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Effect of synbiotics on performance and gut health in broiler chickens submitted to an enteric challenge

Efeito do uso de simbiótico sobre o desempenho e saúde intestinal de frangos de corte submetidos a um desafio entérico

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Highlights

Use of synbiotics as an alternative to performance enhancers.
Improved feed conversion efficiency with synbiotics.
Enteric challenges affect gut mucosal permeability.

Abstract

The aim of this study was to assess the effects of a nutritional program free of performance enhancers when compared to a program using synbiotics, in both enteric challenge and non-challenge conditions, on the intestinal health and performance of broiler chickens. A total of 864 one-day-old male Cobb broiler chicks were used. The birds were distributed in a completely randomized design, using a 2 × 2 factorial scheme (2 diets × 2 health conditions), resulting in 4 diets with 12 replications of 18 birds per cage, totaling 48 experimental units. The diets used were control diet (Diet A); control diet + synbiotic (Diet B); Diet A + enteric challenge; and Diet B + enteric challenge. At 14 days old, an enteric challenge was applied with a commercial coccidiosis vaccine (20 times the manufacturer's recommended dose), followed by inoculation with *Escherichia coli* (ATCC® 8739™). The enteric challenge resulted in worse performance in all the phases assessed and changed the intestinal mucosa morphology five days after the challenge. At 28 days old, two weeks after the enteric challenge, a regenerative process was already occurring. Supplementation with synbiotics improved the feed conversion of the 28-day-old birds, regardless of the experimental challenge. Synbiotic supplementation resulted in greater tensile

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strength, hardness, and elasticity of the jejunal mucosa. These results indicate that it is possible to improve productivity using alternative additives, even in experimental environments where variables are controlled and free from challenges that compromise animal welfare and health.
Key words: Performance enhancers. Microbiome. Synbiotics.

Resumo

O objetivo deste estudo foi avaliar a resposta de um programa nutricional isento de aditivos melhoradores de desempenho comparado a um programa com uso de simbióticos, em situação de desafio entérico ou não, sobre a saúde intestinal e o desempenho de frangos de corte. Foram utilizados 864 pintos de corte, machos de 1 dia de idade, linhagem Cobb. As aves foram distribuídas em um delineamento inteiramente casualizado, esquema fatorial 2 x 2 (2 dietas x 2 condições sanitárias), totalizando 4 dietas com 12 repetições de 18 aves por gaiola, totalizando 48 unidades experimentais. As dietas utilizadas foram dieta controle (Dieta A); dieta controle + simbiótico (Dieta B); dieta A + desafio entérico e dieta B + desafio entérico. Aos 14 dias de idade, foi aplicado o desafio entérico com vacina comercial para coccidiose (20 vezes a dose recomendada pelo fabricante) seguido da inoculação contendo *Escherichia coli* (ATCC® 8739™). O desafio entérico resultou em pior desempenho produtivo em todas as fases avaliadas, e alterou a morfometria da mucosa intestinal, cinco dias após o desafio. Aos 28 dias de idade, duas semanas após o desafio entérico, observou-se um processo de regeneração já em resolução. A suplementação das dietas com o simbiótico melhorou a conversão alimentar das aves aos 28 dias de idade, independentemente do desafio experimental. A suplementação com simbiótico resultou em maior força de ruptura, dureza e elasticidade da mucosa intestinal do jejuno das aves. Esses resultados evidenciam que é possível melhorar a produtividade com o uso alternativo de aditivos zootécnicos, mesmo em ambientes experimentais cujas variáveis são controladas e isentas de desafio que comprometa o bem-estar e a saúde animal.
Palavras-chave: Melhoradores de desempenho. Microbioma. Simbiótico.

Introduction

The global demand for meat has been increasing annually. In addition to meeting market needs, industries must adapt to new standards imposed by consumers, who are seeking quality-certified products that ensure food safety, demonstrating growing public health concerns (Associação Brasileira de Proteína Animal [ABPA], 2021).

Antibiotics have been used for decades in feed not only to control diseases but also to enhance performance and feed efficiency in animals. However, the widespread use of antibiotics over time can contribute to the development of drug-resistant bacteria that combat human infections. As such, the global market has imposed restrictions on products considered risky for public health, leading to the ban on growth-promoting antibiotics (Gadde et al., 2018).

These factors make it essential to develop strategies that allow continued efficient production without using antibiotics as growth enhancers. Nutrition

may be an option, requiring continuous adjustment to achieve better performance while simultaneously ensuring a diet with immunogenic capacity. In this respect, it is often necessary to adopt alternative programs to growth-enhancing antibiotics, typically based on certain feed additives. Non-antibiotic therapies, such as probiotics and prebiotics (which include competitive exclusion mechanisms), as well as essential oils, have demonstrated bacteriostatic properties (Keerqin et al., 2017; H. Wang et al., 2017; Whelan et al., 2018).

Among these additives, the use of probiotics or microorganisms in the diet has attracted significant attention (Lekshmi et al., 2017). Different strains have distinct modes of action, such as competition for nutrients and adhesion to enteric pathogens. Probiotic bacteria help in nutrient digestion and absorption by producing hydrolytic enzymes such as amylase, lipase, and protease. These beneficial bacteria also enhance immunity by modulating the host's immune system and altering microbial activities in the gut (Pourabedin et al., 2015; Hofacre et al., 2019).

Thus, intestinal health is a holistic concept that includes diet, mucosa, microbiome, and the immune system. The diet provides nutrients that help the mucosa maintain intestinal integrity and a stable microbial community in a balanced and healthy environment, which, in turn, keeps the mucosal immune system in a state of defense and tolerance (Wang et al., 2019).

Commercial production is extremely challenging for poultry, since environmental and microbiological stressors are present throughout the production period. The gastrointestinal tract remains in a state of physiological inflammation most of the time, and high pathogen exposure can transform this inflammation into a pathological condition. Thus, rapid regeneration and reconstitution of epithelial integrity are essential due to the multifunctional complexity of this mucosa. A total intestinal cell turnover can occur between 24 and 96 hours, representing 2.5 to almost 10% of the broiler chicken's life cycle (Iseri & Klasing, 2014; Gottardo et al., 2016).

As such, the aim of this study was to assess the effect of a nutritional program using synbiotics as performance enhancers, in enteric challenge and non-challenge situations, on the intestinal quality and performance of broiler chickens.

Material and Methods

The experiment was conducted on an experimental farm in Palotina, Paraná state, Brazil, with all procedures approved by the Animal Ethics Committee of the Federal University of Paraná – Palotina Sector, under process CEUA 18/2020.

A total of 864 male Cobb broiler chicks were randomly distributed in a completely randomized design, with a 2 × 2 factorial scheme (two diets: control and supplemented with synbiotics, and two health conditions: with and without challenge), resulting in four diets with 12 replicates of 18 birds per cage, totaling 48 experimental units. The diets were as follows: Diet 1: control diet; Diet 2: control diet + synbiotics; Diet 3: control diet + enteric challenge; Diet 4: control diet + synbiotics + enteric challenge

The birds were housed in two equal-sized rooms with identical environmental conditions according to diets 1 + 2 and 3 + 4. The experimental cages were equipped with heating lamps, fans, exhausts, and cooling plates controlled by an automated system to maintain ambient temperature within the thermal comfort zone. The bedding consisted of shredded paper, with poultry litter water and feed provided *ad libitum.*

Three diets based on maize and soybean meal were formulated, as follows: pre-initial (days 1 to 7), initial (days 8 to 20), and growth (days 21 to 28), to meet the nutritional requirements of the birds according to the adapted recommendations of Rostagno et al. (2017) (Table 1). The synbiotic used in the diet contained *Bacillus coagulans* (5×10^{-7} CFU g^{-1}), *Bacillus licheniformis* (5×10^{-8} CFU g^{-1}), *Bacillus subtilis* (5×10^{-8} CFU g^{-1}), *Lactobacillus acidophilus* (5×10^{-7} CFU g^{-1}), *Saccharomyces cerevisiae* (2×10^{-7} CFU g^{-1}), and *mannan oligosaccharides* (2 g kg^{-1}). The dosage provided as per the manufacturer's recommendation was 200 g $tonne^{-1}$ of feed in the pre-initial phase and 100 g $tonne^{-1}$ in the subsequent phases.

At 14 days old, the challenged groups (diets 3 and 4) received a commercial coccidiosis vaccine containing *Eimeria acervulina, Eimeria maxima, Eimeria praecox, Eimeria tenella,* and *Eimeria mitis.* The vaccine was administered directly into the crop of each bird at 20 times the recommended dose (±80,000 sporulated oocysts). Two days after vaccine administration, an inoculum containing *Escherichia coli* (ATCC® 8739™) with a concentration of 10^9 CFU day^{-1} $bird^{-1}$ was prepared and inoculated directly into the crop of each challenged bird.

Body weight was recorded weekly during the experiment (days 7, 14, 21, and 28). Average weight gain was calculated by the difference between the weights of each period, divided by the number of birds. Average feed intake was determined by the difference between feed consumption and leftovers for each period, divided by the total number of birds in each replicate. The feed conversion rate (FCR) was calculated as the weight of feed consumed divided by weight gain. There was no need to account for mortality, since no deaths occurred during the experiment.

On days 19 and 28, 24 birds from each diet group were euthanized to collect duodenum, jejunum, and ileum fragments. Samples (approximately 5 cm) were dissected, fixed in 10% buffered formalin, and embedded in paraffin. Sections (5 µm thick) were prepared and stained with hematoxylin and eosin.

Table 1
Nutritional Composition of the experimental diets

	Pre-initial		Initial		Growth	
Diets	1 and 3	2 and 4	1 and 3	2 and 4	1 and 3	2 and 4
Ingredients						
Maize	519.61	519.61	533.33	533.33	602.57	602.57
Soybean meal (46%)	411.08	411.08	400.00	400.00	333.33	333.33
Soybean oil	30.39	30.39	33.92	33.92	33.33	33.33
Calcitic limestone (35%)	12.55	12.55	10.90	10.90	11.59	11.59
Bicalcium phosphate (18%)	11.76	11.76	9.61	9.61	7.18	7.18
Common salt	4.902	4.902	4.902	4.902	4.274	4.274
Lysine	2.451	2.451	1.843	1.843	2.564	2.564
DL-Methionine	3.039	3.039	2.745	2.745	2.427	2.427
L-Threonine	0.735	0.735	0.314	0.314	0.530	0.530
Choline chloride (60%)	-	-	0.235	0.235	-	-
Choline (20.8%)	1.176	1.176	-	-	-	-
B.H.T.	0.100	0.100	0.098	0.098	0.100	0.100
Kaolin	0.200	-	0.100	-	0.100	-
Synbiotic additive	-	0.200	-	0.100	-	0.100
Vit. Premix + Min.[1]	2.00	2.00	2.00	2.00	2.00	2.00
Calculated nutritional levels						
Met. energy,kcal/kg	2.977	2.977	3.024	3.024	3.099	3.099
Raw protein (%)	23.48	23.48	22.99	22.99	20.52	20.52
Raw fiber (%)	3.50	3.50	3.47	3.47	3.25	3.25
Calcium (%)	0.967	0.967	0.855	0.855	0.803	0.803
Available phosphorous (%)	0.461	0.461	0.421	0.421	0.370	0.370
Electrolyte bal., mEq/kg	236.81	236.81	233.19	233.19	203.81	203.81
Digestible lysine (%)	1.303	1.303	1.244	1.244	1.122	1.122
Digestible methionine (%)	0.633	0.633	0.599	0.599	0.537	0.537
Methionine+ Dig. Cystine (%)	0.959	0.959	0.921	0.921	0.831	0.831
Digestible tryptophan (%)	0.262	0.262	0.256	0.256	0.222	0.222
Digestible threonine (%)	0.862	0.862	0.807	0.807	0.740	0.740
Digestible arginine (%)	1.487	1.487	1.457	1.457	1.269	1.269

[1] Vitamin premix and initial mineral (kg per premix): Vitamin A (KUI/kg 6,500.000); Vitamin D3(KUI/kg 1,625.000); Vitamin E (UI/kg 22,500.000) Vitamin K3 (mg/kg 1,250.080); Vitamin B1-Thiamine (mg/kg 749,700); Vitamin B2 – Riboflavin (mg/kg 3,000,000); Vitamin B6 – Pyridoxine (mg/kg 1,500,380); Vitamin B12 – Cyanocobalamin (mcg/kg 6,000,000); Pantatenic acid (mg/kg 5,999,560) Niacin (mg/kg 12,499,900); Folic acid (mg/kg 399,840); Biotin (mcg/kg 75,000,000); Manganese (ppm 39,999,960); Zinc (ppm 40,000,100); Iron (ppm 24.999,900); Iodine (ppm 550,200); selenium (ppm 150,000); BHT (ppm 300,000); Phytase (g/kg 250,000).

For morphometric analysis, images were captured using light microscopy (10x objective) and analyzed with a computer-assisted image analyzer (ImagePro-Plus - Version 5.2 – Media Cybernetics). The length and width of 20 villi were measured from each section. These morphometric measures were used to calculate the mucosal absorption area using the formula proposed by Kisielinski et al. (2002).

Absorption area:

$$\frac{(VW \times VH)+(VW\ 2^{-1} + LC\ 2^{-1})^2 - (VW\ 2^{-1})^2}{(VW\ 2^{-1} + CW\ 2^{-1})^2}$$

Where VW = villus width, VH: villus height, CW: crypt width

Ultrastructural analysis was performed only on ileal sections fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate (pH 7.4). Tissues were washed in buffer, fixed in 1% osmium tetroxide for 1 hour, immersed in 1% tannic acid for 1 hour, and dehydrated in a series of increasing ethanol concentrations. After critical point drying with CO2, fragments were gold ion-coated and photographed using a scanning electron microscope (TESCAN VEGA3).

From the same birds (24 per diet), 8cm ileal fragments were immersed treatment in physiological saline for 24 hours to conduct a flexion test. A perforation testing device adapted to a texture analyzer (Model TA-XT2i, Stable Micro Systems Ltd., Godalming, UK), was used to obtain parameters such as constant strain rate for viscoelastic material, tensile strength (kg), and elasticity (mm) of the ileal mucosa. The parameters used were speed of 1 mm s^{-1}, trigger force of 10 g, and tension of 15 mm.

Intestinal permeability was assessed by measuring the serum levels of fluorescein isothiocyanate-dextran (FITC-d). On day 19, FITC-d (0.55 mg kg^{-1}) was administered orally to one bird per replicate. After 2 hours, the birds were euthanized, blood collected, allowed to clot at room temperature, centrifuged to obtain serum, and stored in freezers for subsequent analysis. Serum FITC-d fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm. FITC-d concentration per mL of serum was calculated based on a standard curve of known FITC-d concentrations.

Cell proliferation activity in the intestines was analyzed by immunohistochemistry for PCNA (Proliferating Cell Nuclear Antigen) in 5 µm jejunal sections, which were cleared in xylene, hydrated in decreasing concentrations of alcohol and treated with 3% hydrogen peroxide, followed by incubation with 10 mM citric acid (pH 6.0) in a microwave. The sections were washed in PBS (Phosphate Buffered Saline), treated with 1% BSA (Bovine Serum Albumin) in PBS for 1 hour, incubated with monoclonal anti-PCNA antibody (FL-261; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and detected with anti-rabbit polyclonal antibody. Three images of the crypt region were captured from each slide (40x objective), and PCNA-positive cells per mm^2 were measured and quantified.Data were submitted to analysis of variance via the GLM procedure of SAS software (Statistical Analysis System Institute [SAS Institute], 2002).

Results and Discussion

At 7 days old, there were no significant differences in any productive parameters between broiler chickens supplemented or not with the synbiotic (Table 2). However, at 14 days, adding the synbiotic to the diet resulted in improved weight gain ($p<0.054$) and FCR ($p<0.001$) (Table 2).

Table 2
Productive performance of broiler chickens from 1 to 7 days and 7 to 14 days old supplemented or not with synbiotic

	Body weight, g		Feed intake, g		Weight gain, g		Food conversion	
Diets	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
Basal	188.27	417.24	139.55	280.29	146.52	225.84[b]	0.952	1.243[a]
Synbiotic	186.73	418.72	140.42	274.51	145.05	233.00[a]	0.965	1.182[b]
CV%	2.14	3.42	3.11	5.00	2.72	5.47	3.31	5.012
p-value	0.2298	0.7213	0.5000	0.1649	0.2416	0.054	0.1761	0.0001

CV: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$).

This improvement in FCR corroborates He et al. (2018) and Leite et al. (2020), who reported that FCR is one of the primary performance indicators in broiler chickens fed diets supplemented with synbiotics or probiotics. This higher FCR is generally attributed to the Bacillus subtilis, Bacillus licheniformis, and Bacillus coagulans strains. These non-pathogenic, spore-forming bacteria are widely used in industrial poultry farming due to their resilience to the high temperatures applied in feed manufacturing, attributed to the longer expiry date of the commercial products, low pH, bile, and other antimicrobial compounds present in the gastrointestinal tract (Ciurescu et al., 2020). Additionally, these strains improve growth by secreting a range of enzymes such as lipase, protease, and amylase, which favor digestion and nutrient absorption, thereby optimizing feed use (Hmani et al., 2017; Hofacre et al., 2019). Authors also report that bacilli enhance bird performance through greater intestinal mucosa development, increased enzyme expression on the brush border, colonization of the gastrointestinal tract, and fimbrial adhesion to intestinal epithelial cells, thereby maintaining intestinal mucosal integrity and functionality (Ma et al., 2018). Improved weight gain and FCR with the synbiotic may also be attributed to an improved gut microbiota that hinders pathogenic bacteria by competing for vital space, intestinal binding sites, and nutrient availability.

On the 14th day of life, the enteric challenge protocol was conducted, and zootechnical data were collected for the following periods: 5 days post-challenge (days 14 to 19), 7 days post-challenge

(days 19 to 21), and the week following the challenge (days 14 to 21). Between days 14 and 19, the enteric challenge affected productive performance, namely, lower weight gain ($p<0.001$), reduced feed intake ($p<0.029$), and worse FCR ($p<0.001$) regardless of the diets. With respect to the experimental diets, the control diet resulted in a better FCR ($p<0.012$). Between days 19 and 21, the same behavior was observed in the enteric challenge. Birds submitted to the challenge, regardless of diet, exhibited lower weight gain ($p<0.001$), reduced feed intake ($p<0.001$), and worse FCR ($p<0.0002$). The diets influenced the FCR, similarly to that observed in the previous period.

The zootechnical performance of the birds corroborates the findings described by El-Sawah et al. (2018), where the best results were from the negative control treatment, characterized by the absence of synbiotic supplementation and enteric challenge. However, these results differ from those described by Leite et al. (2020), which indicate better performance for groups that received probiotic or synbiotic supplementation in the diet up to 21 days. These discrepancies in zootechnical performance are generally attributed to different variables in the experimental design of each study, such as variations in the diet compositions and synbiotic formulations used, synbiotic supplementation concentration, and environmental management conditions, including housing type, bedding, temperature, available space, stresses, and, particularly for this study, the period in which the enteric challenge was applied.

Considering the period between 14 and 21 days, that is, one week post-infection, no significant interactions were observed between the diets and the enteric challenge. However, the challenge affected all the performance variables analyzed independently of the diets, interfering with productive performance and resulting in reduced weight gain ($p<0.001$), lower feed intake ($p<0.0001$), and worse FCR ($p<0.001$) (Table 3). This response may be attributed to the possible energy shift in the challenged birds in developing an immune response and regenerating intestinal mucosa to contain pathogen proliferation.

Table 3
Productive performance of boiler chickens aged between 14 and 21 days, supplemented or not with synbiotics and submitted or not to enteric challenge

	Feed intake, g	Weight gain, g	Food conversion
Diet			
Basal	589.14	435.86	1.365
Synbiotic	590.07	429.47	1.399
Challenge			
Control	607.52[a]	477.85[a]	1.283[b]
Challenged	571.69b	387.47[b]	1.481[a]
Basal diet + control	612.43	484.09	1.267
Basal diet + challenge	565.85	387.62	1.463
Synbiotic diet + control	602.61	471.62	1.300
Synbiotic diet + challenge	577.54	387.31	1.499
CV%	4.87	8.27	5.74
Diet	0.9121	0.539	0.138
Challenge	0.0001	<.0001	<.0001
Diet x Challenge	0.2059	0.559	0.946

CV: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$).

Between days 21 and 28, the negative effect of the enteric challenge on the birds' weight gain persisted ($p<0.0431$). Diet supplementation with the synbiotic product lowered weight gain ($p<0.001$) and feed intake ($p<0.001$). No interaction was observed between enteric challenge and diet for any of the variables analyzed.

The productive performance results observed from 1 to 28 days of age show that there were no differences in average weight and weight gain between birds supplemented with basal or synbiotic diets, regardless of the challenge. However, feed intake and FCR were affected by synbiotic consumption, whereby birds receiving the synbiotic diet had lower feed intake ($p=0.0023$) and better FCR ($p=0.0046$) (Table 4).

As observed, when the birds were supplemented with the synbiotic, feed intake was lower and performance results during the enteric challenge period were unfavorable during some periods. The synbiotic contains probiotic strains that may initially cause an imbalance in the intestinal microbiota, followed by a subsequent microbiota reorganization; however, this requires time. Thus, it is evident that products of this class require prolonged supplementation, that is, until the age of slaughter, approximately 42 to 49 days, to effectively contribute to microbiota reorganization, eubiosis restoration, immune response adjustment, and result in improved performance, as demonstrated by the results over the entire period, as shown in Table 4.

Table 4
Productive performance of boiler chickens aged between 1 and 28 days, supplemented or not with synbiotics and submitted or not to enteric challenge

	Average weight, g	Feed intake, g	Weight gain, g	Feed conversion
Diet				
Basal	1490.18	2066.65[a]	1448.06	1.431[a]
Synbiotic	1498.45	1996.52[b]	1456.82	1.376[b]
Challenge				
Control	1566.07[a]	2038.89	1523.93[a]	1.340[b]
Challenged	1422.56[b]	2024.29	1380.94[b]	1.467[a]
Basal diet + control	1560.65	2080.73	1518.22	1.371
Basal diet + challenge	1419.71	2052.57	1377.90	1.490
Synbiotic diet + control	1571.49	1997.04	1529.64	1.309
Synbiotic diet + challenge	1425.41	1996.00	1383.99	1.444
CV%	4.30	3.70	4.42	4.47
Diet	0.6578	0.0023	0.6392	0.0046
Challenge	<.0001	0.5042	<.0001	<.0001
Diet x Challenge	0.8902	0.5348	0.8865	0.6478

CV: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$).

The hypothesis that probiotics need additional time to be effective was also raised by Nakphaichit et al. (2011). The researchers administered Lactobacillus reuteri to broiler chickens only during the first week of life and then monitored the ileal microbiota through 16S RNA gene sequencing for 6 weeks. The study concluded that probiotics administered in the early phases only resulted in positive effects after 6 weeks, demonstrating significant diversity and abundance of Lactobacillus and a significant reduction in pathogens when compared to the control. These data reinforce the performance results when considering the total period from 1 to 28 days old, where similar weight gain was observed between the basal diet and the diet containing the synbiotic, but with lower feed intake ($p<0.05$) and better FCR ($p<0.05$), favorable to the groups receiving the synbiotic diet (Table 4).

Intestinal permeability analysis was conducted at 19 days old, which revealed no significant interaction between diet and enteric challenge. However, increased permeability was observed in the intestines of challenged birds ($p<0.0001$) (Figure 1; Table 5). This finding corroborates the results obtained in the morphometric parameters, where enteric challenge led to a disruption in the integrity of the intestinal wall, possibly due to the breakdown of tight junctions, resulting in damage to the continuous layer of epithelial cells. It is important to note that intestinal integrity is an indicator of the efficiency of the protective barrier formed by the gastrointestinal tract, which prevents

the paracellular translocation of unwanted substances, such as bacterial toxins and microorganisms from the intestinal lumen to the lamina propria and subsequently to the bloodstream (Morales-Barrera et al., 2016). Disturbances in intestinal integrity drastically affect nutrient absorption, altering the homeostasis of organisms and substrate metabolism and compromising everything from electrophysiological functions to the intestinal barrier, consequently causing nutritional deficiencies (Rodrigues et al., 2016). This nutritional deficiency can be observed as a loss in zootechnical performance in challenged animals, particularly during the challenge period from 14 to 21 days old.

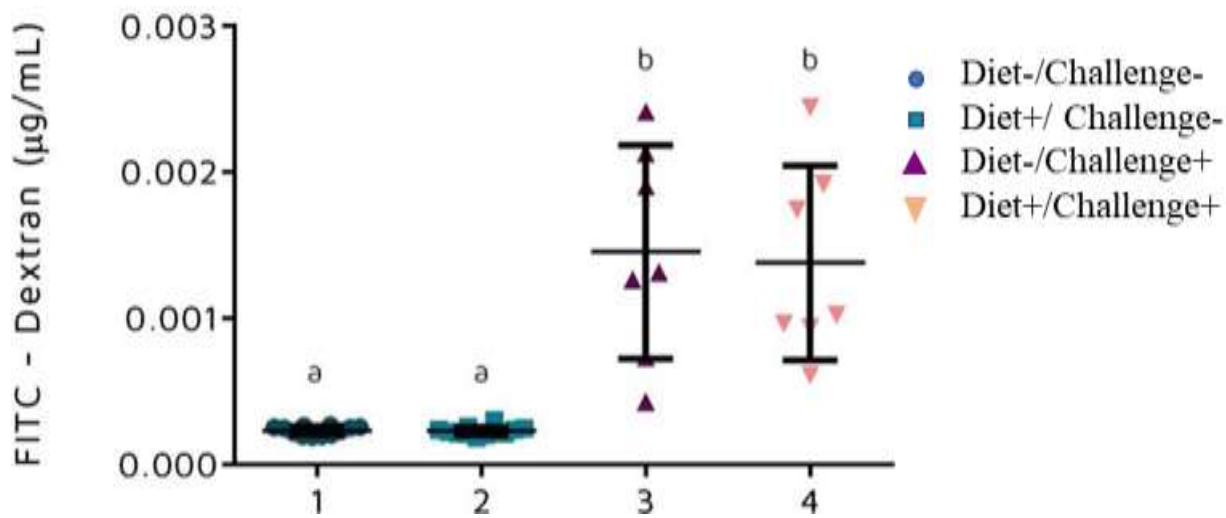

Figure 1. Serum Concentration of Oral FITC-Dextran Marker.
Higher serum concentrations indicate increased intestinal permeability. Each point on the graph represents a sample and the vertical line indicates sample standard deviation.

Table 5
Serum levels of FITC-d and PCNA-positive cell count in the jejunal mucosa of 19-day-old broiler chickens, supplemented or not with synbiotics and submitted or not to an enteric challenge

	FITC-d (µg/mL)	Cells in mitosis/mm^2
Diet		
Basal	0.248	0.458
Synbiotic	0.265	0.436
Challenge		
Control	0.230^b	0.451
Challenged	0.284^a	0.444
Basal diet + control	0.224	0.473
Basal diet + challenge	0.276	0.445
Synbiotic diet + control	0.237	0.430
Synbiotic diet + challenge	0.292	0.443
CV%	13.49	33.11
Diet	0.2149	0.4654
Challenge	<.0001	0.8086
Diet x Challenge	0.8665	0.5051

CV%: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$).

For the PCNA-positive cell count in the crypts of the jejunal mucosa of broiler chickens, there was no significant interaction between diet and enteric challenge, nor any effect of synbiotic supplementation or enteric challenge (Table 5). PCNA-positive cells express antigens during the late G1 phase (first interval) and S phase (synthesis period) of the cell cycle. This cycle is divided into phases with distinct functions and durations. Nuclear division and separation of daughter cells occur in mitosis (M phase); the period between one mitosis and another is called Interphase. DNA replication occurs only in the S phase (synthesis period). The interval between the end of mitosis and the start of DNA synthesis is called the G1 phase, and that between the end of DNA synthesis and the start of mitosis is the G2 phase; these phases provide additional time for cell growth (Schafer, 1998).

The rapid proliferation of epithelial cells is essential for the replacement of the intestinal epithelium, while crypt depth indicates the compensatory capacity or hyperplasia of epithelial cells due to greater aggression against the morphological structure of the intestinal mucosa caused by the enteric challenge (Uni et al., 1998).

At 19 and 28 days old, intestinal integrity was assessed based on parameters of tensile strength, elasticity, and hardness (Table 6). At 19 days old, 5 days after the enteric challenge, no significant differences were observed for the challenge or experimental

diets. However, when the same parameters were assessed at 28 days old, groups that received the synbiotic additive demonstrated greater tensile strength (p=0.0067), elasticity (p=0.0257), and hardness (p=0.0134), indicating that extensive use of the synbiotic improved intestinal quality.

Table 6
Elasticity and toughness of the intestine in broiler chickens at 19 and 28 days old, supplemented or not with synbiotics and submitted or not to an enteric challenge

	Elasticity, mm	Toughness, kg/sec
Diet	19 days of age	
Basal	37.42	1.846
Synbiotic	37.38	1.944
Challenge		
Control	37.36	1.994
Challenged	37.43	1.793
Basal diet + control	38.45	1.883
Basal diet + challenge	36.43	1.807
Synbiotic diet + control	36.23	2.110
Synbiotic diet + challenge	38.58	1.779
CV%	32.79	28.43
Diet	0.9897	0.3753
Challenge	0.9502	0.0719
Diet x Challenge	0.4007	0.2577
Diet	28 days of age	
Basal	34.71[b]	3.405[b]
Synbiotic	39.83[a]	4.076[a]
Challenge		
Control	36.50	3.587
Challenged	38.23	3.868
Basal diet + control	32.73	3.235
Basal diet + challenge	36.69	3.581
Synbiotic diet + control	39.95	3.990
Synbiotic diet + challenge	39.70	4.155
CV%	28.10	33.60
Diet	0.0257	0.0134
Challenge	0.4115	0.3338
Diet x challenge	0.3527	0.7332

CV%: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference (p<0.05).

The effectiveness of probiotics depends on several factors, such as the composition of the mixture, the timing of administration, and the origin of the microorganisms. The microorganisms most widely used as probiotics are mainly Gram-positive bacteria from the Bifidobacterium group and lactic acid bacteria (LAB), including the genera Enterococcus, Lactobacillus, Pediococcus, Streptococcus, Lactococcus, Leuconostoc, and Bacillus. In addition to bacteria, fungi and yeast strains, primarily from Saccharomyces cerevisiae and Kluyveromyces species, are also used as probiotics (Markowiak & Slizewska, 2018; Zommiti et al., 2020).

According to Clavijo and Flórez (2018), strains isolated directly from chicken intestines are more effective than those from other sources. Additionally, the composition of the probiotic may be beneficial for specific enteric challenges, and its effect depends on when it is administered and how and when the effects on intestinal mucosal regeneration are assessed.

Morphometric analyses of the duodenal, jejunal, and ileal mucosa were performed at 19 and 28 days old. No significant interaction was observed between diet and enteric challenge for any of the parameters assessed. In the duodenal mucosa morphometric analysis at 19 days old (Table 7), the challenge increased villus width ($p=0.0213$) and crypt depth ($p<0.0001$) and decreased villus ratio ($p=0.0225$), regardless of synbiotic inclusion in the diet. These morphological changes indicate a regeneration process and an attempt to return to intestinal homeostasis. The performance results at this age showed lower weight gain and average weight, and worse feed conversion, demonstrating that the enteric challenge model used in this study can be applied as a model for assessing nutritional additives.

Table 7
Morphometry of the duodenal mucosa in broiler chickens at 19 and 28 days old, supplemented or not with synbiotics and submitted or not to an enteric challenge

	VL, µm	VW, µm	CD., µm	CW., µm	MLT, µm	V:C	AA, µm²
Diet				19 days			
Basal	570.11	69.32	81.59	24.84	78.19	7.04	18.50
Synbiotic	588.82	69.41	80.52	25.11	80.90	7.18	18.58
Challenge							
Control	564.41	66.74[b]	74.97[b]	24.63	79.52	7.40[a]	18.60
Challenged	594.52	72.00[a]	87.14[a]	25.32	79.56	6.82[b]	18.48
Basal diet + control	561.45	65.48	75.72	24.57	76.73	7.32	18.86
Basal diet + challenge	578.77	73.16	87.46	25.11	79.65	6.75	18.13
Synbiotic diet + control	567.36	68.00	74.22	24.68	82.32	7.47	18.33
Synbiotic diet + challenge	610.27	70.83	86.82	25.53	79.47	6.89	18.84
CV%	18.27	15.21	13.60	14.15	20.90	15.40	13.98
Diet	0.4211	0.9667	0.6457	0.7207	0.4404	0.5531	0.8806
Challenge	0.1966	0.0213	<.0001	0.3493	0.9906	0.0225	0.8477
Diet x Challenge	0.5818	0.2822	0.8523	0.8322	0.4108	0.9943	0.2932
Diet				28 days			
Basal	707.25	77.89	62.85	22.09	81.82	11.20	22.08
Synbiotic	711.68	77.30	63.68	22.64	85.06	11.15	22.41
Challenge							
Control	710.77	81.01[a]	61.19[b]	22.63	79.33[b]	11.37	21.33[b]
Challenged	708.11	74.30[b]	65.22[a]	22.12	87.34[a]	10.98	23.15[a]
Basal diet + control	703.16	80.67	60.20	22.30	76.98	11.36	21.04
Basal diet + challenge	710.66	75.35	65.27	21.90	86.25	11.04	23.12
Synbiotic diet + control	718.38	81.35	62.19	22.96	81.67	11.38	21.62
Synbiotic diet + challenge	705.56	73.25	65.17	22.33	88.44	10.92	23.20
CV%	9.89	16.19	10.41	9.46	16.68	16.19	14.58
Diet	0.7364	0.7869	0.4889	0.2184	0.2346	0.9056	0.6258
Challenge	0.8595	0.0113	0.0039	0.2406	0.0064	0.2995	0.0076
Diet x challenge	0.4997	0.5933	0.4432	0.7838	0.6640	0.8517	0.7156

CV%: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$). VL: villus length, LV: villus width, CD: crypt depth, CW: crypt width, MLT: muscle layer thickness, V:C: villus length: crypt depth ratio, AA: absorption area.

At 28 days old (Table 7), two weeks after the enteric challenge, regeneration was already underway, since the villus:crypt ratio was no longer altered when compared to 19 days old. Additionally, larger mucosal absorption area in the duodenum ($p<0.0076$), greater thickness of the muscle layer ($p=0.0064$), increased crypt depth ($p=0.0039$), and decreased villus width ($p=0.0113$) were observed in the challenged birds when compared to the control group.

Morphometric analyses of the jejunal mucosa at 19 days old (Table 8) showed that the basal diet resulted in greater villus length ($p<0.0285$) compared to the diet supplemented with the synbiotic. This may be attributed to the microbiota reorganization caused by the enteric challenge, along with the supplementation of probiotic strains, which require more time for cellular and microbial intestinal reorganization. With respect to the enteric challenge, the challenged group exhibited greater villus width ($p=0.0008$), crypt depth ($p<0.0001$), crypt width ($p<0.0001$), and muscle layer thickness ($p=0.0013$), resulting in a lower villus:crypt ratio ($p<0.0001$) and a smaller absorption area ($p<0.0001$).

For jejunal mucosa morphometry at 28 days (Table 8), regeneration was more advanced, given that the only difference observed was in villus width ($p=0.0122$), where challenged groups still exhibited a smaller width. It is important to underscore that at 19 days, this segment was the most affected by the enteric challenge (Table 8). The lack of differences in this parameter indicates that intestinal mucosa recovery occurred due to the microbiota reorganization caused by administration of the synbiotic to the birds (up to 28 days old).

Morphometric assessment of the ileal mucosa, five days after the challenge (Table 9), indicated that the challenge affected all parameters except villus length. Greater villus width ($p=0.0360$), crypt depth ($p<0.0001$), crypt width ($p<0.0001$), and decreased muscle layer thickness ($p=0.0405$), villus:crypt ratio ($p<0.0004$), and absorption area ($p<0.0038$) were observed in the challenged group.

Table 8
Morphometry of the jejunal mucosa in broiler chickens at 19 and 28 days old, supplemented or not with synbiotics and submitted or not to an enteric challenge

	VL, µm	VW, µm	CD., µm	CW., µm	MLT, µm	V:C	AA, µm²
Diet				19 days			
Basal	355.06a	51.75	67.41	23.36	75.01	5.34	13.28
Synbiotic	328.54[b]	53.29	67.89	23.70	75.15	5.05	12.61
Challenge							
Control	353.19	50.81[b]	59.26[b]	20.52[b]	70.14[b]	5.95[a]	14.27[a]
Challenged	330.42	54.23[a]	76.05[a]	26.55[a]	80.02[a]	4.45[b]	11.63[b]
Basal diet + control	364.49	49.91	59.77	20.45	69.83	6.01	14.45
Basal diet + challenge	345.63	53.59	75.05	26.27	80.20	4.68	12.12
Synbiotic diet + control	341.89	51.70	58.74	20.59	70.46	5.88	14.09
Synbiotic diet + challenge	315.20	54.87	77.05	26.82	79.84	4.23	11.14
CV%	16.91	8.53	14.58	12.65	18.68	20.03	19.06
Diet	0.0285	0.1226	0.8176	0.5903	0.9639	0.1872	0.2055
Challenge	0.0592	0.0008	<.0001	<.0001	0.0013	<.0001	<.0001
Diet x Challenge	0.7431	0.7960	0.4658	0.7433	0.8670	0.4742	0.5580
Diet				28 days			
Basal	429.23	51.55	51.54	21.01	84.24	8.38	17.52
Synbiotic	415.78	50.64	51.72	20.54	85.12	8.13	17.24
Challenge							
Control	423.64	52.53[a]	51.55	20.77	84.19	8.27	17.12
Challenged	420.91	49.56[b]	51.75	20.79	85.26	8.22	17.63
Basal diet + control	425.13	53.02	51.92	21.00	85.14	8.22	17.05
Basal diet + challenge	432.99	50.07	51.18	21.03	83.34	8.53	17.98
Synbiotic diet + control	422.15	52.05	51.17	20.54	83.24	8.32	17.19
Synbiotic diet + challenge	408.84	49.04	52.33	20.55	87.17	7.91	17.29
CV%	17.92	10.86	11.36	9.15	23.17	18.96	17.47
Diet	0.3929	0.3908	0.8714	0.2396	0.8144	0.4333	0.6771
Challenge	0.8634	0.0122	0.8644	0.9613	0.7953	0.8816	0.4233
Diet x challenge	0.5047	0.9795	0.4372	0.9779	0.4856	0.2756	0.5192

CV%: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$). VL: villus length, LV: villus width, CD: crypt depth, CW: crypt width, MLT: muscle layer thickness, V:C: villus length: crypt depth ratio, AA: absorption area.

Table 9
Morphometry of the ileal mucosa in broiler chickens at 19 and 28 days old, supplemented or not with synbiotics and submitted or not to an enteric challenge

	VL, µm	VW, µm	CD., µm	CW., µm	MLT, µm	V:C	AA, µm²
Diet				19 days			
Basal	261.72	57.77	63.65	22.80	113.92	4.22	9.94
Synbiotic	249.30	58.50	62.17	22.58	111.23	4.09	9.46
Challenge							
Control	251.48	56.87[b]	57.86[b]	20.31[b]	117.94[a]	4.44[a]	10.24[a]
Challenged	259.55	59.39[a]	67.96[a]	25.08[a]	107.20[b]	3.86[b]	9.16[b]
Basal diet + control	256.52	57.13	59.37	20.69	121.81	4.40	10.31
Basal diet + challenge	266.92	58.40	67.93	24.91	106.02	4.03	9.57
Synbiotic diet + control	246.44	56.62	56.34	19.92	114.07	4.49	10.17
Synbiotic diet + challenge	252.17	60.38	67.99	25.25	108.39	3.69	8.76
CV%	15.39	9.69	16.05	15.10	21.88	18.19	17.74
Diet	0.1316	0.5360	0.4807	0.7559	0.6042	0.4221	0.1871
Challenge	0.3254	0.0360	<.0001	<.0001	0.0405	0.0004	0.0038
Diet x Challenge	0.7749	0.2952	0.4618	0.4373	0.3305	0.1695	0.3554
Diet				28 days			
Basal	292.83	56.09	49.20	19.59	105.77	5.96	11.96
Synbiotic	291.45	56.00	48.85	19.53	104.19	6.06	12.02
Challenge							
Control	287.69	55.61	50.03	19.99[a]	102.28	5.83[b]	11.78
Challenged	296.40	56.46	48.04	19.14[b]	107.60	6.18[a]	12.20
Basal diet + control	287.33	56.30	49.50	19.68	100.84	5.81	11.63
Basal diet + challenge	298.10	55.88	48.92	19.51	110.51	6.11	12.29
Synbiotic diet + control	288.06	54.91	50.56	20.29	103.71	5.86	11.92
Synbiotic diet + challenge	294.69	57.04	47.15	18.78	104.69	6.26	12.10
CV%	16.64	10.38	12.26	9.90	20.09	13.95	14.75
Diet	0.8940	0.9281	0.7741	0.8770	0.7362	0.5813	0.8900
Challenge	0.3880	0.4828	0.1094	0.0363	0.2244	0.0464	0.2569
Diet x challenge	0.8370	0.2966	0.2522	0.0968	0.3208	0.7605	0.5169

CV%: Coefficient of variation. Means with different lowercase letters in the same column indicate a difference ($p<0.05$). VL: villus length, LV: villus width, CD: crypt depth, CW: crypt width, MLT: muscle layer thickness, V:C: villus length: crypt depth ratio, AA: absorption area.

The alteration in ileal mucosa morphometry shows that the enteric challenge disrupted microbiota balance and resulted in increased diameter due to gases produced by the fermentative process, caused mainly by the increased presence of *C. perfringens* (Paiva & McElroy, 2014), which explains the decline in ileal muscle layer thickness.

At 28 days old (Table 9), the same morphometric behavior of the ileal mucosa as that of the duodenum and jejunum was observed. The smaller crypt width in challenged birds (p=0.0363) may indicate lower basal cell proliferation in the crypts due to improved absorption capacity, which resulted in better productive performance at 28 days (Table 4). These observations are confirmed by the higher (p=0.0464) villus ratio in the ileal mucosa of challenged birds when compared to controls..

The enteric challenge may have favored greater pathogen adhesion to the intestinal mucosa as well as bacterial translocation, possibly leading to weakened intestinal integrity followed by an increased rate of cellular renewal of the intestinal mucosa with interference in the extrusion rate. This resulted in greater villus width and crypt depth in challenged birds due to hyperplasia caused by mitotic activity (Ohland & MacNaughton, 2010).

Additionally, the potential loss of tissue functionality is discussed. Correlating the morphometric results of the intestinal mucosa with zootechnical findings, the challenged birds from 14 to 21 days exhibited lower weight gain, lower feed intake, and worse FCR when compared to control birds.

Recovery of the intestinal epithelium was observed in challenged birds at 28 days, where the duodenal absorption rate showed improved results, indicating tissue functionality recovery and improved feed conversion. Moreover, a reduction in villus width was observed, enabling a possible decrease in extrusion rate, and greater crypt depth indicated maintenance of epithelial renewal. Mucosal thickness exhibited better results in challenged animals, reflecting a higher number and/or volume of intestinal cells (Aleixo et al., 2011).

The images captured by scanning electron microscopy illustrate the previously presented data, underscoring that the proposed enteric challenge was effective and caused characteristic lesions in the intestinal epithelium of the challenged birds. The epithelial extrusion points evident in the challenged birds (Figure 2 – C and D) when compared to the control group (Figure 2 – A and B) reflects the performance and morphometric parameter findings found.

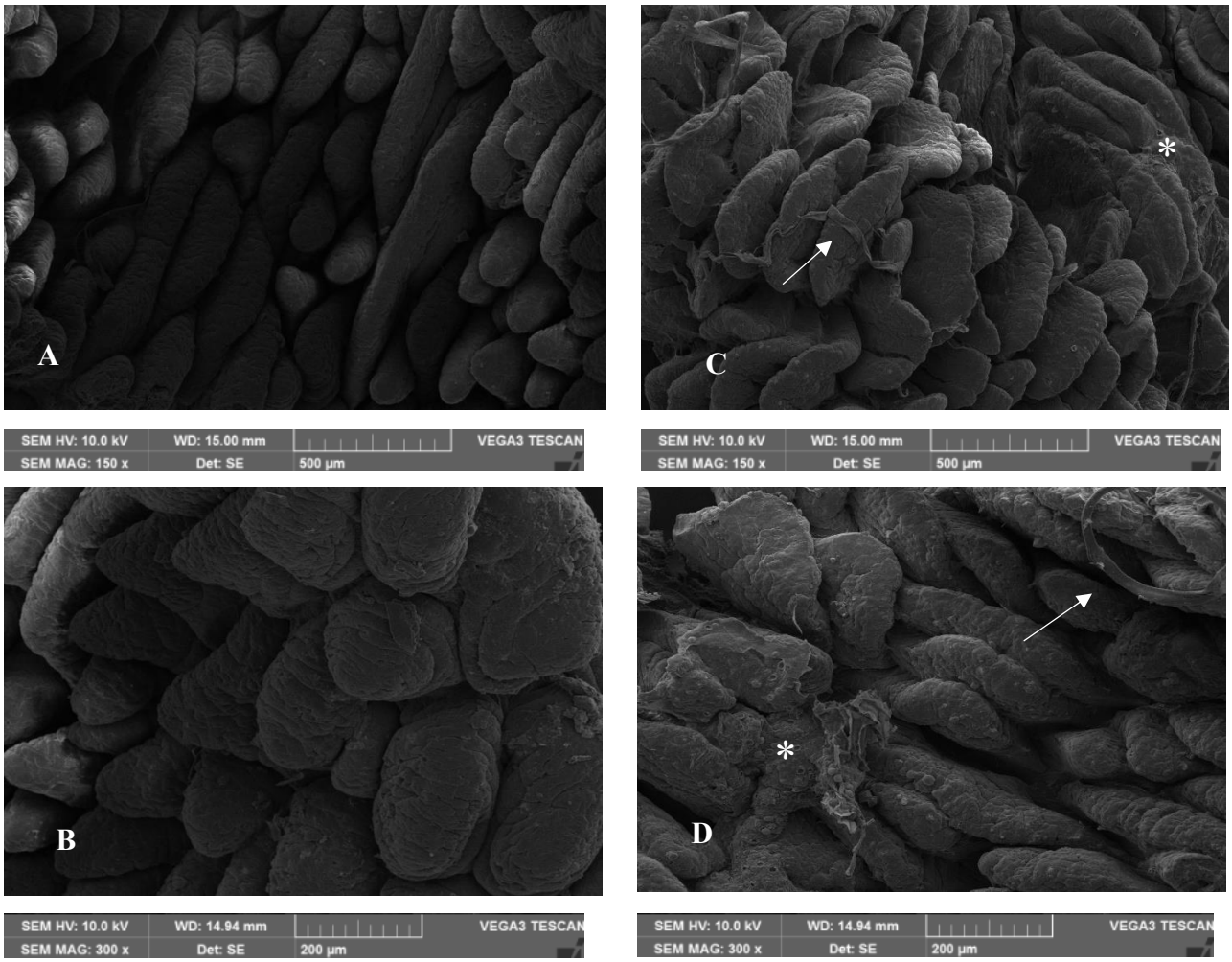

Figure 2. Representative scanning electron microscope images of the ileum of the control group (A – 150x and B – 300x) and challenged group (C – 150x and D – 300x). C-D: Segmented filamentous bacteria (*), and abundance of mucus and goblet cells (→).

Conclusions

Supplementing the diet with the synbiotic led to increased weight gain and improved feed conversion in the first two weeks of the birds' life, prior to the experimental challenge.

The experimental challenge was found to compromise performance, in addition to altering the permeability and integrity of the birds' intestinal mucosa.

From 1 to 28 days old, synbiotic supplementation resulted in better feed conversion, regardless of the experimental challenge. At 28 days old, supplementation with the synbiotic led to recovery of intestinal mucosa integrity, as well as improved tensile strength, hardness, and elasticity of the jejunal mucosa.

These results demonstrate that productivity can be improved through the alternative use of zootechnical additives,

even in experimental environments whose variables are controlled and free from challenges that compromise animal welfare and health.

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