digestibility, rumen health, and gains in DMI, ADG and FE, however, the performance benefits may be of low magnitude [6]. In this context, the authors suggest that new technologies in yeast products, in terms of specific strains and combinations with other technologies, can be developed in order to complement the current benefits and obtain greater gains in animal performance [6].

The higher concentrate intake (9.5%) in animals receiving ADV, in relation to the MON treatment, may be related to the expected reduction in intake with the use of monensin in relation to a diet without feed additives [1]. This is in addition to a possible stimulus to consumption with the use of ADV, due to the action of yeast benefitting rumen health [6]. Monensin has been responsible for changes in the rumen microbiota, greater propionate production and changes in satiety mechanisms, which can result in a reduction in the amount of feed ingested and an increase in the frequency of meals [32]. In fact, in our data regarding the behavior of supplement intake, it was observed that animals receiving MON had a higher number of visits per day to the supplement and a shorter time spent feeding per visit. However, the total time spent feeding per day was not different between MON and ADV, due to the fact that although ADV generated fewer visits, the time spent feeding also increased, a behavior that, associated with higher supplement intake in this treatment, indicates that the animals in ADV had a higher consumption of concentrate per visit to the feeder. This behavior could favor the occurrence of metabolic disorders associated with high concentrate intake, such as the occurrence of subacute ruminal acidosis (SARA) [33]. However, the high intake of concentrate in ADV and the good animal performance in this treatment indicates that the feed additive was able to maintain the rumen health of the bulls, since one of the main problems in the occurrence of SARA is irregular intake and reduced performance [33]. Yet, studies evaluating rumen pH and fermentative profile using the present blend of yeasts and organic minerals are necessary to test this hypothesis.

During the periods of evaluation of forage intake and digestibility (d 28 to 56 and d 84 to 112), greater intake of concentrate supplement in ADV was also observed. However, the forage intake was not different between treatments, demonstrating that within the production model of this study, the results comparing feed additives reflect only on the consumption of the concentrate. Few studies to date have evaluated forage intake by cattle finished on pasture with high concentrate supplementation. Simioni et al. [15] evaluated pasture intake in beef cattle of different genotypes (Nellore, $\frac{1}{2}$Angus and $\frac{1}{2}$Senepol) finished with supplementation level similar to that adopted in the present study, and observed that the participation of forage in the diet ranged from 6.26 to 14.5%. In the present study, the proportion of forage in the diet ranged from 16.5 to 19.8% in ADV and MON, respectively. The difference observed between the experimental periods on forage intake may be related to the occurrence of rain during the final experimental period, which led to the emergence of green leaves available for grazing, which may have favored the ingestion of pasture in the final evaluation period.

The evidence in this study indicates that the greater total DM intake observed with the use of ADV in relation to the use of MON, can be attributed to the greater intake of concentrate. Consequently, the consumption of diet components (OM, CP, NDF, NFC) was

higher in ADV. Shen et al. [34] also reported higher intake of DM, OM, NDF and starch in finishing beef heifers with the use of a *Saccharomyces cerevisiae* fermentation product compared to the use of antibiotics (monensin + tylosin). In addition to the higher intake, the use of ADV generated greater digestibility of DM, OM, CP and tended toward greater NFC digestibility. In fact, one of the most consistent benefits of using yeast products has been the improvement in diet digestibility [6,35,36]. Many of these benefits have been attributed to an improvement in the rumen environment, where the addition of yeast can stimulate specific ruminal microorganisms [37]. Increased concentrations of ruminal fibrolytic bacteria have been observed using yeast supplementation [38,39]. In this context, although it is frequent in studies using yeast products to observe an effect on NDF digestion [6,35,36], in the present study, the use of ADV did not affect NDF digestibility. In fact, Batista et al. [6] observed, in the meta-analysis study, high heterogeneity between studies in NDF digestibility responses and attributed, as a possible source of heterogeneity, factors such as the type of forage used, the amount of fiber in the diet, as well as the use of fiber-rich co-products. In the present study, the lack of effect on NDF digestibility is probably related to the low concentration of NDF in the concentrate, consequently little NDF ingested from it and the low quality of the fiber from the pasture.

The higher average daily gain observed with the use of ADV in the post-adaptation periods and the tendency for greater ADG when evaluated throughout the experimental period may be associated with the greater concentrate supplement intake in these periods, as well as the greater digestibility of nutrients observed with the use of ADV. In the same way that ADG was similar between treatments in the first experimental period, concentrate supplement intake was not different between treatments. It is worth mentioning that in this period there was a gradual increase in the supply of concentrate for 15 days in order to adapt the animals to the high-concentrate diet. Therefore, there was no ad libitum offer of the concentrate during this period. Thus, with the limited supply of concentrate in part of the period, associated with changes in rumen dynamics, passage rate, and consequently a change in the amount of gastrointestinal content, due to the transition from a diet high in forage to a diet with high concentrate proportion [40], may have influenced the possibility of observing the effect of treatments on ADG measurement in this first period.

According to our knowledge, so far, this was the first study to evaluate the use of an additive based on live yeasts and organic minerals as an alternative to monensin for beef cattle finished on pasture with high concentrate supplementation. Batista et al. [6] reported greater ADG in beef cattle with the use of several yeast products, however, in magnitudes lower than those observed in the present study (0.036 kg/d or 2.3% in relation to the study database). Likewise, the use of technologies in organic minerals has presented variable responses in performance and health of beef cattle [7]. Nevertheless, Dorton et al. [41] reported increased feedlot receiving ADG when beef cattle were supplemented with organic complexed Zn, Cu, Mn and Co during 30 days post-weaning and 28 days post receiving of feeder cattle. In this context, the combination of technologies, such as live yeasts + organic sources of trace minerals used in ADV, showed benefits of higher magnitudes on ADG of Nellore cattle under the conditions of the present study. The use of a blend of live yeast and organic minerals instead of monensin or a diet without additives also increased milk and solids yield of dairy cows during the hot season [13].

The similar result between treatments on the feed efficiency of concentrate supplement intake demonstrates that ADV was effective in promoting feed efficiency in beef cattle. As demonstrated by Duffield et al. [1], among the greatest benefits of using monensin is the ability to generate feed efficiency through reduced intake without affecting or increasing ADG. Although, in this study, a higher concentrate supplement consumption in the ADV treatment in relation to MON was observed, in the same way that ADV promoted higher ADG, a probable reason for the similarity on supplement G:F between treatments. Additionally, the ADV diet contained the trace mineral Cr which was absent in the MON treatment. It was previously demonstrated that the supplementary use of a zinc amino acid complex, in association with chromium methionine, improved feed efficiency of Nellore

bulls finished in confinement, compared with zinc amino acid supplementation alone or control treatment with all trace minerals from inorganic sources [12]. Budde et al. [42], observed with Cr propionate supplementation in diets containing 90 mg/kg of DM of supplemental Zn from Zn hydroxychloride, greater final BW, ADG and hot carcass weight in feedlot steers. Little is known about Cr absorption and metabolism [43]; however, Cr is known to be an important trace mineral that is associated with glucose metabolism [43] and potentiates the action of insulin in insulin-sensitive tissues [44].

Although a greater ADG in live weight was observed with the use of ADV, the ADG in carcass and the final HCW was not different between the treatments, despite the numerical difference of 8.3% in carcass ADG, which generated the numerical difference of 9.4 kg in the final HCW. However, the trend towards greater LM area (5.8%) observed with the use of ADV, may be indicative of greater muscularity and commercial yield of the carcasses as a result of possible higher cold carcass weight [45]. However, the fat thickness was similar between treatments. Recent studies evaluating the use of yeast products (Live yeast and yeast fermentation product) have not shown effects on HCW, LM area and fat thickness [3,5,36]. On the other hand, in agreement with the present results, supplementation strategies with organic sources of trace minerals have shown benefits in generating greater LM area, without affecting fat thickness, as with zinc proteinate supplementation to the detriment of zinc oxide [46], and with zinc amino acid complex and chromium methionine [12].

The similarity between the treatments on the liver concentrations of Cu, Mn and Se, indicate that the lower supplementary inclusion levels of trace minerals from organic sources present in the treatment with ADV was sufficient to maintain similar hepatic concentrations of these minerals compared to higher inorganic inclusion levels. It is worth mentioning that in the ADV treatment, the supplementary amount of these minerals was, respectively, 55.5, 29.2 and 33.3% lower for Cu, Mn and Se minerals in relation to the inorganic sources, whose supplementary amount of these minerals was established to meet or exceed NASEM recommendations [8]. This result demonstrates the possibility of reducing the level of supplementation with these trace minerals with the use of ADV, without a negative effect on the status of these minerals in cattle during a finishing period with high concentrate, since the liver is the organ that often represents the status of several trace elements in animals [47]. This result was probably due to the recognized higher bioavailability of organic source of trace minerals, with the possibility of generating greater absorption, retention and biological activity compared to inorganic minerals [9–11]. Additionally, the hepatic concentrations of the minerals evaluated in this study, in both treatments, are considered to be within adequate ranges [8,48,49].

The trend of lower zinc concentration observed In ADV was probably due to a lower supplementary amount of Zn (64.9% less than in the supplementation with inorganic sources) in relation to the inorganic sources of trace minerals. Nonetheless, the maintenance of liver Zn concentrations within the range established as adequate [8,48] and the greater ADG and LM area observed with the use of ADV demonstrates that the supplemental amount of Zn from zinc proteinate was sufficient to support the demand for tissue growth. Interestingly, Niedermayer et al. [50] observed that, at the end of a period of 125 days of finishing beef steers, a tendency of lower concentration of Zn in the liver of steers receiving 100 mg/kg of supplemental Zn (industry recommendation of trace minerals) in relation to steers receiving 30 mg/kg supplemental Zn [8], from zinc sulfate. However, supplementation of 100 mg/kg generated higher ADG and HCW [50]. Carmichael-Wyatt et al. [51], supplementing Zn at five times current NASEM recommendations [8], had no effect on liver Zn concentrations, supporting the assertation that liver Zn is insufficiently sensitive to distinguish among cattle with adequate or greater Zn status.

In the present study, there was no difference in chemical composition of the meat from bulls fed with ADV or MON. There was also no difference in most meat quality traits, except for the L* and b* values, which were greater for bulls fed ADV than for those fed MON. The greater lightness observed with the use of ADV suggests the benefit of

producing less dark meat, which is more attractive to the consumer, as this is an important attribute in the visual evaluation of meat [16]. Greater yellowness observed in meat has been associated with increased fat deposition [52]. Differences observed in meat lightness may be related to factors such as changes in the concentration of heme pigment, which can be generated due to a change in muscle fiber types and oxidative metabolism [53]. However, this hypothesis still needs to be investigated in relation to the treatments evaluated here. The backfat thickness, which is also an attribute that can influence meat L*, due to the effect of protecting the carcass during cooling during the postmortem period, was similar between MON and ADV and greater than 3 mm, which is a threshold value reported to effectively protect the carcass during cooling [54]. The trend towards higher carcass pH observed with the use of ADV may also be a factor influencing meat L*, however in the opposite direction, as higher pH has been correlated with lower L* [16]. Nonetheless, the pH observed in both treatments was below 5.8, which is considered for the meat industry as adequate to generate positive attributes in the meat [55]. In addition, Vestergaard et al. [53] reported differences in meat L* even at a similar meat pH, with darker meat color for bulls fed roughage compared to concentrate. According to Moholisa et al. [56], tenderness is one of the most important meat characteristics related to consumer satisfaction. In the present study, the Warner–Bratzler Shear Force was similar across treatments. The observed values, being greater than 42.8 kg, which was considered an upper limit to classify beef as tender [57], are probably due to the age of the animals used in this study [58].

5. Conclusions

Sodium monensin and inorganic sources of trace minerals were able to be removed from the diet of beef cattle finished on pasture with high concentrate supplementation, without impairing feed efficiency, and with benefits on animal growth and meat color, when a blend of live yeast and organic trace minerals were included in the diet. The greater ADG observed is likely be related to the higher intake of concentrate and higher digestibility of the diet with the use of live yeast and organic minerals. The use of trace minerals from organic sources, even with reduced supplementary levels, made it possible to maintain adequate mineral status during the finishing period. This study contributes to the current knowledge about the use of technologies in feed additives for beef cattle, demonstrating that the blend of live yeasts and organic minerals is an interesting nutritional alternative. However, further studies are required to evaluate the effects of this technology on ruminal metabolism and the mechanisms by which it was possible to improve meat color attributes.

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Institutional Review Board Statement: This study was carried out in accordance with the ethical principle established by the Brazilian Council for the Control of Animal Experimentation and approved by the Ethics Committee for the Use of Animals of the Sao Paulo State University, College of Agricultural and Veterinary Sciences, Sao Paulo, Brazil (process no. 014782/19).

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Conflicts of Interest: Some of the co-authors are associated with the manufacturer of the feed additive tested.

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Combined Effects of Parsnip Fermented Juice and Hawthorn Extract Regarding Pork Mince Stability: Physico-Chemical and Microbiological Aspects

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Abstract: Parsnip fermented juice (PFJ) and hawthorn extract (HE) were identified as natural nitrite and antioxidant sources for pork mince. This study aimed to determine the effects of varying levels of HE added to a constant concentration of PFJ on lipids stability, heme pigment conversion degree, residual nitrite content, and spoilage bacteria growth, during refrigeration, compared with the combined effect of synthetic nitrite and sodium ascorbate (SA). Pork mince was formulated in six different ways with sterile distilled water (NC), 100 ppm synthetic nitrite and 50 ppm SA (PC), PFJ in the concentration of 100 ppm NO_2^- (T1), constant level of PFJ (100 ppm NO_2^-), and increased level of HE, 50, 25 and 10 ppm GAE (T2, T3 and T4). During the experiment, pH increased for all the treatments, but the addition of PFJ alone or in combination with HE, it was maintained below the NC pH value. The lowest TBARS values and the highest PUFA concentrations were found in the T3, T4, and PC treatments. Of all the samples, the lowest residual nitrite values were found for T2. The highest NO-heme values were found for T2 and PC. After 9 days of storage, TVC results were higher than 5.69 logs CFU/g for all treatments. Overall, the obtained results showed that the combination of HE and PFJ could be a promising natural preservative for minced meat that could replace synthetic preservatives.

Keywords: parsnip fermented juice; hawthorn extract; natural nitrite; natural antioxidant; lipids stability; spoilage bacteria; heme pigment conversion degree; bioactive compounds

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1. Introduction

Oxidative processes and meat spoilage bacteria are the most important factors in decreasing the shelf life of meat and meat products. Meat oxidation starts during slaughtering and continues in post-slaughtering conditions, handling, processing, and storage [1,2]. Meat is susceptible to spoilage bacteria due to its favourable growth conditions, high water content, nitrogen-containing molecules, carbohydrates, lipids, lactic acid, vitamins, and minerals. These degradative processes result in the development of off-odours, off-flavour, off-taste, loss of colour, loss in nutritional value, slim formation, and toxic compounds generation, making the meat undesirable for human consumption [3–5]. In the meat industry, synthetic antioxidants and nitrates and nitrites inhibit oxidative processes, bacterial growth, and input attractive colour. Lipid oxidation may be slowed down by synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), propyl gallate (PG), and sodium ascorbate and ascorbate.

To inhibit the grow spoilage bacteria and improve meat colour, synthetic additives, such as potassium/sodium nitrite and potassium/sodium nitrate are used. According to commission regulation (EU) No 1129/2011, the amount of nitrite permitted for use as a food additive in cured meat is currently 150 mg kg^{-1}. Many studies have suggested the toxicity and carcinogenicity of synthetic antioxidants with phenolic structures [6–8] and synthetic nitrites and nitrates [9,10] and for this reason, the researchers interested in replacing these synthetic substances with natural ones has increased in recent decades [11–16]. Many research studies have demonstrated the antioxidant and antimicrobial activity of plant extracts rich in phenolic compounds [14]. Vegetables are important sources of natural nitrates, but nitrate concentrations vary widely among plants, plant parts, and growing conditions. During the fermentation process, nitrates from plant juices can be converted into nitrites and, after concentration, concentrated fermented vegetable plant juices or fermented plant powders rich in natural nitrites result. The high nitrate content in the fermented vegetable juice/powder led researchers to study it as a nitrite natural source for the meat industry [17]. In recent years, consumer preferences have shifted towards requiring health-friendly, high-quality, nutrient-rich natural products. Parsnip fermented juice appears to be highly compatible with processed meat products because it has a high content of nitrates, very little vegetable pigment, and a mild, pleasant flavour profile. On the other hand, extracts of hawthorn berries are sources of natural antioxidants with previously proven preservative effects for meat products [5]. In fact, the present research highlights the preservative activity of the active compounds in hawthorn and nitrite-rich fermented juice. This study emphasizes the effects of varying levels of hawthorn berries ethanolic extract (HE) added to a constant concentration of parsnip fermented juice (PFJ) on lipids stability, heme pigment conversion degree, residual nitrite content, and spoilage bacteria growth in pork mince during refrigeration, compared to the combined effect of synthetic nitrite and sodium ascorbate. This study aimed to evaluate the combined effects of parsnip fermented juice and hawthorn phenolics on lipids and colour stability, and on spoilage bacteria growth in pork mince.

2. Materials and Methods

2.1. Parsnip Fermented Juice (PFJ) Obtaining

Parsnip roots were bought from a local market near Bucharest. The parsnip roots were cut into small pieces (1 × 1 cm) and then placed in a homogenizer with sterile distilled water in a ratio of 1:2 (*w*/*v*), and left to rest for 3 h at 4 °C. After homogenization, filtration and sterilization *Staphylococcus xylosus* (ATCC 29971) were added at 10^8 cfu/mL in a shaker incubator at 37 °C for 36 h [18]. Next, the mixture was filtered through Whatman No. 1 filter paper and evaporated using a rotary evaporator (Heidolph Laborota 4000). The concentrated fermented parsnip juice had a pH of 5.31 and 6237.5 ppm nitrite content. The nitrite concentration was assayed by the AOAC method [19]. The concentrated juice was kept in the refrigerator until used.

2.2. Hawthorn Extract (HE) Obtaining

The dried hawthorn fruits were collected from the forests near Câmpulung Muscel, Argeș county. After grinding, using a kitchen milling machine, the obtained powder was mixed with 60% ethanol (*v*/*v*), in an extraction rapport of 1:10 (*w*/*v*). Then was vigorously mixed and left to stand for 5 h. After that, the extraction was continued in a water bath (GFL 1092) at 60 °C for 3 h. The mixture was filtered through Whatman No. 1 filter paper and concentrated with a rotary evaporator (Heidolph Laborota 4000) to a temperature lower than 80 °C. The total phenolic content was assayed using Folin Ciocalteu reagent [20] and was expressed as mg gallic acid equivalent/mL (mg GAE/mL).

2.3. Experimental Setup and Preparation of Meat Samples

Fresh pork leg was purchased from a local butcher. After washing with distilled sterile water, all subcutaneous and intramuscular fat and visible connective tissue were removed

with a knife. The meat was manually chopped into cubes of approximately 2 cm^3 and then minced in a grinder equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. The meat was divided into 6 portions of one kilogram. The samples were treated with sodium nitrite (SN) and sodium ascorbate (SA), parsnip fermented juice (PFJ), and hawthorn extract (HE) at different concentrations, as shown in Table 1. Sodium ascorbate and sodium nitrite were analytical reagents and were obtained from Sigma-Aldrich, St Louis, MO, USA.

Table 1. Pork mince treatments with sodium nitrite (SN), sodium ascorbate (SA), parsnip fermented juice (PFJ), and hawthorn extract (HE).

Additives / Treatments	SN (ppm NO_2^-)	SA (ppm)	PFJ (ppm NO_2^-)	HE (ppm GAE)
NC	0	0	0	0
PC	100	50	0	0
T1	0	0	100	0
T2	0	0	100	50
T3	0	0	100	25
T4	0	0	100	10

Abbreviation: SN, sodium nitrite; SA, sodium ascorbate; PFJ, parsnip fermented juice; HE, hawthorn extract; NC, negative control; PC, positive control; GAE, gallic acid equivalent; ppm, parts per million and it expresses milligrams per kg (mg/kg); T1, treatment 1; T2, treatment 2; T3, treatment 3; T4, treatment 4.

For the pork-minced formulation, the ingredients (SN, SA, PFJ, HE) were dissolved in cold (4 °C) sterilized distilled water in a 100 mL final volume. For the negative control, 100 mL of sterilized distilled water was used. All meat samples were homogenized using a food processor (Moulinex DP 700), packed in plastic film, and stored at 4 °C for 9 days.

2.4. Physicochemical Analysis

pH Value

The pH value of all samples was measured using a pH meter (Hanna Instruments, Cluj Napoca, Romania) by direct measurement with a glass electrode calibrated with the phosphate buffers 4.0 and 7.0 at room temperature (21 °C). Ten grams of sample was homogenized with distilled water in a ratio of 1:100 (w/v) for 30 min. After filtration, the pH of the filtrate was measured [21].

2.5. Chemical Analysis

2.5.1. Chromatographic Profile of Fatty Acids

Lipid Extraction and Fatty Acid Methyl Esters

The fatty acids profile of lipids from minced meat was determined as fatty acids methyl esters (FAME). Lipid extractions were made according to [22] method and FAME was prepared by transmethylation using 2 M KOH in methanol and normal heptane according to the method described by [23].

Gas-chromatographic Analysis of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAME) were quantified by gas chromatography (GC) using a Perkin-Elmer-Clarus 500 system with a flame ionisation detector (FID), capillary column injection system and a silica capillary column SGE (BPX70, 60 m; 0.25 mm inner diameter, 0.25 µm film, Agilent). Gas-chromatographic conditions were according to the procedure described by [5]. Each fatty acid was expressed as g/100 g fatty acid methyl esters (FAME).

2.5.2. Thiobarbituric Acid Reactive Substances (TBARS) Value

Lipid oxidation in the minced pork was monitored by measuring thiobarbituric acid reactive substances (TBARS) every 3 days during refrigeration storage. TBARS value was assayed by the method described by [24]. Briefly, 0.5 g minced meat was treated with 2.5 mL thiobarbituric acid solution. After homogenisation, the tube with the mixture was

immersed in a boiling water bath for 10 min. After cooling under running tap water, sonication for 30 min, and centrifugation, the absorbance of the supernatant was read at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane and the concentration ranging from 0 to 10 ppm. TBARS value was expressed as mg of malondialdehyde equivalents/kg of the sample (mg MDA/kg).

2.5.3. Nitrosyl Hemochrome, Total Pigment Content, and the Heme Pigment Conversion Degree

Nitrosyl hemochrome and total pigments were measured after extraction with acetone and acidified acetone [25]. For quantification of the nitrosyl hemochrome, a 10 g sample was mixed with 43 mL of acetone-water solution (acetone: water, 40.5:2.5, v/v) in reduced light. After 5 min, the mixture was filtered through Whatman No. 1 paper, and the filtrate absorbance was measured at 540 nm using acetone water solution (80% acetone, 20% water) for blank. NO-heme pigment concentration was calculated using formula (1).

$$\text{NO-heme concentration (ppm hematin acid)} = \text{sample A540} \times 290 \quad (1)$$

For total pigment measurement, a 10 g sample (65% water) was mixed with 40.4 mL acetone, 1.6 mL water and 1 mL of concentrated HCl. The mixture was stored in dark at the room temperature, stirring from time to time. After 1 h, the homogenates were filtrated and the absorbance of the filtrate was read at 640 nm against the same solvent mixture used for the homogenate preparation. The optical density read at 640 nm was multiplied by 680, according to formula (2), to express the total pigment concentration.

$$\text{Total pigment concentration (ppm hematin acid)} = \text{A640} \times 680 \quad (2)$$

The heme pigment conversion degree was calculated using formula (3).

$$\%\text{Conversion} = (\text{NO-Heme/Total-Heme}) \times 100 \quad (3)$$

2.5.4. Residual Nitrite Level

Residual nitrite levels in the minced samples were assayed using the method recommended by [26]. For nitrite extraction, 5 g of sample was mixed completely with 40 mL hot water and quantitative transferred to a 500 mL volumetric flask. After the addition of about 300 mL, the volumetric flask was immersed in a shaking water bath for 2 h at 80 °C. Then, the mixture was cooled at room temperature, diluted to volume with water, mixed and filtrated. The colourimetric method using Griess diazotization was performed. Residual nitrite level was determined by comparison with the prepared standard curve and was expressed as ppm NO_2^- (μg NO_2^-/g sample).

2.5.5. Volatile Basic Nitrogen (VBN) Value

To determine the extent of protein deterioration during refrigeration storage, volatile basic nitrogen value was performed by the method of [27]. Briefly, 5 g of sample were mixed with 30 mL of 5% (w/v) trichloroacetic acid (TCA). The homogenate was made up to 50 mL of final volume with 5% (w/v) TCA and filtered using the Whatman filter paper No. 1. One mL of filtrate and 1 mL of borate buffer were placed in the outer and inner Conway dish, respectively. After 100 min incubation at 37 °C, the inner solution was titrated with 0.01 N HCl and the titration volume was recorded. The results were expressed as mg%.

2.5.6. Microbiological Analysis

Total viable count (TVC) was determined in plate count agar by the pour-plate method [28]. A total of 25 g of sample was aseptically weighed and homogenized with sterile 0.1% peptone water in the ratio 1:9 (w/v) for 1 min using a stomacher (400 circulators Seward Ltd.UK) at a speed of 6000 rpm. The homogenized sample was serially diluted (1:10) in sterile 0.1% peptone water. One mL sample of serial dilutions was plated into plate

count agar and then incubated at 35–37 °C for 48 h. Microbiological data were expressed as the logarithm of the number of colony-forming units (logCFU/g). All counts were performed in triplicate.

2.6. Statistical Analysis

The effect of treatments on the fatty acids profile of minced pork was performed using one-way ANOVA (XLStat, Addinsoft, New York, NY, USA). The effect of treatments and storage time on pH value, TBARS, NO-heme, total heme, heme pigment conversion degree, residual nitrite level, volatile basic nitrogen, and the total viable count was performed using two-way ANOVA (XLStat, Addinsoft, New York, NY, USA). The Tuckey test was used to predict differences among the criteria; the effects were considered significant if $p < 0.05$. The statistical model included the fixed effects of treatments (NC, PC, T1, T2, T3, T4) and storage time (0, 3, 6, 9 days) and their interactions. For correlations between parameters, Pearson's correlation was used.

3. Results and Discussions

3.1. Physico-Chemical Analysis

pH Value

Several studies demonstrated that increased refrigeration storage increased raw meat's pH [29–31]. It was suggested that increases in pH during refrigeration are due to the volatile basic compounds, such as ammonia, methylamine, dimethylamine and trimethylamine, and microbial catabolites, which result in meat spoilage [4,32,33]. The pH variations for meat samples according to the storage time are shown in Table 2.

Table 2. Changes in pH values of pork mince depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	5.788 [a]	0	5.460 [c]	Treatments	<0.0001
0	5.52 [a]	5.40 [b]	5.43 [ab]	5.45 [ab]	5.47 [ab]	5.49 [ab]	PC	5.495 [d]	3	5.492 [c]	Time	<0.0001
3	5.59 [a]	5.43 [b]	5.45 [b]	5.47 [b]	5.49 [b]	5.52 [ab]	T1	5.628 [b]	6	5.625 [b]	Treatments × time	<0.0001
6	5.81 [a]	5.47 [c]	5.7 [b]	5.51 [c]	5.55 [c]	5.71 [b]	T2	5.533 [d]	9	5.867 [a]		
9	6.23 [a]	5.68 [d]	5.93 [b]	5.7 [d]	5.81 [c]	5.85 [bc]	T3	5.580 [c]				
							T4	5.643 [b]				

[a–d] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1 –treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4– treatment with PFJ +HE, 10 ppm GAE.

On day 0, a slight difference was found between the positive control pH values and others, due to the residual acidity of hawthorn phenolics and PFJ. After 3 days of refrigeration, the pH increased for all samples, but the highest increase was found in the NC. The increase in the pH during storage was lower in the first 3 days and higher in the last 6 days. On the 6th day, T2 and T3 pH values were similar to PC. After 9 days of storage, the pH value of T1 was significantly lower than the pH value found in NC ($p < 0.05$). T2 pH value was significantly lower than the pH values found in T3 and T4 ($p < 0.05$). HE addition in pork mince inhibited pH values increasing in a relation depending on HE levels; thus, after 9 days of refrigeration storage, the pH values decreased in order T4, T3, and T2. By comparison of pH values for samples treated with PFJ + HE, it can be seen that samples treated with 25 and 10 ppm GAE (T3 and T4) resulted in a greater increase in pH ($p < 0.05$); formulation with 50 ppm GAE (T2) resulted in an increase in pH values similar to PC. The slight increase in the pH in the meat samples treated with the PFJ and PFJ + HE treatments (T1, T2, T3 and T4) is attributed to the inhibitory effect of antimicrobial compounds found both in HE and PFJ on the growth and proliferation of spoilage microorganisms that metabolize basic nitrogen compounds, such as amino acids, L-carnitine, lecithin, and choline [30,31].

3.2. Chemical Analysis

pH Value

Chromatographic Profile of Fatty Acids

Lipid deterioration is the major cause of the loss of bioavailability and sensory quality of the meat and is due to fatty acids oxidation and, to a lesser extent of fatty acids catabolism by spoilage bacteria. Lipid oxidation in muscle systems is initiated at the membrane level in the intracellular phospholipid fractions, with high polyunsaturated fatty acids (PUFA) content [34]. In minced meat, PUFA oxidation is the result of the interaction with endogenous prooxidants, such as metalloproteins [35], and with exogenous prooxidants, such as iron from grinding machines and singlet oxygen. The effects of PFJ and PFJ + HE, in concentrations 50, 25, and 10 mg GAE/kg, on the fatty acids profile, after 9 days of refrigeration are shown in Table 3.

Table 3. Fatty acid profile of pork mince with different treatments after 9 days of refrigeration.

Fatty Acid [1] (g/100 g)	NC	PC	T1	T2	T3	T4	SEM	*p* Value
C8:0	0.18 ^{ab}	0.17 ^{abc}	0.19 ^{a}	0.16 ^{bc}	0.14 ^{c}	0.16 ^{abc}	0.006	0.0001
C10:0	0.18 ^{a}	0.17 ^{b}	0.18 ^{ab}	0.17 ^{b}	0.05 ^{c}	0.17 ^{b}	0.007	<0.0001
C12:0	0.10 ^{cd}	0.11 ^{d}	0.14 ^{a}	0.11 ^{d}	0.13 ^{bc}	0.14 ^{ab}	0.005	<0.0001
C14:0	1.72 ^{c}	0.75 ^{b}	1.68 ^{a}	1.66 ^{a}	1.68 ^{a}	1.67 ^{a}	0.014	<0.0001
C15:0	0.17 ^{a}	0.17 ^{a}	0.11 ^{b}	0.17 ^{a}	0.11 ^{b}	0.07 ^{c}	0.010	<0.0001
C15:1	0.07 ^{bc}	0.08 ^{ab}	0.07 ^{bc}	0.10 ^{a}	0.05 ^{c}	0.06 ^{bc}	0.008	0.0001
C16:0	23.94 ^{b}	23.35 ^{f}	24.35 ^{a}	23.44 ^{e}	23.66 ^{c}	23.58 ^{d}	0.021	<0.0001
C16:1	3.67 ^{a}	3.39 ^{c}	3.58 ^{b}	3.59 ^{b}	2.73 ^{e}	3.08 ^{d}	0.016	<0.0001
C17:0	0.18 ^{c}	0.19 ^{bc}	0.22 ^{b}	0.19 ^{bc}	0.26 ^{a}	0.20 ^{bc}	0.010	<0.0001
C17:1	0.20 ^{a}	0.19 ^{a}	0.19 ^{a}	0.18 ^{ab}	0.04 ^{c}	0.15 ^{b}	0.011	<0.0001
C18:0	10.61 ^{a}	10.13 ^{c}	10.40 ^{b}	10.20 ^{c}	10.42 ^{b}	10.32 ^{bc}	0.062	<0.0001
C18:1	44.67 ^{b}	44.6 ^{c}	44.79 ^{a}	44.75 ^{a}	44.58 ^{c}	44.45 ^{d}	0.021	<0.0001
C18:2n−6	12.21 ^{d}	13.80 ^{a}	12.24 ^{d}	12.99 ^{c}	13.52 ^{b}	13.52 ^{b}	0.044	<0.0001
C18:3n−6 (γ)	0.14 ^{b}	0.22 ^{a}	0.16 ^{b}	0.17 ^{b}	0.20 ^{a}	0.20 ^{a}	0.009	<0.0001
C18:3n−3(α)	0.54 ^{c}	0.71 ^{a}	0.55 ^{c}	0.55 ^{c}	0.65 ^{ab}	0.60 ^{bc}	0.021	<0.0001
C20:2n−6	0.64 ^{a}	0.56 ^{b}	0.43 ^{d}	0.49 ^{cd}	0.54 ^{bc}	0.46 ^{d}	0.082	<0.0001
C20:3n−6	0.45 ^{bc}	0.57 ^{a}	0.30 ^{d}	0.50 ^{b}	0.46 ^{bc}	0.43 ^{c}	0.017	<0.0001
C20:4n−6	0.43 ^{c}	0.72 ^{a}	0.39 ^{d}	0.52 ^{b}	0.7 ^{a}	0.69 ^{a}	0.014	<0.0001
Others fatty acids	0.08 ^{a}	0.07 ^{a}	0.03 ^{b}	0.06 ^{ab}	0.03 ^{b}	0.05 ^{ab}	0.008	0.001
∑FA	100	100	100	100	100	100	-	-
∑SFA	40.98 ^{d}	35.04 ^{e}	35.71 ^{a}	36.10 ^{c}	36.46 ^{b}	36.31 ^{bc}	0.037	<0.0001
∑MUFA	44.61 ^{a}	48.26 ^{b}	48.63 ^{a}	48.62 ^{a}	47.40 ^{d}	47.74 ^{c}	0.038	<0.0001
∑PUFA	14.41 ^{e}	16.67 ^{a}	14.30 ^{f}	15.22 ^{d}	16.12 ^{b}	15.90 ^{c}	0.060	<0.0001

[1] FAME—fatty acids methyl esters. FA—fatty acids; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids. $^{a–f}$ Means in a row without a common superscript letter significantly differ ($p < 0.05$). NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

In all samples, the monounsaturated fatty acids (MUFA) showed the highest degree (44.61–48.63%), with the oleic acid (C18:1) being the most abundant fatty acid (44.58–44.79%), whereas polyunsaturated fatty acids (PUFA) showed the lowest percent (14.30–16.67%), with linoleic acid (C18:2n−6) the most abundant PUFA (12.21–13.80%). Saturated fatty acids (SFA) were found in the intermediate degree (35.04–40.98%), with palmitic (C16:0) and stearic (C18:0) acids the most abundant (23.35–24.35% and 10.13–10.61%, respectively). The results are in agreement with the results obtained by [36] that found a similar fatty acid profile in pork meat. Results show differences in fatty acid profile for all treatments, although some of them were statistically significant. For PFJ + HE treatments, (T2, T3 and T4), the total PUFA degree was significatively higher than the degree found in NC ($p < 0.05$), on the last day of determination. The highest total PUFA degree was found in PC

samples, treated with sodium nitrite and sodium ascorbate (PC). The total PUFA degree in sample T3, treated with PFJ + HE, 25 ppm GAE, follows an increasing path as PC ($p < 0.05$). Except for the formulation with PFJ + HE 50 ppm GAE/kg (T2), the other treatments with PFJ + HE (T3 and T4), significantly ($p < 0.05$) increased the stability of unsaturated fatty acids to oxidation, compared with samples formulated with PFJ alone (T1). The lowest degree for total PUFA was found for T1. Comparing the PUFA results obtained for T2, T3, and T4, a prooxidant effect on minced pork lipids by HE added at 50 ppm GAE/kg can be suggested. According to the results from the present study, the concentrations of 25 and 10 mg GAE/kg, seem to protect the essential fatty acids, linoleic (C18:2n−6), linolenic (C18:3n−3), and arachidonic (C20:4n−6) acids against oxidative damage, comparatively with T1. Some authors have shown that colonial-type salami treated with rosemary extract alone or in association with celery powder had a higher PUFA content compared with control samples. [37]. Some authors used Mediterranean berries' ethanolic extracts as antioxidants in pork burgers subject to cooking and chilled storage and found that the protective effect of ethanolic extracts on PUFA decreased in the following order: *Rosa canina*, *Rubus ulmifolius*, *Arbusto unedo*, and *Crataegus monogyna*. The highest level of linoleic acid they found in patties treated with the ethanolic extract obtained from *Arbusto unedo* berries [38].

3.3. Thiobarbituric Acid Reactive Substances (TBARS) Value

Generally, lipid oxidation affects bioavailability and sensory quality in minced meat. A good indicator of lipid oxidation level is the TBARS value. It was suggested that the TBARS value at 0.5 mg/kg was a threshold value for rancidity perception by consumers [39]. The results regarding the changes in TBARS values for the six evaluated treatments are presented in Table 4.

Table 4. Changes in thiobarbituric acid reactive substances values of minced pork depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	0.845 [a]	0	0.123 [b]	Treatments	<0.0001
0	0.121 [a]	0.122 [a]	0.121 [a]	0.130 [a]	0.122 [a]	0.121 [a]	PC	0.198 [e]	3	0.328 [a]	Time	<0.0001
3	0.650 [a]	0.17 [d]	0.45 [b]	0.27 [c]	0.16 [d]	0.27 [c]	T1	0.523 [b]	6	0.632 [d]	Treatments × time	<0.0001
6	1.340 [a]	0.22 [e]	0.87 [b]	0.65 [c]	0.27 [e]	0.44 [d]	T2	0.373 [c]	9	0.557 [c]		
9	1.27 [a]	0.28 [e]	0.65 [b]	0.44 [c]	0.35 [d]	0.35 [d]	T3	0.226 [e]				
							T4	0.295 [d]				

[a–e] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1 –treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4– treatment with PFJ +HE, 10 ppm GAE.

The analysis of variance for the TBARS data indicates that the TBARS values for pork mince were influenced by the refrigeration period ($p < 0.001$) and treatments. Overall, TBARS values increased during refrigeration storage, but the lowest TBARS values were found in PC pork mince ($p \leq 0.05$). The sample treated with PFJ alone (T1) had lower TBARS values than NC, but higher than PC ($p \leq 0.05$). The addition of HE in the minced pork affects the TBARS values in a concentration-dependent manner, after 3 days of refrigeration. The best antioxidant activity was found in the sample treated with PFJ + HE in a concentration equal to 25 ppm GAE (T3), followed by the samples treated with PFJ + 10 ppm GAE HE (T4), on the 6th day of the determination. On the last day of the investigation (9th day), the samples treated with PFJ + 50 ppm HE (T2) showed the highest TBARS values, comparatively with T3 and T4. The result obtained showed that the capacity of PFJ + HE to inhibit lipid oxidation in minced pork depends on the amount of HE used. HE in concentrations of 50 ppm GAE/kg meat has prooxidant activity, but HE in a concentration of 25 and 10 mg GAE/kg influenced the antioxidant activity as the association of sodium nitrite with sodium ascorbate (PC).

Another study showed the TBARS values after the preparation of beef patties using resveratrol as an antioxidant in concentrations as 110 μmol/kg meat and 550 μmol/kg meat, during 9 days of refrigeration. The highest TBARS values were found in samples treated with 550 μmol/kg meat, comparatively with TBARS values found in samples treated with 110 μmol/kg meat, but both were lower than those found in control samples [40]. It was reported different TBARS values for cooked pork patties, prepared with ethanolic berry extracts, and stored by refrigeration than those found in control samples (1.071 mg MDA/kg) [38]. So, higher TBARS values were reported for *Crataegus monogyna* (0.181 mg MDA/kg) and *Rosa canina* (0.143 mg MDA/kg) treatment extracts, but lower TBARS values for *Rubus ulmifolius* (0.082 mg MDA/kg) and *Arbusto unedo* (0.113 mg MDA/kg) treatment extracts.

3.4. Nitrosyl Hemochrome, Total Pigment Content, and the Heme Pigment Conversion Degree

Nitrosyl hemochrome (NO-heme), the pink pigment of cured meat, is formed mainly from NO(II)Mb resulting in the reaction of myoglobin (Mb) with NO generated from nitrite conversion in the presence of ascorbate [41]. Some studies suggested that the addition of ascorbate in the muscle reduces met(III)Mb to deoxy(II)Mb and the simultaneously generated NO from nitrite binds to deoxy(II)Mb or can reduce nitrosylate met(III)Mb under anaerobic conditions, resulting in the formation of NO(II)Mb [41].

The studies regarding the effect of pH on the stability of nitrosyl hemochromogen suggest that NO-Heme is extremely unstable at weakly acid pH, and this explains why pH affects the apparent colour of cured meat products with storage time [42]. Table 5 showed the results obtained for nitrosyl hemochrome (NO-Heme) values of minced pork formulated with parsnip fermented juice (PFJ) alone, parsnip fermented juice + hawthorn extract (PFJ + HE) compared to untreated minced pork (NC), and synthetic added compounds (PC).

Table 5. Changes in NO-heme value, total heme value, and heme pigment conversion degree of minced pork depending on treatment and storage time.

Treatments	NO-Heme (ppm Hematin Acid)	Total Heme (ppm Hematin Acid)	Heme Pigment Conversion Degree (%)
0 days			
NC	0.00 ^{c}	121.23	0.00 ^{c}
PC	26.23 ^{a}	120.99	21.68 ^{a}
T1	20.50 ^{b}	121.45	16.87 ^{b}
T2	26.20 ^{a}	123.09	21.30 ^{a}
T3	26.00 ^{a}	121.40	21.41 ^{a}
T4	25.89 ^{a}	120.97	21.42 ^{a}
3 days			
NC	0.00 ^{d}	120.46	0.00 ^{c}
PC	44.60 ^{a}	120.90	36.68 ^{a}
T1	26.70 ^{c}	123.77	21.76 ^{b}
T2	30.50 ^{b}	123.09	23.99 ^{b}
T3	30.70 ^{b}	121.41	24.47 ^{b}
T4	27.98 ^{bc}	121.56	23.02 ^{b}
6 days			
NC	0.00 ^{e}	119.00 ^{b}	0.00 ^{e}
PC	55.60 ^{a}	120.35 ^{ab}	46.11 ^{a}
T1	32.5 ^{d}	123.56 ^{a}	26.31 ^{d}
T2	45.81 ^{b}	122.67 ^{ab}	37.34 ^{b}
T3	43.21 ^{bc}	120.50 ^{ab}	34.70 ^{bc}
T4	40.76 ^{c}	121.67 ^{ab}	33.50 ^{c}

Table 5. *Cont.*

Treatments	NO-Heme (ppm Hematin Acid)	Total Heme (ppm Hematin Acid)	Heme Pigment Conversion Degree (%)
9 days			
NC	0.00 ^{d}	118.00	0.00 ^{d}
PC	60.50 ^{ab}	119.30	50.71 ^{ab}
T1	38.45 ^{c}	120.15	32.00 ^{c}
T2	63.50 ^{a}	120.75	52.58 ^{a}
T3	43.21 ^{ab}	119.50	50.79 ^{ab}
T4	40.76 ^{b}	119.59	47.99 ^{b}
Main effects Treatments			
NC	0.000 ^{e}	119.672 ^{c}	0.000 ^{e}
PC	46.733 ^{a}	120.438 ^{bc}	38.794 ^{a}
T1	29.540 ^{d}	121.482 ^{ab}	24.235 ^{d}
T2	41.503 ^{b}	122.400 ^{a}	33.803 ^{b}
T3	40.153 ^{b}	120.703 ^{abc}	32.843 ^{bc}
T4	38.008 ^{c}	120.950 ^{abc}	31.482 ^{c}
Time			
0	20.803 ^{d}	121.522 ^{a}	17.113 ^{d}
3	26.747 ^{c}	121.364 ^{a}	21.653 ^{c}
6	36.315 ^{b}	121.327 ^{a}	29.660 ^{b}
9	46.758 ^{a}	119.550 ^{b}	39.012 ^{a}
p value			
Diet	<0.0001	0.001	<0.0001
Time	<0.0001	0.0001	<0.0001
Diet × time	<0.0001	0.769	<0.0001

$^{a–e}$ Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1–treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4–treatment with PFJ +HE, 10 ppm GAE

The highest value for NO-Heme during 9 days of refrigeration was found in PC. In the sample formulated only with PFJ (T1), NO-Heme content was significantly lower, compared with PC and samples formulated with PFJ + HE (T2, T3 and T4) ($p < 0.05$). The formulation of the minced meat with PFJ and HE increased NO-Heme content, in a relation dependent on the HE level in samples (Table 5).

After 9 days of refrigeration storage, in the samples treated with PFJ + HE, the content of NO-Heme increased in the order: T2, T3, and T4. Obtained results showed that HE accelerates the heme pigments to react with NO in a concentration-dependent manner. The nitrosylation reaction stimulated by HE and SA may be due to the lowering of the pH values on day 0 and of the reaction between phenolics and nitrite [43]. It was reported that a pH decreasing only 0.2 unit, doubles the rate of colour formation through nitrite-myoglobin reaction [44]. It was suggested that the final colour properties of nitrite also increase with decreasing pH and this constitutes the basis for product formulation with acidulants to accelerate curing [44,45].

Table 5 presents the results regarding total heme pigment values obtained for all treatments during 9 days of refrigeration. In all samples, a slight decrease in total heme pigment was found after 9 days of storage. Significant differences ($p < 0.05$) in total heme pigment were found in the treated minced pork after 9 days of refrigeration storage compare with previous determinations (0, 3 and 6 days).

Heme pigment conversion degrees for all minced pork treatments are presented in Table 5. After 9 days of refrigeration, the results showed that the minced pork with PFJ (T1) produced an increase in the heme pigment conversion % equal to 32.00%. When HE was

added to PFJ, even the lowest HE concentration, 10 ppm GAE (T4) produced an increase in heme pigment conversion degree (47.99%), compared to the formulated sample with PFJ alone. So, compared to PC, the samples formulated with PFJ + HE provided increases in the heme pigment conversion degree in T2 and T3 or slower in T4 (Table 5). Moreover, PFJ + HE and SN + SA have the same effect on the heme pigment conversion degree, meaning an increased percentage. Results found for pH values, NO—heme and heme pigment conversion degree show important aspects related to sample formulation. The results can be attributed to the reducing and acidic properties of SA or HE that favour met(III)Mb reduction, NO generation, and the reaction of heme pigments with NO also.

3.5. Residual Nitrite Level

Nitrite added in the meat reacts especially with heme proteins (myoglobin, haemoglobin), and less with nonheme proteins, lipids, and carbohydrates. Unreacted nitrite, named residual nitrite, can be depleted by the formation of nitrogen gasses [46]. Nitrite depletion in cured meat is influenced by pH, temperature, time, meat type, salt, and nitrite concentration. The residual nitrite level in meat treated with PFJ and PFJ + HE is shown in Table 6. For all samples, residual nitrite level decreased during refrigeration storage, but the most rapidly decreasing was found after 3 days of storage. Throughout the refrigeration, the lowest residual nitrite level was found in the negative control (NC), without adding any nitrite, and the highest level was found in pork mince treated with PFJ alone (T1). The addition of HE to the PFJ favours the decrease in the residual nitrite level. Therefore, the residual nitrite level in minced pork treated with PFJ + HE decreased with increasing levels of HE. In the pork mince treated with the PFJ + 50 ppm HE (T2), the residual nitrite level was significantly ($p < 0.05$) lower than those found in the positive control. Regarding the residual nitrite level in the minced pork treated with the PFJ + 50 ppm HE (T3), the rate of nitrite depletion with storage time was similar to the one found for the positive control. The residual nitrite depletion was a bit slower in the meat treated with PFJ + HE, at level 10 ppm GAE (T4), compared with the PC. The addition of HE showed a statistically significant effect in the nitrite depletion with storage time, and so, the decrease in the residual nitrite level ($p < 0.001$).

Table 6. Changes in residual nitrite level and volatile basic nitrogen of minced pork depending on treatment and storage time.

Treatments	Residual Nitrite Level (ppm)	Volatile Basic Nitrogen (VBN) mg%
	0 days	
NC	0.00 ^{b}	12.89
PC	99.67 ^{a}	12.82
T1	99.33 ^{a}	12.89
T2	98.67 ^{a}	12.84
T3	100.00 ^{a}	12.81
T4	98.33 ^{a}	12.88
	3 days	
NC	0.00 ^{e}	23.55 ^{a}
PC	20.30 ^{c}	16.84 ^{c}
T1	80.50 ^{a}	19.89 ^{b}
T2	13.50 ^{d}	17.87 ^{bc}
T3	25.60 ^{c}	18.02 ^{bc}
T4	33.50 ^{b}	20.10 ^{b}

Table 6. *Cont.*

Treatments	Residual Nitrite Level (ppm)	Volatile Basic Nitrogen (VBN) mg%
	6 days	
NC	0.00 ^{d}	30.45 ^{a}
PC	16.00 ^{c}	18.86 ^{c}
T1	75.00 ^{a}	23.78 ^{b}
T2	10.50 ^{c}	10.87 ^{bc}
T3	15.50 ^{c}	21.17 ^{bc}
T4	23.00 ^{b}	21.70 ^{bc}
	9 days	
NC	0.00 ^{d}	46.7 ^{a}
PC	10.00 ^{bc}	20.11 ^{d}
T1	69.00 ^{a}	33.70 ^{b}
T2	5.80 ^{c}	22.39 ^{cd}
T3	13.50 ^{b}	24.50 ^{c}
T4	14.00 ^{b}	31.80 ^{b}
	Main effects Treatment	
NC	0.000 ^{e}	28.398 ^{a}
PC	36.492 ^{c}	17.158 ^{d}
T1	80.958 ^{a}	22.565 ^{b}
T2	32.117 ^{d}	18.493 ^{c}
T3	38.650 ^{c}	19.125 ^{c}
T4	42.208 ^{b}	21.620 ^{b}
	Time	
0	82.667 ^{a}	12.855 ^{d}
3	28.900 ^{b}	19.378 ^{c}
6	23.333 ^{c}	22.805 ^{b}
9	18.717 ^{d}	29.867 ^{a}
	p value	
Diet	<0.0001	<0.0001
Time	<0.0001	<0.0001
Diet × time	<0.0001	<0.0001

$^{a–e}$ Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

These results were in agreement with those found by other researchers [47,48], who reported continuous depletion of the residual nitrite level in cured meat during the storage time. A more pronounced decrease in residual nitrite level in minced pork formulated with SA and HE, compared to the sample formulated with PFJ alone may be the result of the pH decreasing and the interaction of nitrite with the SA and the HE. Some researchers reported a decrease in free nitrite in aqueous solutions of nitrite and sodium ascorbate [49]. Other results suggested that NO resulting in the reaction of free nitrite with SA is responsible for nitration reactions and residual nitrite depletion during meat curing [50]. A good correlation ($p < 0.001$) was found between pH value and residual nitrite depletion rate and between HE concentrations and residual nitrite depletion rate. Similar results were achieved in different research regarding cured meat products [51–54].

3.6. Volatile Basic Nitrogen (VBN) Value

Volatile basic nitrogen is a product of bacterial spoilage and endogenous enzyme action [55]. VBN is mainly composed of ammonia and primary, secondary, and tertiary

amines [56] resulting from amino acid degradation [57,58]. The changes in the VBN value of all samples with storage time are shown in Table 6.

The results show the progressively increasing VBN for all samples. The highest VBN values were found in NC and the lowest in the PC, throughout the refrigeration period. In pork mince formulated with PFJ alone, VBN values were lower than those found in NC but higher than those found in PC. Meat formulation with PFJ + HE decreased VBN values in total phenolics in HE concentration-dependent manner, the lowest values were found for PFJ + HE, in the concentration of 50 ppm GAE (T2) and the highest for PFJ +HE, 10 ppm GAE (T4). In the T2 samples, the geometry of the VBN values concentration dependence was similar to one obtained for PC, but the values were higher by an average of 11.14%. A previous study used fermented spinach for pork loin curing and reported similar results [15]. VBN values in samples treated with fermented spinach were higher than those found in the negative control and lower than those found in the positive control treated with synthetic nitrite.

3.7. Microbiological Analysis

The effect of PFJ and PFJ + HE on total viable count (TVC) is shown in Table 7. The initial number of bacteria in samples was between 2.60 log CFU/g and 2.77 log CFU/g which indicated the good quality of pork used in this study. For all samples, TVC increased with storage time and the value of NC and T1 increased faster than PC, T2, T3, and T4. From day 0 to day 3, there were strong significant differences between NC and T1 ($p < 0.05$). After 6 days of storage, significant ($p < 0.05$) differences between NC and T1, which indicated that PFJ inhibited the growth of TVC alone, were found. After 6 days of storage significant differences ($p < 0.05$) between T1 and samples T2 were found and these results indicated that HE added in PJF increased the antimicrobial activity of PFJ. A significant correlation was established between PC and samples T2, T3, and T4 ($p < 0.001$).

Table 7. Changes in total viable count of minced pork depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	7.325 [a]	0	2.687 [d]	Treatments	<0.0001
0	2.77	2.67	2.67	2.75	2.6	2.66	PC	3.863 [d]	3	4.143 [c]	Time	<0.0001
3	5.2 [a]	2.87 [c]	4.66 [a]	3.34 [bc]	4.22 [ab]	4.57 [a]	T1	5.945 [b]	6	5.890 [b]	Treatments × time	<0.0001
6	8.22 [a]	4.11 [e]	6.89 [b]	4.56 [de]	5.55 [cd]	6.01 [bc]	T2	4.358 [d]	9	8.400 [a]		
9	13.11 [a]	5.8 [d]	9.56 [b]	6.78 [cd]	7.2 [c]	7.95 [c]	T3	4.893 [c]				
							T4	5.298 [c]				

[a–e] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

After 9 days of storage in all samples, TVC was higher than 5.69 logs CFU/g and so, not acceptable for safe consumption, but TVC values were lower for T1, T2, T3, and T4 than NC. Similar results were reported by the literature, showing that fermented spinach extract, celery juice powder, and cherry juice powder can inhibit the growth of microorganisms in different meat models [15,59]. Other researchers tested the antimicrobial activity of the ethanolic extracts obtained from rosemary and cloves and found important antimicrobial activity for extracts used alone or in combination [31].

3.8. Correlations between Analysed Parameters

The Pearson correlation coefficient (r) is the simplest way to measure a linear correlation. It takes values between −1 and 1 and indicates the relationship between two variables in strength and direction. When one variable changes, the other variable also changes. In this study, we analysed the effects of various concentrations of HE in combination with a constant PFJ concentration on the pH, fatty acids profile, TBARS value, NO-heme, total heme pigment, heme pigment conversion degree, residual nitrite, VBN, and TVC. pH value is a very important parameter for pork mince and was influenced by VBN value (r = 0.2488,

$p < 0.0001$) and TVC, in a positive and linear manner. NO-heme is a parameter related to meat colour stability. According to the results, a weak positive relationship was found between NO-heme and TBARS (r = 0.9349, $p < 0.0001$), but negative correlations were found between TBARS value and MUFA (r = −0.7275, $p < 0.0001$), and between TBARS value and PUFA (r = −0.8643, $p < 0.0001$). Colour stability is influenced by TBARS and could be related to fatty acids profile. A negative correlation was found between NO-heme and residual nitrite (r = −0.7786, $p < 0.0001$). The pH values determined in the present study established positive correlations with heme conversion degree, (r = 0.5848, $p < 0.0001$), and NO-heme (r = 0.5787, $p < 0.0001$) (data in Table S1).

4. Conclusions

The use of PFJ in combination with HE exerted an inhibitory effect on the growth of spoilage bacteria that metabolize nitrogen compounds and significantly increased the stability of unsaturated fatty acids to oxidation in minced pork. The addition of HE in the minced pork decreased the TBARS values compared to T1. At any time during the experiment, NO-heme increased in a concentration-dependent manner with HE. So, HE may influence the NO-heme concentration. The concentration of HE of 25 ppm together with PFJ was the most effective to delay the FA oxidation in pork mince, whereas the higher concentration of HE (50 ppm GAE) had decreased the total PUFA and increased TBARS, suggesting a prooxidant effect on the minced pork lipids. Colour and lipids stability may depend on the HE and PFJ combination. However, T1, T2, T3, and T4 showed lower TVC values than NC. Between them, T2 was the most effective against microbial growth. In conclusion, these combinations of natural sources of nitrites and antioxidants could be considered promising meat alternatives to synthetic nitrites and antioxidants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020432/s1, Table S1: Pearson's correlation coefficients of analysed parameters.

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Review

Transforming *Rhodotorula* sp. Biomass to Active Biologic Compounds for Poultry Nutrition

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Abstract: In broiler chick-rearing, the color is usually acquired by synthetic carotenoids in addition to broiler diets (25–80 mg/kg feed), often represented by β-apo-8′-carotenal. In the past fifteen years, the demand for organic food products originating from free-range reared chicks started to grow, with a more directed awareness of the quality of meat and egg. Various investigations have been reporting microorganisms, such as the oleaginous red yeasts genus *Rhodotorula* sp., as fast-growing unicellular eukaryotes able to synthesize natural pigments. *Rhodotorula* sp. represents a perfect choice as a natural resource due to the capacity to adapt easily to the environment valuing low-cost sources of nutrients for their metabolism and growth. The biodiversity and the ecology effects establish novel boundaries regarding *Rhodotorula* sp. productivity enhancement and control of biological risks. It is, therefore, necessary to review the current knowledge on the carotenoid synthesis of *Rhodotorula* sp. In this paper, we aimed to address the pathways of obtaining valuable yeast carotenoids in different conditions, discussing yeast biosynthesis, bioengineering fermentative evaluation, carotenoid extraction, and the techno-economic implication of valuable pigment additives on poultry nutrition. Finally, the pro-existent gaps in research are highlighted, which may clear the air on future studies for bio-carotenoid engineering.

Keywords: artificial pigment alternative; broiler nutrition; carotenoids; health; pigment additives; vegetal waste

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1. Introduction

Carotenoids are soluble pigments classified as tetraterpenoids divided as primary (hydrocarbons, carotene) and secondary as their oxidation product (xanthophylls). Widely, around 1100 different carotenoids [1] are synthesized in plant, algae, and fungi species. As natural lipophilic pigments [2], they are often characterized by a range of colors, starting from a pale and creamy yellow, light-pink, strong yellow, pink, and orange until strong red pigmentation and a rare, purple color [3]. Under natural circumstances, carotenoids have a multitude of roles, including sustaining photosynthesis, ensuring photoprotection [4,5], antioxidant capacity [6], reproductive enhancement [7], embryonal development [8], cell maturation [9], and immune system protection [10]. Birds cannot synthesize carotenoids hereby; carotenoids must be included in dietary intake. Dietary feed ingredients used in

commercial poultry feeding formulas are often processed in pelleted and extruded form, with consideration to nutrient availability and economic efficiency [11]. Mechanical procedures such as palletization or extrudate are currently employing high-temperature and pressure applied directly to feeding ingredients [12], thus affecting the retinol and retinol precursor by degradation, cumulating the vitaminic losses through handling and storage [13].

Carotenoids represent significant resources of retinol precursors (0.66 μg β-carotene = 1 U of retinol acid), with large implications for healthfulness and quality [14] products. Poultry is the most successful livestock sector around the globe and tends to grow due to the increasing consumption of poultry products. By the year 2020, the poultry sector generated almost 101 metric tons of meat and 1.65 billion eggs [15]. The great potential of carotenoid sources in industries (including food, feeds, nutritional supplements, pharmaceutics, and cosmetics) will have increased the forecasted market value to around $2.0 billion by 2022 [16]. The most commonly used food and feed colorant additives in poultry nutrition are xanthophylls (lycopene, canthaxanthin, astaxanthin, and zeaxanthin) that originate from almost 90% mainly synthetic resources. Annually, the market for pigment additives tends to grow by 8.2% percent during the forecasted period 2022–2032 [17] due to the increasing consumption of poultry products (meat and eggs). Currently, there has been a growing interest in obtaining organic pigment additives from non-conventional resources (algae, bacteria, and yeasts). The composition and the stability of the natural resources might be undefined and wide because of the complexity of biochemical metabolism and biological variability that is often associated with the cell structure. Great consideration was attributed to the carotenoid biosynthetic pathways of yeast, understanding the carotenoid yield, as productivity and integrity, with a view regarding product improvement and industrial scalability. In non-phototrophic microorganisms, carotenoids present a clear advantage in obtaining natural pigments [18]. One of the most important attributes is the capacity of microorganisms to use industrial waste as raw material substrate [19], hence increasing profitability and lowering the related costs of production. Many microorganisms synthesize carotenoids and present a valuable industrial potential (Table 1), although the data concerning *Rhodotorula* sp. yeast pigment application on livestock nutrition are few.

Table 1. Carotenoid-producing microorganisms.

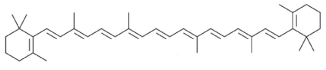
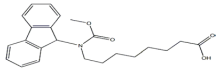
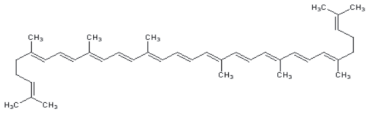
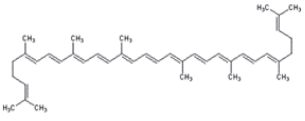
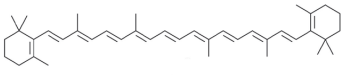
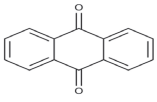
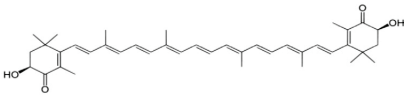
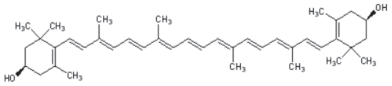
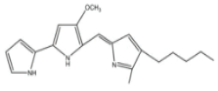
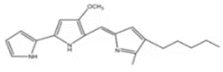
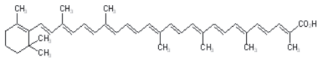
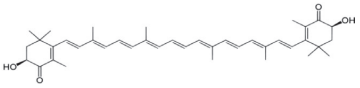
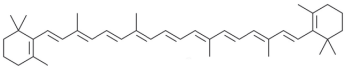
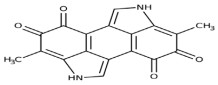
Microorganism	Carotenoid	Structure	Reference
		Funghi	
Neurospora crassa	β-carotene		[20]
Monascus sp.	Monascorubramin		[21]
Blakeslea trispora	Lycopene		[22]
Fusarium sporotrichioides	Lycopene		[23]
Aspergillus sp.	β-carotene		[24]
Pacilomyces farinosus	Anthraquinone		[25]

Table 1. *Cont.*

Microorganism	Carotenoid	Structure	Reference
		Bacteria	
Paracoccus carotinifaciens	Astaxanthin		[26]
Staphylococcus aureus	Zeaxanthin		[27]
Zooshikella sp.	Prodigiosin		[28]
Serratia marcescens	Prodigiosin		[29]
		Yeast	
Rhodotorula glutinis	Torularhodin		[30]
Xanthophyllomyces dendrorhous	Astaxanthin		[31]
Rhodototula mucilaginosa	β-carotene		[32]
Saccharomyces neoformans	Melanin		[33]

The current paper aims to highlight the multitude of approaches to obtaining valuable yeast carotenoids in different conditions, discussing yeast biosynthesis, bioengineering, fermentative evaluation, carotenoid extraction, and the techno-economic implication of valuable pigment additives on poultry nutrition.

2. *Rhodotorula* sp. General Aspects

The genus *Rhodotorula* sp. covers more than 165 species [34]. Morphologically, *Rhodotorula sp.* is a polyphyletic-shaped yeast [35] forming fast-growing colored colonies [36]. The proliferation of the *Rhodotorula* genus is generally regarded as asexual [37]; however, some strains belonging to the genus present sexual reproductive traits [38]. *Rhodotorula sp.* ecology and biodiversity cover a board of environmental varieties using a large variety of carbon resources, including glycerol [39], glucose [40], sucrose [41], galactose [42], and maltose [43], often encountered as dominant in yeast microflora (water, soil, vegetal, and animals) [44].

Yeast such as *Rhodotorula* sp. represents a perfect choice as a natural resource of secondary metabolites (Figure 1): carotenoids [45], lipids [46], and extracellular enzymes (Table 2). Saprophytic and ubiquitously found, the *Rhodotorula* genus possesses a full capacity for intracellular carotenoid biosynthesis [47] (provitamin A precursors, such as β-carotene and γ-carotenoid) [47,48], although the main carotenoids are torulene and torularhodin [49].

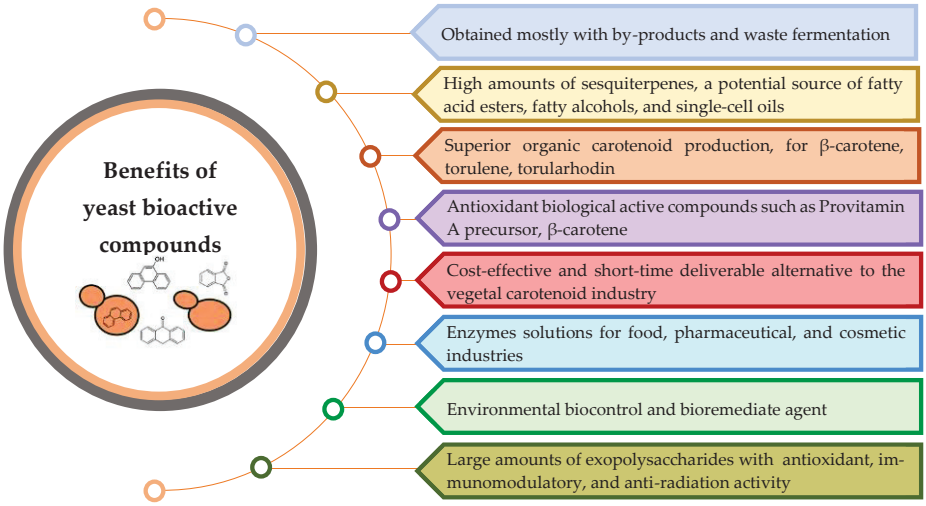


Figure 1. Major benefits of bioactive compounds from *Rhodotorula* sp.

Table 2. *Rhodotorula* sp. biomass active compounds.

Yeast Strain	Assay Conditions and Results	Reference
	Oil←lipids→fatty acids	
Rh. mucilaginosa IIPL32	Fed-batch C/N ratio = 40; Fed-batch C/N ratio = 60; Scalability: 50 mL→50 L; lipid yield for C/N ratio fed-batch = 40: 0.4 g→1.3 g/L lipid yield for C/N ratio fed-batch = 60: 0.45 g→1.8 g/L	[50]
Rh. mucilaginosa IIPL32	(Lipids as FAME) 72 h→97.23 mg/g dry cell weight; 35–55%, MUFA C18:1 and C16:1 (oleic and palmitoleic acids)	[51]
Rh. mucilaginosa CCT3892	The total amount of lipids obtained in the molasses medium was similar to the synthetic medium (15.36% ± 1.36% and 16.50% ± 0.68%, respectively), thus, the production of the metabolites was higher in the molasses medium.	[52]
	Enzymes	
Rhodotorula mucilaginosa CBMAI 1528	Aspartic protease—pepsin family	[53]
Rhodotorula mucilaginosa	Invertase—the invertase with greater cell-structural stability and nystose productivity	[54]
Rhodotorula sp. Y-23	Lipase (Lip-Y23)—low-temperature applications	[42]
R. mucilaginosa Y-1	Carboxylase—Acetyl coenzyme A carboxylase (ACC1)	[55]
	Carotenoids	
Rhodotorula glutinis	β-carotene, torularhodin	[47,56]
Rhodotorula mucilaginosa KC8	β-carotene, torularhodin	[57]

Carotenoids are mainly synthesized via successive condensation (Figure 1) attributed to isoprenoid units such as isopentenic pyrophosphate (IPP) isomerized in dimethylallyl pyrophosphate (DMAPP) [4,58–63]. Particularly, yeasts such as *Rhodotorula* sp. possess the ability to transform lycopene into cyclic carotenoids like β-carotene (under lycopene β-cyclase action) and γ-carotene (conversion supported by lycopene cyclase).

The γ-carotene unit represents the main precursor for yeasts' carotenoid formations, as shown in Figure 2. β-carotene ($C_{40}H_{56}$) is the most common and abundant

precursor for retinol [64], strong-orange-red colored, chemically classified as isoprenoid (synthesized from eight isoprenoid units) [65]. Torularhodin is regarded as a xanthophyll ($C_{40}H_{52}O_2$—3′,4′didehydro β, ψ-carotene-16′oic acid) due to the presence of the carboxyl group [66] and represents the prevailing chemical structure in *Rhodotorula* sp. total carotenoid yield [67]. Torulene is classified as a carotenoid. The torulene molecule includes only hydrogen and carbon atoms [49], $C_{40}H_{54}$, 3′,4′ dihydro-β, ψ-carotene.

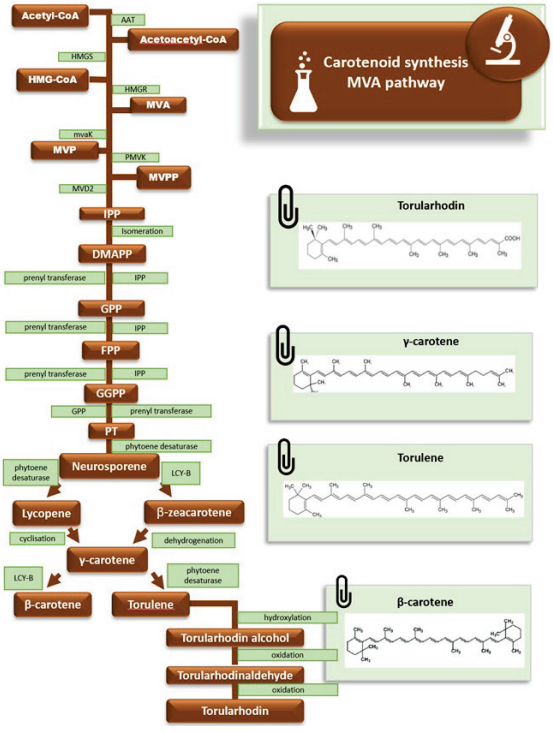


Figure 2. Rhodotorula carotenoid synthesis via MVA pathway; adapted after [68].

Concerning pigment bio-applications, *Rhodotorula* sp. yeast owns valorous advantages: fast-growing capacity, usually on organic waste materials (carbon-rich materials), and cost-effective production and harvesting (minimized human/process-associated interventions), which is more than suitable for large-scaling pigment industrial production and directly competing within the pigment market.

The vegetal sector is the most important resource concerning natural pigment industries. Factors such as poor soils [69] and climate-changing conditions [70] are currently affecting the industries, leading to long delays and negative economic implications within the pigment-related industries (food, feed, pharmaceutical, and cosmetic) [71]. Furthermore, the legislation and regulation of synthetic pigment use in food and feedstuff are narrowing the offer. Microbial pigment additives are still at the developing stage, up-scaling as the future organic source, an alternative to conventional resources, proving higher pigment capacity, in a shorter time. Recent studies regarding pigment-producing yeasts such as *Rhodotorula* sp. show an improvement in carotenoid productivity and componence that might be modulated [72] by mutagenesis [73] combined with other techniques, including different controlled stressors, such as temperature [74,75], substrate composition [76], lightning conditions [77] and aeration [78]. β-carotene, torularhodin, and torulene are valorous compounds synthesized exclusively by fungi and yeast [79], found in lipid bodies

of cellular biomass, and they are light unstable. The yeast carotenoid compounds present highly anti-oxidative characteristics [80].

3. Factors Affecting Pigmentation

3.1. Yeasts Nutrition

The productivity efficiency is generally based on the interdependency of the specific pathway involved and the culture medium (organic or inorganic compounds), strain specificity, and the growing conditions [81]. It is essential to balance the yeast requirements and cultivation conditions, to support the carbon-efficient use and yeast growth rates, thus optimizing secondary metabolites yields [46]. Moreover, individual metabolites yield (pigment quality and quantity) depends on the enzymatic complex employed in the yeast metabolic system [82]. Carbon and nitrogen have been considered the main sources of energy [83], and growth support of microorganisms, metabolite enhancement, and carotenoid producibility could be modulated by balancing the substrate composition, although, there are studies that show the induced stress by nutritive limitation (nitrogen sources) enhances carotenoid productivity (up to 0.75 mg/g dry cell weight) in Rhodotorula toruloides by suppressing the cell growth [84].

The influence of nutritional carbon/nitrogen resources on yeast fermentation and carotenoid yield might differ (Table 3) under the strain pigmentation capacity. Moreover, the nutritional carbon source might modulate the yeast carotenoid profile. Li et al. [83] showed that a medium containing glucose had more than 93% torularhodin in the total yeast carotenoid profile. There are also differences between the utilization of organic and inorganic compounds. Organic nitrogen resources such as yeast extract improve carotenoid yield up to 987 g/L [83], although using residual waste such as fruit and vegetable pulp/peel [85] and beer sludge represent hardly recycled market resources in developing countries, presenting worthy yeast nutritional potential.

Table 3. Factors affecting the carotenoid metabolism in yeast.

Species	Factors Affecting Pigmentation Capacity and Productivity		Results	References
	Mutagenesis			
R. toruloides NP11	Atmospheric exposure, 30 °C and plasma technique followed by chemical mutagenesis with nitrosoguanidine		*R. toruloides* XR-2, colonies of dark-red colored Nitrogen limitation conditions induced slower growth with high carotenoid yields	[84]
Rhodotorula sp. Strains	T-DNA insertional mutagenesis gene discovery in *R. toruloids*		Twenty-seven mutant yeast phenotypes for lipid and carotenoid metabolism	[81]
R. toruloides NP11	An *Agrobacterium tumefaciens*-mediated transformation (ATMT) to change its carotenoid production and profiles		Selected three new phenotypes and mutants with different colors Characterized their carotenoid products	[86]
Rhodotorula toruloides CBS 14 and NCYC 1585	Cloning strategy; four inducible promoters for control gene expression in *Rhodotorula toruloides* to obtain molecular genetic tools for manipulation		Directed genetic and gene expression for carotenoid and lipid yields in *Rhodotorula toruloides*	[87]
	Cultivation medium			
	Carbon	Nitrogen		
Rhodotorula sp.	-	Threonine (0.1, 0.2, 0.3%) glutamic acid (0.1, 0.2, 0.3%)	Both amino acid stimulation enhanced yeast growth parameters and total carotenoid formation	[76]

Table 3. *Cont.*

Species	Factors Affecting Pigmentation Capacity and Productivity		Results	References
Rhodotorula mucilaginosa	Waste from the olive oil industry (Alperujo water, AE) in different aqueous solutions at concentrations: of 5, 10, 20, and 30%		The volumetric carotenoid production significantly increases in 20 and 30% AE concentration (up to 7.3 ± 0.6 mg/L total carotenoids)	[67]
Rhodotorula sp. RY1801	Sucrose, lactose, maltose, fructose and glucose	Inorganic nitrogen: ammonium sulfate, ammonium nitrate; Organic nitrogen: yeast extract, urea	Carbon sources: glucose; carotene yield up to 962 μg/L Nitrogen sources: yeast extract; carotene yield up to 987 μg/L	[83]
Rhodotorula glutinis (AS 2.703)	-	Peptone (PEP), yeast extract (YE), and ammonium sulfate	The highest biomass accumulation was 12.2 g/L after 144 h (YE)	[88]
Rhodotorula mucilaginosa	Onion and potato (skin)	Mung bean (husk) and pea (pods)	The highest carotenoid yield was archived by using onion peel extract and mung bean (up to 717.82 μg/g)	[79]
		Lightning conditions		
Rhodotorula mucilaginosa	Irradiation UV-C—254 nm		Metabolite production by psychro-tolerant *Rhodotorula mucilaginosa* produced up to 56.9 ± 3.2 (μg/g^{-1}.dry weight) of total carotenoids	[89]
R. glutinis (CGMCC No. 2258)	LED lamp's light exposure intensities (4000 lx and 8000 lx)		The lipid and β-carotene production enhancement by using light exposure and sodium acetate componence substrate in *R. glutinis*	[56]
R. mucilaginosa K-(1)	Two lighting shakers: Shaker 1: 1700 lx; Shaker 2: 3500 lx. Settled as 12 h dark: 12 h light		Illumination intensity increases the carotenoid yield (1700 lx); High illuminating intensity (3200 lx) inhibits yeast glucose metabolism. Thus, cell growth	[77]
R. glutinis (CGMCC No. 2258)	Two groups: without and with continuous irradiation (3400 lx)		Continuous irradiation might positively affect the lipid and carotenoid content	[90]
Rhodotorula mucilaginosa	Stress conditions: ultraviolet (UV) light and photoperiods		Optimum conditions for stimulating the carotenoid productivity were 1 min of UV exposure combined with 0.5 mg/L magnesium sulfate and 18:6 h lighting conditions	[91]
		Thermic conditions		
Rhodotorula sp. RY1801	Incubation temperature ranges from 20 to 37 °C		Optimum incubation temperature at 28 °C	[83]
Rhodotorula glutinis	Incubation temperature ranges from 25, 30, 35 to 40 °C		Optimum incubation temperature at 30 °C	[92]
Rhodotorula mucilaginosa	Incubation temperatures 15, 20, 25, 28, 30, 35, and 40 °C		The most suitable temperature for culture growth and carotenoid production was 28 °C	[93]

Table 3. *Cont.*

Species	Factors Affecting Pigmentation Capacity and Productivity	Results	References
R. mucilaginosa ATCC 66034 *and R. gracilis* ATCC 10788	Incubation temperature 20 °C and 28 °C	Optimum incubation temperature at 20 °C	[39]
[74]	Low temperature (16 °C) treatment Control temperature (25 °C) treatment	At 16 °C, the carotenoid yield was significantly increased	[75]
	Aeration conditions		
Rhodotorula glutinis NRRL Y-12905	Different conditions of agitation (150 to 250 rpm) and aeration [(2.5 to 5.0 of flask volume-to-medium volume ratio (vvm)]	Agitation and aeration at 250 rpm and 5.0 optimal conditions (high yeast cell concentration)	[78]
Rhodotorula rubra PTCC 5255	Aeration levels: 0.115, 0.345 and 0575 vvm	The optimum carotenoid concentration was found at an aeration rate of 0.469 vvm, having the substrate initial pH of 6.48, and light intensity of 1757.84 lx	[94]
Rhodotorula mucilaginosa MTCC-1403	Different conditions of agitation: 80, 110, and 140 rpm	Elevation of up to 100 µg carotenoids per g of dry biomass	[79]
	Metabolizable salts and microelements addition		
Rhodotorula glutinis CCT 2186	Different experimental levels of: glucose, KH_2PO_4, $MgSO_4$, NH_4NO_3, and pH	Combined sources of inorganic and organic nitrogen sources had high productivity yields	[45]

Sharma and Ghoshal [79] used onion peel, mung bean, and pea (agro-industrial wastes) as a substrate for pigment production on *Rhodotorula mucilaginosa*, obtaining the best carotenoid productivity (27.4 mg/L) on onion peel extract. The olive oil industrial waste (20%-culture media) improved the total volumetric carotenoid production (up to 5.5 g/L) [67]. Carrot peels or starch in the potato feed industry is a typical example of recoverable fractions either as solids or as sludge which, after drying and sterilization, can be included directly in yeast bioprocess as sources of carbohydrates [95]. By recycling vegetal waste and thus improving the culture media for yeast growth, the productive-related costs are reduced to a minimum or absent in the development of the market economy. The costs involved mainly in recovering profitable nutrients from food waste processing; the credit played is derived from nutritional applications useful in all agriculture branches. At the same time, the economy of waste conversion and valuable byproducts generates a new secondary-industry domain, with new jobs and skills at the place of production [96].

3.2. Yeasts Fermentation Conditions

Carotenoid yield related to obtaining secondary specific metabolites might be an induced response generated by different stressors applied to the *Rhodotorula* sp. growth (nutritive limitation, aeration, and temperature) and could influence (by delaying or accelerating) the carotenoid synthesis. It has been demonstrated that the yeast carotenoid yields maximum values within reaching the cell's mature development [97]. Furthermore, the variability of the carotenoid yield componence proportions within the mature cells variates depending on the temperature and time of cultivation, 144 h on yeast malt, 252.99 µg/g total carotenoids [98], 120 h on yeast malt, 223.5 µg/g total carotenoids production [99].

The temperature parameter is a critical cultivation factor in the first place, affecting culture viability and biomass productivity and active bio compounds quality. Temperature correlates with metabolic functions and influences enzymatic activity [100] with carotenoid productivity, hence, effective regulation between cyclic carotenoids synthesis,

followed by precursors. An indirect metabolic synthesis between the low temperature of β-carotene synthesis and the opposite [101], increasing xanthophyll's and β-carotene precursors concentrations at higher temperature values, is probably by the low-temperature enzymatic activity of lycopene β-cyclase. Recent research points out that higher values of β-carotene production were recorded-by at 20 °C 250 mg/L representing 92% of total carotenoids compared with 30 °C, 125 mg/L, and the amount of 60% from total carotenoids and 35 °C, with less than 19% β-carotene and torulene encountered in biomass; although at 35 °C the torularhodin synthesis increased, leading up to 78% of total carotenoids in biomass [29,39,102].

Yeasts such as *Rhodotorula* sp. have naturally developed a light-sensitive response to environmental lightning conditions, protecting the yeast cells by synthesizing a large amount of β-carotene. White light irradiating trials were conducted on 21 strains of *Rhodotorula* sp., and the results concluded that the amount of carotene is twofold higher by irradiation (14.2 mg/100 g dry weight biomass). At the same time, light irradiation as a photo-regulative measure could modulate yeast growth and biochemical componence to enhance carotenoid productivity [77], although strong light exposure could negatively affect the yeast cultures, inhibiting their growth.

Rhodotorula sp. is an oxygen-dependent yeast [103] affecting both viability and productivity. Recent studies regarding the oxygen demand have demonstrated that the yeast cell growth and metabolism are strongly crisscrossed with yeast phenotype and the yeast-applied stressors, confirmed by the secondary metabolite's yields [30] and other bioactive compounds such as hemoproteins [104].

Besides the photo-protective role, yeast carotenoid active compound has an oxidative protection function facing the oxidant agents before yeast cell wall attack [105]. Oxygen supply, through aeration, agitation, or airlift bioreactors, is crucial to yeast metabolite productivity. Yeast oxygen requirements concerning carotenoid productivity were studied, and the results show an increase of end-metabolites synthase (torularhodin) expected from cyclic carotenoid oxidation [106].

4. Yeasts Pigment Extraction and Quantification

Yeast carotenoid yield determinism is directly modulated by the yeast phenotype and the engineering approach via metabolites enhancement. Despite the progress achieved in the biotechnological yeast carotenoids synthesis optimization, there is a permanent need for research efforts to constantly adapt and improve the in-process efficiency and minimize the economic implication. The yeast fermentative process is followed for the quantification of productivity determinations: preparative (harvesting, cell biomass disintegration) and quantitation methods (extraction, separation, and evaluation).

Harvesting viable cells, carotenoid extraction and purification of the carotenoid components are the most expensive procedures in techno-economic analysis. There are many ways to process yeast carotenoids. Harvesting cell biomass can be easily achieved by mechanical, chemical, or biological strategies. The centrifuge separation is the conventional mechanical method used in yeast industries, employed at 8000–10,000 rpm during a period of 7–10 min [107–109]. Current innovative methods concerning biomass harvesting are flocculation, pre-concentration techniques, high-pressure filtration, flotation, osmosis, bubble columns, and exploitation of hydrophobicity/hydrophobicity yeast proprieties [110]. The appropriate harvesting method is generally chosen through yeast proprieties such as cell size, biomass density, production volume, and final product specificity. Consecutive in yeast recovery, the yeast cell purification techniques, as successive washing with solvent and filtration cycles with the purpose of cell biomass clear separation. There are many carotenoids extractive methods [30,111–113] for samples and pure specific carotenoid quantification. Microbial carotenoids are secondary metabolites [114], present in almost 95% of the cell. Cell wall disruption and disintegration are needed as preparative procedures in carotenoid extraction. The most common approach is an organic/solvent-free mechanical breakage [115,116] combination between sonication/pressure treatments or

freeze-thawing/sonication [117,118] without having major losses on the yeast cell biomass compared to the synthetic chemical disruption that might generate artifacts or radicals [119], artificial condensation (acetonides) [120] or at worst, generating radioactive components (aldehydes) [121]. There are cell breakage methods that are less harmful, involving hydrolysis, supercritical CO2 [122], or enzymatic digestion extraction [123,124], having superior recovery rates, and implying extra financial costs. Carotenoids are non-polar chemical compounds characterized by water insolubility. A more hydrophilic carotenoid form is represented by their derivates, xanthophylls, due to the hydroxyl radical on the chemical structure. The commonly used extractive processes imply the reagents (acetone, cyclohexane, dimethyl sulfoxide, chloroform, petroleum ether, and ethyl acetate) usage as extractive solvents to separate the pigment compound in the partitioned liquid of analysis [125,126]. Carotenoids and xanthophylls are chemical compounds having more than nine double bonds that are capable of light absorption, detected between visible/UV wavelengths range of violet and blue-green spectra (450–550 nm) [127], naturally reflecting red, orange, and yellow color shades. Carotenoid detection and quantification have various protocol approaches, employing spectrophotometry or spectroscopy determinations. Pigment quantification assays are practically based on a comparative determination against pure chemically carotenoid materials (commercially available standard references, as 95–99% pure, for specific determinations), lab standardized as etalons curves, as for accuracy, reproducibility, and repeatability (as for peaks, retention time and area of peak) that later on might be interpreted as values using conversion formulae [128]. Carotenoid UV-Vis assay is a feasible method of quantification but needs a long time to determine because carotenoids obey the law of Lamber–Beer (the compound concentration is directly proportioned with the compound spectral absorbance) [129]. The UV-Vis conducted assays evaluate the liquid carotenoid sample (up to 3.0 mL) compound against the pure carotenoid standard reference substance with the intention of total carotenoid measurement [130]. The disadvantage of employing the UV-Vis method is the mediocre specificity consisting of the incapacity of distinction between individual carotenoids (similarity of peaks and absorbance wavelength around 459–500 nm for more than four distinct carotenoids). Quantitation is possible only by mathematical determination by using specific carotenoid partition coefficients [131]. A more precise approach is using the HPLC method (high-pressure liquid chromatography). Despite the time and costs regarding reagents and capillary system components, carotenoids are detected and measured simultaneously and accurately quantified individually [132] needing no more than 1.5 mL of liquid sample, injected (40 µL) with high pressure, carried (flow rate: 0.5 mL/min) with the eluent (A: acetonitrile: water, 9:1 and B: 1% formic acid ethyl acetate) to the stationary component (column C18, 250 mm × 4.6 mm, 5 µm) and detected (UV detector) [88]. Moreover, it highlights the labor exercise that lies in the systematic examination of the spectral signature, which is no longer just that of the compound of interest, needing specific determination to identify and quantify impurity componence. The FTIR (Fourier transform infrared spectroscopy) is capable of simplifying the total carotenoid quantitation, not only by time (less than 150 s) and cost but also by accuracy, dividing them as chemical structures [133]. RAMAN spectroscopy is the superior method of determination, analyzing at the same time light absorbance and matter structure of the sample only by photon laser interaction with the small sample size, in a very short time determination—based on the relation of light interaction on all materials, scattering the same amount of energy as incidence light [134].

5. Yeast Carotenoids in Poultry Nutrition

5.1. Retinol Requirements and Retinol Precursors in Poultry

The challenge regarding poultry vitamin requirements is and will be an actual research domain due to the genetic abundance and oscillational nutritional aspects between various factors that appear in poultry-intensive sectors (health status, veterinary medications, feed, breed, age, housing aspects, and rearing technology). Poultry specialized hybrids have exigent vitamin A requirements (Table 4), solidly correlated with the breed's purpose and

rearing management recommendation. In poultry, both layers and meat broilers have an excessive level of vitamin A feed supplementation starting from 10,000 International Units (IU)/kg diet up to 13,000 IU/kg diet, according to supplier recommendation, despite the requirements profile established by the National Research Council (NRC, 1994) colorant additives should not exceed 4500 IU/kg-fed meat broilers and 2500 IU/kg-fed layers. Moreover, vitamin supplementation is recommended to be equal to or more than birds' requirements [135], hence avoiding vitamin deficiency. In poultry, provitamin A and retinol deficiency could be a consequence of malabsorption or the impossibility of metabolic conversion, often regarded as biologically available [136]. The effects concerning retinol deficiencies are complex and affect a large range of metabolic activities: weight loss cumulated with slowing down the growth processes and negative performance rates [137], follicular hyperkeratosis, epithelial lesions, xerophthalmia [138], keratomalacia, hemeralopia, reproductive system malfunction [139], and gastrointestinal disorders. Exceeding retinol and provitamin A in poultry leads to xanthomatotic disorders [140] and hypercalcemia, followed by bone system disorders. The vitamin A origin and the stability within the dietary intake is a current challenge, although most of the commercial feeding formulas are developed and balanced by adding artificial vitamins along with micro and macro elements and by not taking into account the vegetal raw material vitamin content, thus the vitamin antagonistic [141] or destructive compounds [142]. Additionally, naturally occurring vitamins in feed and forages are presenting stability issues [61] due to inadequate feed manipulation and storage, often causing vitamin oxidation and frequent bacterial infestation [143], implying constant economic depreciation and loss [144]. Furthermore, the treatments such as insecticides and pesticides administrated to livestock crops are interfering with and affecting the feed vitamin concentration, leading to toxic traces within grain cultures [145].

Table 4. Broiler chicks and laying hens' pigment additive (IU/kg fed) in dietary-fed formulas *.

Broiler Chicks				
Hybrid	**0–11 Days**	**12–23 Days**	**24–42 Days**	**References**
Cobb 500	Up to13,000	10,000	10,000	[146]
Ross 308	13,000	13,000	13,000	[147]
Arbor acres	13,000	10,000	10,000	[148]
Hubbard	13,000	13,000	13,000	[149]
Laying Hens				
Hybrid	**0–6 Weeks**	**7–12 Weeks**	**12–18 Weeks**	**>18 Weeks**	
Hy-line W36	5700	5700	5700	5700	[150]
ISA chick	15,000	15,000	13,500	13,500	[151]
Lohmann	10,000	10,000	10,000	10,000	[152]
Turkeys				
Hybrid		**0–42 Weeks**	**43–84 Weeks**	**>84 Weeks**	
Hybrid Grade Maker male turkey		9000–12,000	9000–12,000	8000–11,000	[153,154]
Hybrid meat turkey		13,500	12,000	11,000	[155]
Ducks				
Hybrid		**Starter**	**Grower/Finisher**	
Longyan laying ducks		10,000	8000/12,000	[156]
Pekin		10,000	10,000	[157]

* As supplier nutritional guidelines.

5.2. Carotenoid Absorption in Poultry

The physiologic and biochemical roles of provitamin A and retinol precursor cover multiple functions. In poultry, in the starter growing phase, retinal and provitamin A stimulates the growth processes and normal development of the reproductive system [158]. Provitamin A is the most important in preventing epithelial disorders (conferring elasticity

and anti-infectious resistance) and maintaining homeostasis of the visual function [159]. In poultry physiology, carotenoid synthesis is absent. Therefore, an exogenous intake is required. Birds can synthesize retinol from β-carotene through the retinal enzyme [90,160], β, β-carotene-15,15′-monooxygenase, capable of separation into two retinal symmetrically molecules [161]. The vitamin A precursor, β-carotene is an indispensable nutrient for reproduction, growth, and production (the biological activity is almost 60% of retinol activity). β-carotene absorption and bio-disposable variates by the bird's metabolism, the bird's absorptive capacity, and the forage quality related to formula stability and biochemical characteristics. Physiologically (Figure 3), β-carotene is a long-term absorption compound (up to three days until retinol conversion) that combines into chylomicrons in the small intestine mucosa (duodenum) and is carried further to the liver through the portal vein [162]. Oil presence enhances the vitamin A precursors absorption and liver metabolization [163], combined with lipoproteins in triglycerides (VLDL and LDL) and transferred to a specific tissue (skin, meat, fat, ovary, and egg yolk). Retinol in excessive quantity is moreover deposited in the liver and blood, then in muscle, fat, eggs, or skin [164]. Egg yolks' carotene deposits vary between 40–50% of total carotenoid intake [165]. However, most of them are lycopene, canthaxanthin, astaxanthin, and zeaxanthin, and lidding the β-carotene yolk concentration less than 1% due to the higher xanthophyll absorption in the bird's digestive tract [166]. Moreover, the bioavailability of carotenoids is mostly influenced by the matricidal food structure, carotenoid compound chemical structure, and interaction with other dietary nutrients.

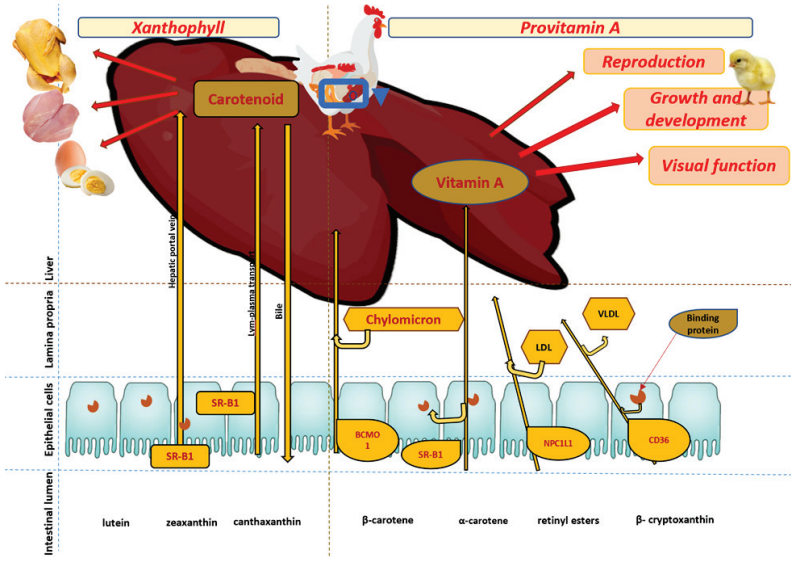


Figure 3. Carotenoids metabolism in poultry physiology; adapted after [167].

5.3. Poultry Feed Sensorial Additives

Nine pigment additives (Table 5) are regulated and used widely in the EU (indexed as pigment additives E160, E161, E162, and E163) [168] as appropriate for poultry nutrition labeled (EU Council directive 96/23/EC, 2010) as dietary pigment additives (SafeFood, 2022), for improving the egg yolk skin and meat product color.

Table 5. Pigment additives used in poultry nutrition [1].

Pigment Additive [1]	Code [2]	Meat *	Egg *	Origin
β-Apo-8′-carotenal	E 161e	80	80	artificial
Cryptoxanthin	E 161b	80	80	natural
Lutein	E 161b	80	80	natural
Ethyl ester of β-apo-8′-carotenoid acid	E 160f	80	80	artificial
Zeaxanthin	E 161h	80	80	natural
Violaxanthin	E 161e	80	80	natural
Citranaxanthin	E 161i	-	80	artificial
Canthaxanthin	E 1601g	25	8	artificial
Capsanthin	E 160c	80	80	natural

[1] Pigment additives regulation in poultry feed approved by the E.U.; [2] additives encoding by Council directive 70/524/EEC; * expressed as mg/kg diet.

The use of commercial carotenoids in poultry feed formulations is expensive and originates from around 90% artificial sources. Most of the pigment additives are approved for dietary inclusion up to 80 mg/kg in broiler chicks feeding formula [11], except canthaxanthin. Canthaxanthin levels are restricted and should not exceed more than 8 mg/kg for laying hens and 25 mg/kg feed for broiler chicks [169]. As the sole pigment additive used in human, fish, and poultry nutrition, canthaxanthin [170] dietary overdosage leads to residual pigment tissue deposits that expose the final consumer to a pigment intake that exceeds the Acceptable Daily Intake (ADI, 0.03 mg/kg body) [171], and might negatively affect the consumer's health (high risk of toxicity). Research concerning natural carotenoid sources as an alternative to commonly used synthetics for livestock nutrition shows that using natural resources such as maize and pasture (fresh or preserved) [172,173] and genetically modified organism (GMO) or non-GMO (plants, algae, and yeasts) could serve as superior native carotenes used pigment additives [174–176]. Few studies regarding the microbial piments additives on broiler meat [45,177,178]. Dietary inclusion of red yeast *Phaffia rhodozyma* (10–20 mg/kg feed) on broiler chicks positively affected the broiler chicks' performances and immune response, presenting 10 times stronger pigment capacity [179]. Moreover, in broiler nutrition, pigment additives are often employed along with oils [180,181] to mitigate the spontaneous oxidative effects on fat deposits and to improve the carcass's oxidative stability [182]. Furthermore, dietary carotene addition shows controversial effects via vitaminic metabolism, showing antagonistic [183] and synergic action [175] and might have an opposite role as an antioxidant [184] and pro-oxidant factor [185], depending on factors such as dietary formulation (inclusion or addition). The antagonism between vitamin E accumulation and β-carotene was studied, and the results show that the presence of β-carotene in broiler breast meat tends to limit vitamin E accumulation [186]. However, the dietary addition of lycopene and vitamin E improves the broiler chicks' growth performance and tight meat oxidative stability and also presents a synergic benefic effect on thigh meat cholesterol content. In laying hens, diets include distinct amounts of corn and alfalfa meal, contributing to the content of native pigments in the diet [6]. Intensive rearing systems diets are low in native xanthophylls. Therefore, the egg yolk is often characterized by a pale-yellow color [6] due to rich amounts in barley, rice, or wheat that are supplemented with artificial pigments (β-apo-8ícarotenoic-acid-ethyl ester) to satisfy the range of color scores required by the European egg producers and to meet the consumer's expectations [187].

6. Conclusions

As a directed movement in the food and feed markets guided for more natural products, the demand for organic ingredients is rising. Feed formulation recipes using natural and organic additives are the new trend in livestock nutrition research, using not only active principles that affect vegetal but also microorganisms for valuable active bio compounds. Yeast pigments are outstanding sources of natural color, covering a wide range of nutri-

tional and medicinal properties. Both carotenoid yield and total carotenoid structure are important aspects that could be optimized depending on strategy, adopting strain genetic engineering and process development, and employing cheap organic substrates. Further studies are required to establish biological and chemical proprieties, and yeast carotenoid mechanisms, enhancing yeast carotenoid productivity, stability, and marketability as alternatives to classic synthetic pigments. Data generation concerning a highly productive yeast process involving scalability for large-scale adaptability to fermentation aspects (fermentation design and bioreactor types) is essential. Furthermore, studies regarding the effects of value-added yeast pigment additives on livestock health, productivity, and product quality are important in validating nutritional and medicinal potential. Not last, consumers' perceptions and preferences in buying animal products obtained with microbial pigment additives firmly increase the need for knowledge.

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