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Effect of Probiotic and β-Mannanase Supplementation on the Productive Performance and Intestinal Health of Broiler Chickens Challenged by *Eimeria maxima* and *Clostridium perfringens*

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Abstract: The use of antibiotics in poultry farming has been associated with bacterial resistance in humans, leading to a ban on their inclusion in chicken diets. Therefore, the objective was to evaluate the effects of probiotics and β-mannanase on the growth performance and intestinal health of broiler chickens challenged by *Eimeria maxima* and *Clostridium perfringens*. For this, 2100 one-day-old male Ross 308 chicks were used. The treatments were as follows: T1—Negative control (NC) unchallenged birds; T2—Positive control (PC) challenged with *E. maxima* + *C. perfringens*; T3—PC + Antibiotic (Enramycin 8%-125 g/ton); T4—PC + β-mannanase (Hemicell^{HT}; 300 g/ton); T5—PC + probiotic (Protexin^{TM}; 150 g/ton); T6—PC + β-mannanase + probiotic. Significant differences ($p < 0.05$) were observed from 1 to 42 days in the variables body weight, body weight gain and feed intake, and the NC treatment presented higher values compared to the PC and PC + probiotic groups. The villus/crypt ratio in the duodenum increased in the PC + β-man + prob treatment, differing from the NC, PC and PC + probiotic ($p < 0.05$) treatments. The use of β-mannanase, probiotics or both together is effective to mitigate the effects of production challenges, through the maintenance of the intestine by modulating action on the cecum microbiome and intestinal morphometry.

Keywords: coccidiosis; intestinal permeability; necrotic enteritis; natural alternative

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1. Introduction

Coccidiosis is a parasitic disease caused by an apicomplexan parasite of the genus *Eimeria*, which infects different parts of the intestinal tract depending on the species of *Eimeria* [1]. *Eimeria* spp. invade intestinal cells and cause physical damage to the intestinal epithelium, producing hemorrhagic diarrhea and disrupting the normal functioning of the intestinal tract [2]. This leads to impaired nutrient absorption and compromised gut health [3]. On the other hand, necrotic enteritis (NE) is a significant and widespread bacterial disease in poultry that causes economic losses in commercial production [4]. This disease is caused by *Clostridium perfringens*, a Gram-positive bacterium, in which the disease causes necrotic lesions of enterocytes [5].

Both diseases can occur in tandem and establish a symbiotic relationship, exacerbating the negative effects on gut integrity and leading to dysbiosis [6]. The damage caused by coccidia weakens the gut barrier and makes the birds more susceptible to *C. perfringens* infection [7]. Coccidial parasitism creates an environment favorable to the proliferation of *C. perfringens* and the production of its toxins, leading to the development of NE, resulting

in increased mucogenesis, severe intestinal lesions, decreased intestinal permeability and, in more serious cases, death of birds [8–10].

Years ago, antibiotics and anticoccidial medications were used to prevent and control many infectious diseases in birds. As such, the ban on antibiotics as growth promoters has led to the development and evaluation of alternative additives [11,12]. One of these alternatives is the use of probiotics in the diet to prevent and control coccidiosis and NE [13]. Some probiotics create a physical barrier by occupying binding sites on the intestinal mucosa, thus excluding pathogenic bacteria through competitive exclusion [14]. Additionally, probiotics exhibit antimicrobial activity by producing substances such as bacteriocins, carbon dioxide, hydrogen peroxide and organic acids (acetic and lactic acids). Probiotics compete with pathogenic bacteria for nutrients, effectively reducing their colonization and growth in the intestinal mucosa [13,15]. The mechanism of microbial balance improved by probiotics has a beneficial effect on gut health and, consequently, on nutrient absorption [16]. Many commercial probiotics are currently used in practical poultry production; however, probiotics composed of multiple beneficial microorganisms in an adequately balance can have better gut integrity protection. In our study, we used a commercial probiotic with a complex microbial composition to enhance the beneficial effect.

On the other hand, the utilization of enzymes in feed has demonstrated an improvement in nutrient absorption by reducing antinutritional components and having a beneficial effect on gut integrity [17]. Supplementation of β-mannanase can be an alternative to improve nutrient utilization [17]. β-mannanase is an endohydrolase enzyme that breaks down β-mannans, which are non-starch polysaccharides (NSPs), making previously indigestible nutrients available [18]. β-mannanases have been used to act on specific polysaccharide targets and break down β-mannans found in plant cell walls of the many ingredients that are part of the feed formula.

The negative effects of β-mannans impact nutrient absorption and nutrition, increasing the particularity of intestinal mucus, leading to reduced absorption and negative effects on intestinal integrity. They also interfere with intestinal microorganisms, damaging the intestinal microbiota [19,20]. As reported by [21,22], a β-mannanase was found to have microbial modulating activity in broiler chickens fed corn and soy diets and challenged with *C. perfringens* and *Eimeria*. β-mannanase decreased the magnitude of *Eimeria* and *Clostridium* infection, resulting in a significant body weight gain and a reduction in some intestinal lesions compared to the enzyme-free diet. Supplementation of β-mannanase at the level of 200 and 400 ppm in the poultry diet beneficially improved the homeostasis of anabolic hormones, blood glucose, digestible energy, digestible amino acids and feed conversion [23]. The authors of [24] found that β-mannanase supplementation at the level of (200 or 400/ton) improved ileal digestible energy, provided intestinal vitamins and increased the growth of broiler chickens.

The additive interaction between β-mannanase and probiotics has not been explored for broiler chickens challenged by an NE model. In this sense, we hypothesize that supplementing broiler diets with β-mannanase may result in reduced viscosity. β-mannanase used in conjunction with a probiotic blend can serve as a robust alternative for improving gut health by contributing to the development of beneficial microbial communities, improving nutrient absorption, and ultimately leading to better growth performance. Therefore, this study aims to evaluate the effects of the concomitant application of probiotics and β-mannanase on intestinal integrity, microbiota establishment and productive performance responses in broiler chickens challenged by *E. maxima* in association with *C. perfringens.*

2. Materials and Methods

2.1. Ethics Approval

The experiment was carried out at the Laboratory of Poultry Science, São Paulo State University, Jaboticabal. This study received approval (Protocol no: 1061/22) and was conducted in strict adherence to the guidelines established by the Animal Ethics Committee of the Faculty of Animal Science and Veterinary Medicine, São Paulo State University, Brazil.

2.2. Management and Facilities

The experiment was conducted within a controlled-environment poultry house, with environmental conditions adjusted in accordance with the recommendation of the genetic management guidelines [25]. The birds were allocated to collective pens with a stocking density of 39 kg/m^2 throughout the test period, using new wood shavings as bedding material. The birds were provided free access to fresh water via nipple-type drinkers and ad libitum feeding. A lighting regime was implemented as recommended by the lineage manual [25]. Throughout the experimental period, daily monitoring and recording of environmental temperature and relative humidity were conducted (Thermohygrometer HTC-2 Temperature and Humidity Meter—Radical TechArt, Mercado livre, São Paulo, Brazil), resulting in an average temperature of 24.6 °C and an average relative humidity of 52.1%.

2.3. Birds, Experimental Design and Dietary Treatments

A total of 2100 one-day-old sexed male Ross 308 broiler chicks were obtained from a local commercial hatchery. These birds were subject to the vaccination program, including immunization against Marek on their first day and subsequent vaccinations against Newcastle and Gumboro diseases at seven days of age. The birds were randomly allocated to 60 pens, each measuring 3.0 m × 1.5 m, consisting of experimental units that guarantee the same body weight (42 g ± 0.5). Each unit comprised 35 birds per pen. Subsequently, each experimental unit was assigned one of the six experimental treatments, with ten replicates for each treatment. The feeding regimen encompassed three phases: the initial (days 1–14), grower (days 15–28) and finisher (days 29–42). Across all experimental treatments, the feed consisted of corn–soybean meal basal diets, formulated to meet the nutritional requirements as recommended by [26] (Table 1). A representative feed sample was collected from each batch for further analysis.

The six experimental treatments were as follows: T1—Negative control (NC) (non-challenged birds without antibiotics or additives); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens* without antibiotic or additive); T3—PC + Antibiotic; T4—PC + β-mannanase; T5—PC + probiotic; and T6—PC + β-mannanase + probiotic. The antibiotic used was enramycin, administered at a rate of 125 g/ton. β-mannanase was supplemented at a rate of 300 g/ton, while the probiotic was included at 150 g/ton during the starter phase, 100 g/ton during the grower phase, and 50 g/ton during the finisher phase, following the brand recommendations (Elanco®, SP, Brazil). β-mannanase is the active ingredient, and its source is through a patented enzyme produced from the fermentation of the bacteria *Paenibacillus lentus* (Elanco®, SP, Brazil). All additives were administered from the birds' first day of life until the end of the test. The probiotic used was composed of *Lactobacillus acidophilus* (ATCC 4356) (2.06 × 10^8 CFU/g), *Lactobacillus bulgaricus* (ATCC 11842) (2.06 × 10^8 CFU/g), *Lactobacillus rhamnosus* (ATCC 7469) (2.06 × 10^8 CFU/g), *Lactobacillus plantarum* (ATCC 14917) (1.26 × 10^8 CFU/g), *Bifidobacterium bifidum* (ATCC 29521) (2.0 × 10^8 CFU/g), *Enterococcus faecium* (ATCC 19434) (6.46 × 10^8 CFU/g), *Streptococcus thermophilus* (ATCC 19258) (4.10 × 10^8 CFU/g) (Elanco®, SP, Brazil).

Table 1. Composition of diets provided throughout the trial (%).

Ingredients	Start (1–14 d)	Grower (15–28 d)	Finisher (29–42 d)
Corn (7.88% CP)	54.88	56.29	61.30
Soybean meal (45% CP)	35.00	33.30	29.00
Meat and bone meal (48% CP)	6.55	5.76	4.67
Soybean oil	2.00	3.34	3.71
Limestone	0.15	0.11	0.20
Salt	0.42	0.41	0.40
DL-Methionine (99%)	0.35	0.30	0.25
L-Lysine HCl (78%)	0.29	0.16	0.16
L-Threonine (98%)	0.07	0.04	0.03
[1] Vitamin Premix	0.10	0.10	0.10
[2] Mineral Premix	0.10	0.10	0.10
Choline Chloride (60%)	0.05	0.05	0.05
[3] Innert or tested products	0.05	0.04	0.04
Nutritional composition			
Metabolizable Energy, (Kcal/kg)	3000	3100	3175
* Crude Protein, (%)	23.79 (24.14)	22.62 (23.35)	20.50 (20.90)
Lysine (%)	1.282	1.190	1.069
Met + Cys (%)	0.948	0.881	0.791
Methionine (%)	0.653	0.596	0.526
Threonine (%)	0.846	0.786	0.706
Valine (%)	0.957	0.916	0.830
Tryptophan (%)	0.245	0.235	0.706
Isoleucine (%)	0.864	0.827	0.746
Arginine (%)	1.467	1.396	1.247
Leucine (%)	1.730	1.673	1.560
Histidine (%)	0.531	0.511	0.468
Phenyl + tyrosine (%)	1.755	1.685	1.535
Gly + serine (%)	2.150	2.032	1.811
Calcium (%)	0.925	0.818	0.714
Available Phosphorus (%)	0.441	0.397	0.335
Sodium (%)	0.222	0.213	0.203
Electrolytic balance	214	197	177

[1] Content/kg of premix= Vitamin A (min) 11,000,000 IU; Vitamin D3 (min) 4,000,000 IU; Vitamin E (min) 55,000 IU; Vitamin K3 (min) 3000 mg. Vitamin B1 (min) 2300 mg. Vitamin B2 (min) 7000 mg, Pantothenic Acid (min) 12 g. Vitamin B6 (min) 4000 mg. Vitamin B12 (min) 25,000 mcg. Nicotinic Acid (min) 60 g. Folic Acid (min) 2000 mg. Biotin (min) 250 mg. Selenium (min) 300 mg; [2] Content/kg of premix = iron (min) 100 g. Cuprum (min) 20 g. Manganese (min) 130 g. Zinc (min) 130 g. Iodine (min) 2000 mg; [3] Inert: washed sand, Antibiotic: Enramycin, β-mannanase and probiotic: The probiotic was composed of *Lactobacillus acidophilus* (2.06×10^8 CFU/g), *Lactobacillus bulgaricus* (2.06×10^8 CFU/g), *Lactobacillus rhamnosus* (2.06×10^8 CFU/g), *Lactobacillus plantarum* (1.26×10^8 CFU/g), *Bifidobacterium bifidum* (2.0×10^8 CFU/g), *Enterococcus faecium* (6.46×10^8 CFU/g), *Streptococcus thermophilus* (4.10×10^8 CFU/g); * Values calculated and analyzed, respectively.

2.4. Eimeria maxima and Clostridium perfringens Challenge Protocol

The health challenge protocol entailed individual inoculation of the challenged treatments at 14 days of age with 1 mL of inoculum containing *E. maxima* at a concentration of 7×10^3 sporulated oocysts/mL (Centro de amparo a pesquisa veterinaria Ltd., Capev, SP, Brazil), which was diluted in a phosphate-buffered saline solution. Subsequently, at 18, 19 and 20 days of age, the chickens received individual daily inoculations of 1 mL each, containing *C. perfringens* at a concentration of 2.5×10^6 CFU/mL (Microbiology Laboratory, São Paulo State University, Brazil). All inoculations were administered orally, using a syringe attached to a probe inserted into the birds' beaks.

2.5. Productive Performance Collection

The productive performance variables, covering body weight (BW) and feed intake (FI), were recorded at 14, 21, 28 and 42 days of age, in which all birds in the trial were weighed to obtain body weight (n = 2100) (Balança 2124/—Serie: 13237319—Toledo do

Brasil Indústria de Balanças Ltd., São Bernardo do Campo, SP, Brazil). Subsequently, the body weight gain (BWG) for this designated period was calculated and employed in the determination of the feed conversion rate (FCR). At the same time, daily mortality checks were carried out and integrated into the FCR correction, following the methodology elucidated by [27] in which the procedure generally applied consisted of recording the day of death, the number of dead birds and their weights, respectively.

2.6. Oocyst Count Analysis

Oocyt counting was conducted at six days post-inoculation (dpi) with *E. maxima*. To do this, fresh excreta were sampled from various areas within the pen and stored in labeled plastic bags at −4 °C for subsequent analysis. Oocyst counting was carried out at the Laboratory of Parasites (LabEPar, São Paulo State University, Brazil), using 2 g of homogenized sub-samples to determine oocysts per gram of feces (OPGs). This process followed the protocol described by [28], which involved sub-sample dilution in 28 mL of NaCl solution (Sodium Chloride 99%—Êxodo Científica-Sumaré, SP, Brazil), homogenization, and allowing it to settle for 15 min. Subsequently, an aliquot of the solution was taken and placed in the McMaster chamber, and oocysts were counted in each area using an electronic microscope with a 10× objective (Olympus CX31, Evident Corporation, Tokyo, Japan). The final count was multiplied by 50 to determine the number of OPGs.

2.7. Intestinal Morphometry

The assessment of intestinal morphology was conducted at both 21 and 42 days of age. To perform this assessment, one bird per pen was euthanized via cervical dislocation. Following the opening of the celomic cavity, fragments from various segments of the gastrointestinal tract were carefully collected to prevent tissue autolysis.

Sampling from each gut section (duodenum, jejunum and ileum) was executed with precision to ensure uniformity, with each section measuring approximately 1.5 cm to 2.0 cm. These sections were selected within the middle two-thirds of each respective segment. The gathered samples were meticulously cleansed with a 10% formaldehyde (Êxodo Científica-Sumaré, SP, Brazil) solution to eliminate intestinal contents. They were then placed in labeled containers filled with formalin solution, ensuring complete immersion to maintain sample integrity. Subsequently, the samples were transported to the laboratory (Mercolab®, Cascavél—Paraná, Brazil) for further analysis.

For analysis purposes, the collected samples underwent three distinct sectioning techniques: hemicylinder, transverse and longitudinal, each with a thickness of approximately 1 mm. Additionally, the longitudinal sections were 15 mm in length, as recommended by [29]. The samples were subsequently subjected to a dehydration process using ascending PA ethyl alcohol concentrations (Synth, Diadema, SP, Brazil), followed by clarification in PA xylene (Synth, Diadema-São Paulo, Brazil), impregnation, and embedding in paraffin (Pró-cito, Porto Alegre-Rio Grande do Sul, Brazil). Microtomy was employed to produce semi-serial sections with a thickness of 4 µm, and these sections were subsequently stained with hematoxylin in Harry (NewProv, Pinhas-Paraná, Brazil) and eosin (Allkimia, Campinas, SP, Brazil), following the method of [30].

Microscopic observations were carried out using an optical microscope (Olympus CX31, Evident Corporation, Tokyo, Japan), with the examination of fields at a 10× objective. To measure villus height and crypt depth, the Toup View Software (version: x64, 3.7.2270) was utilized, with 10 readings being taken on complete villi for each fragment evaluated.

2.8. Gut Microbiota of the Cecum

The determination and evaluation of the cecal microbiota in birds, at 22 and 43 days of age, involved the collection of samples of cecal content. One bird per box was used at the respective ages. The cecal content was sampled and subsequently stored at −80 °C for further analyses.

For microbial community identification, DNA extraction from the samples was executed using the "ZR Quick DNA Fecal/Soil Microbe MiniPrep™" commercial kit provided by Zymo Research Corporation (D6010), USA, following the manufacturer's prescribed protocol. The quantification of extracted DNA was performed using spectrophotometry at 260 nm (Microvolume spectrophotometer-NanoDrop Lite/Thermo Scientific NanoDrop Products, Brazil). To assess DNA integrity, all samples underwent 1% agarose gel electrophoresis.

The amplification of the V3V4 hypervariable region of the ribosomal 16S rRNA gene was achieved using universal primers as detailed in the methodology of PCR [31]. The amplified samples were employed to construct the metagenomic library utilizing the "Nextera DNA Library Preparation Kit" commercial kit by Illumina®. The pooled samples were subsequently subjected to sequencing on the Illumina® "MiSeq" sequencer [32].

The dataset obtained from the sequencer underwent analysis using the QIIME2 platform (Quantitative Insights into Microbial Ecology) [33]. The analysis workflow included the removal of low-quality sequences, filtration, elimination of chimeras, and taxonomic classification. Sequences were categorized into bacterial genera via Amplicon Sequence Variants (ASVs) by comparing them against a dataset. Specifically, the SILVA database of ribosomal sequences [34], particularly the 2019 update (SILVA 138), was utilized for sequence comparison. To generate bacterial community classifications based on ASV identification, a normalization of 20,536 reads per sample was applied to ensure data comparability among samples with varying read numbers.

2.9. Health Tracking System Analysis

The Health Tracking System analysis (HTSi) was conducted on two birds per enclosure at both 21 and 42 days of age, following the methodology established by Elanco Animal Health [35]. This analysis employs a quantitative metric referred to as "intestinal integrity" (I^2), which serves as an index comprising various intestinal lesions impacting avian health. These lesions encompass gizzard erosion, oral cavity lesions, proventriculitis, intestinal tone, excessive intestinal fluid, conditions of the small intestine and large intestine, excessive bile, excessive mucus, cell desquamation, food passage, necrotic enteritis, hyperemia and intestinal hemorrhage. Additionally, the presence of *Eimeria* genus (*E. acervulina, E. maxima* and *E. tenella*) was evaluated based on the typical lesions produced by these three genera.

Each condition is allocated a weight, with scores ranging from 0 to 1 (indicating presence/absence) for most of the aforementioned items, 0 to 2 for proventriculitis and necrotic enteritis, 0 to 3 for gizzard erosion and intestinal hemorrhage, and 0 to 4 for the severity of lesions across all species of *Eimeria*. The initial score of the evaluated individuals is 100, meaning a healthy digestive system. The presence of each lesion listed corresponds to a decrease in the score, and therefore the lower the score, the less healthy the bird is [35].

2.10. Intestinal Permeability

On day 42, the chickens underwent oral administration of a marker known as FITC-dextran to evaluate intestinal permeability. Serum levels of FITC-dextran were quantified using the methodology proposed by [36]. This trial was performed using one bird per experimental unit selected according to a body weight close to the average body weight of the pen (±10%).

Two hours and thirty minutes before blood sampling, each bird received a 500 µL (0.5 mL) dose of FITC-dextran (PM 3000–5000; Sigma Aldrich Co., St. Louis, MO, USA) through oral gavage and this content was allocated on the crop with a fine and flexible oral tube. On sequence, the blood sample collection was taken from the jugular artery following the administration of the FITC-dextran marker. To prevent clotting, the collected blood (2 to 3 mL) was gently homogenized 5 to 8 times.

To detect the presence of FITC-dextran in the serum, the blood was left at room temperature for 3 h to facilitate clot formation, after which it was centrifuged at 500× *g* for 15 min to separate the serum. Fluorescence levels of the diluted serum (1:1 in PBS)

were measured using an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, multimode microplate reader, BioTek Instruments, Inc., VT, USA). The concentration of FITC-dextran per mL of serum was determined based on a standard curve [36]. Consequently, a higher concentration of FITC-dextran in the plasma/serum indicates a greater degree of intestinal permeability and intestinal damage.

2.11. Statistical Analysis

Statistical analyses were carried out utilizing SAS version 9.4 statistical software. The data were subject to analysis of variance (ANOVA) via the PROC MIXED procedure. The normality of errors and homoscedasticity was checked before ANOVA was performed. Mean comparisons between treatments were performed using the Tukey test, with a significance level set at $p < 0.05$. Non-parametric methods were employed to analyze oocyst count. The Kruskal–Wallis test was initially applied, followed by the Dwass–Steel–Critchlow–Fligner (DSCF) multiple comparison test. Significance was attributed to $p < 0.05$.

In the case of gut microbiome analysis, alpha diversities were calculated using the "phyloseq" package [37] and the "vegan" library [38] and were compared using the Kruskal–Wallis non-parametric test [39], followed by the post hoc test by Dunn [40]. Results with $p < 0.05$ were considered statistically significant. Beta diversity was assessed using permutational multivariate analysis of variance (perMANOVA) through the QiimeII pipeline, involving 10,000 permutations. All additional numbers and statistical analyzes were performed in the R software 4.2.0 environment.

3. Results

3.1. Productive Performance

The performance responses are presented in Table 2 and are detailed for each evaluation period.

During the period from 1 to 14 days of age, significant differences ($p < 0.05$) were observed in BW, FI and BWG between experimental treatments. The PC + Antibiotic treatment showed higher BW, BWG and FI values compared to the PC group. Meanwhile, the combination of β-mannanase and probiotics resulted in a higher FI, like the PC + Antibiotic treatment.

From 15 to 28 years old, corresponding to the most critical period of the challenge, significant differences ($p < 0.05$) were observed in productive performance responses. For the BW variable, the NC treatment presented a higher value compared to the PC and PC + probiotic groups. Furthermore, non-challenged birds (NCs) showed higher BWG and FI than all other treatments. For the FCR variable, PC birds achieved improved conversion compared to PC + β-mannanase and PC + probiotic treatments.

In the final period, from 29 to 42 days of age, only the BWG variable did not differ between treatments ($p > 0.05$). For the variables BW and FI, birds in the NC group presented higher values compared to birds in the PC and PC + probiotic groups ($p < 0.05$). The feed conversion of birds in the PC + Antibiotic treatment was lower compared to the NC, PC and PC + probiotic groups.

Significant differences ($p < 0.05$) in all productive variables were observed throughout the entire trial (1 to 42 days). For the variables BW, BWG and FI, birds in the NC treatment exhibited higher values compared to the PC and PC + probiotic groups. However, the FCR of birds that received PC + Antibiotics was lower compared to birds from the NC group (Table 3).

Table 2. Performance of broiler chickens during growth phases.

Treatments	1 to 14 d				15 to 28 d				29 to 42 d			
	BW [1] 14 d (kg)	**BWG [2] (kg)**	**FI [3] (kg)**	**FCR [4] (kg/kg)**	**BW 28 d (kg)**	**BWG (kg)**	**FI (kg)**	**FCR (kg/kg)**	**BW 42 d (kg)**	**BWG (kg)**	**FI (kg)**	**FCR (kg)**
NC	0.489 ^{abc}	0.446 ^{abc}	0.490 ^{ab}	1.101	1.769 ^{a}	1.250 ^{a}	1.743 ^{a}	1.393 ^{ab}	3.365 ^{a}	1.596	2.889 ^{a}	1.810 ^{a}
PC	0.481 ^{c}	0.438 ^{c}	0.481 ^{b}	1.096	1.701 ^{b}	1.203 ^{b}	1.644 ^{b}	1.368 ^{b}	3.208 ^{b}	1.509	2.731 ^{b}	1.814 ^{a}
PC + Ant	0.507 ^{a}	0.465 ^{a}	0.510 ^{a}	1.098	1.730 ^{ab}	1.200 ^{b}	1.655 ^{b}	1.376 ^{ab}	3.313 ^{ab}	1.590	2.774 ^{ab}	1.745 ^{b}
PC + β-man	0.492 ^{abc}	0.449 ^{abc}	0.501 ^{ab}	1.116	1.707 ^{ab}	1.188 ^{b}	1.661 ^{b}	1.400 ^{a}	3.257 ^{ab}	1.546	2.767 ^{ab}	1.793 ^{ab}
PC + prob	0.486 ^{bc}	0.443 ^{bc}	0.494 ^{ab}	1.114	1.698 ^{b}	1.178 ^{b}	1.647 ^{b}	1.398 ^{a}	3.223 ^{b}	1.517	2.741 ^{b}	1.809 ^{a}
PC + β-man + prob	0.502 ^{ab}	0.460 ^{ab}	0.504 ^{a}	1.095	1.711 ^{ab}	1.190 ^{b}	1.656 ^{b}	1.390 ^{ab}	3.260 ^{ab}	1.554	2.757 ^{ab}	1.775 ^{ab}
SEM	0.002	0.002	0.002	0.005	0.007	0.005	0.008	0.003	0.015	0.010	0.016	0.006
***p*-value**	0.003	0.003	0.002	0.705	0.023	<0.001	<0.001	0.002	0.013	0.064	0.028	0.008

[1] Body weight; [2] Body weight gain; [3] Feed intake; [4] Feed conversion rate. ^{abc} Different letters in the same column represent statistical difference by the Tukey test (p-value < 0.05 was considered statistically different). Means that do not follow a letter are significantly different. Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic; SEM: Standard error of the mean.

Table 3. Performance of broilers throughout the trial (1 to 42 days old).

Treatments	BW [1] (kg)	BWG [2] (kg)	FI [3] (kg)	FCR [4] (kg/kg)
NC	3.365 [a]	3.322 [a]	5.134 [a]	1.545 [a]
PC	3.208 [b]	3.165 [b]	4.856 [b]	1.534 [ab]
PC + Ant	3.331 [ab]	3.269 [ab]	4.928 [ab]	1.508 [b]
PC + β-man	3.258 [ab]	3.215 [ab]	4.941 [ab]	1.537 [ab]
PC + prob	3.222 [b]	3.180 [b]	4.883 [b]	1.536 [ab]
PC + β-man + prob	3.261 [ab]	3.218 [ab]	4.916 [ab]	1.527 [ab]
SEM	0.015	0.015	0.023	0.003
***p*-value**	0.015	0.015	0.004	0.021

[1] Body weight; [2] Body weight gain; [3] Feed intake; [4] Feed conversion rate. [ab] Different letters in the same column represent statistical difference by the Tukey test (p-value < 0.05 was considered statistically different). Means that do not follow a letter are significantly different. Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic; SEM: Standard error of the mean.

3.2. Oocyst Count

Figure 1 presents the results of the oocyst count analysis, revealing statistical significance ($p < 0.05$). The NC treatment demonstrated the absence of oocysts, indicating the effectiveness of the physical barriers and the biosafety procedure adopted to avoid potential cross-contamination. However, *E. maxima* oocysts were detected in the excreta of the challenged treatments, with greater abundance in the PC + Antibiotic group and lower numbers of oocysts in the PC, PC + probiotic and PC + β-man + prob groups.

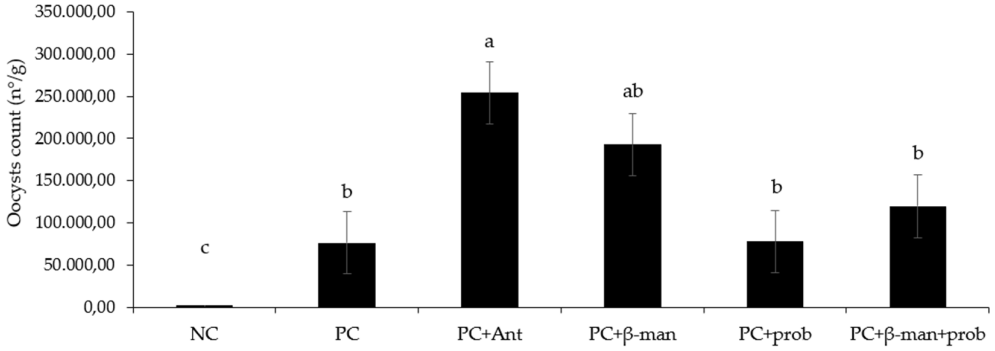

Figure 1. Boxplot of oocyst count (oocyts/g of feces) of 20-day-old broilers fed diets supplemented or not with β-mannanase, Probiotic, Antibiotic and challenged or not with oocysts of *Eimeria maxima* and *Clostridium perfringens*. [abc] Different letters represent statistical difference by a non-parametric Kruskal–Wallis test. Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic.

3.3. Intestinal Morphometry

The morphometric variables of the intestine sections (duodenum, jejunum and ileum) at both collection ages (21 and 42 days of age) were statistically significant ($p < 0.05$) (Table 4). At 21 days of age, the variable villous height (VH) in the duodenum was higher in the NC and PC + probiotic groups, in relation to the PC, PC + β-man and PC + β-man + prob treatments; also, in the jejunum, the NC was higher among all treatments. However, in the ileum, the VH was higher in birds that received PC + β-man + prob. The variable crypt depth (CD) at 21 days indicates that in the duodenum, NC birds had the lowest value among treatments, but in contrast, in the jejunum, they obtained the highest value, and the PC and PC + β-mannanase treatments had the lowest values. Also, the CD in the ileum was lower in the PC group compared to the PC + Antibiotic and PC + β-man + prob groups. In addition, the results of the villus/crypt ratio at this age showed that in the three segments of the intestine, the NC birds obtained higher values between treatments.

Table 4. Morphometric measurements of the mucosa of the duodenum, jejunum and ileum of broilers at 21 days and 42 days of age fed diets supplemented or not with Antibiotic, β-mannanase, probiotic and challenged or not with oocysts of *Eimeria maxima* and *Clostridium perfringens*.

	21 Days									42 Days								
Intestinal Segment	**Duodenum**			**Jejunum**			**Ileum**			**Duodenum**			**Jejunum**			**Ileum**		
Treatments	**VH [1] (µm)**	**CD [2] (µm)**	**V:C [3] (µm)**	**VH (µm)**	**CD (µm)**	**V:C (µm)**	**VH (µm)**	**CD (µm)**	**V:C (µm)**	**VH (µm)**	**CD (µm)**	**V:C (µm)**	**VH (µm)**	**CD (µm)**	**V:C (µm)**	**VH (µm)**	**CD (µm)**	**V:C (µm)**
NC	1.914 ^{b}	0.159 ^{c}	12.363 ^{a}	1.115 ^{a}	1.119 ^{a}	8.139 ^{a}	0.824 ^{bcd}	0.812 ^{bc}	7.042 ^{a}	2.524 ^{a}	2.507 ^{a}	14.720 ^{b}	1.386 ^{a}	1.389 ^{a}	9.071 ^{a}	1.043 ^{a}	1.043 ^{a}	6.788 ^{a}
PC	2.067 ^{a}	0.194 ^{a}	10.872 ^{b}	0.916 ^{d}	0.928 ^{c}	5.968 ^{bc}	0.793 ^{d}	0.794 ^{c}	5.140 ^{c}	2.577 ^{a}	2.577 ^{a}	15.082 ^{b}	1.240 ^{b}	1.259 ^{b}	7.463 ^{bc}	0.956 ^{b}	0.938 ^{bc}	6.033 ^{abc}
PC + Ant	1.998 ^{ab}	0.199 ^{a}	10.435 ^{b}	1.032 ^{bc}	1.004 ^{b}	6.475 ^{b}	0.859 ^{ab}	0.890 ^{a}	6.274 ^{b}	2.515 ^{ab}	2.512 ^{a}	15.984 ^{ab}	1.443 ^{a}	1.440 ^{a}	8.117 ^{ab}	1.012 ^{a}	1.012 ^{a}	6.604 ^{ab}
PC + β-man	1.923 ^{b}	0.179 ^{b}	11.110 ^{b}	0.980 ^{c}	0.948 ^{c}	6.114 ^{bc}	0.814 ^{cd}	0.814 ^{bc}	5.420 ^{c}	2.396 ^{b}	2.347 ^{b}	15.872 ^{ab}	1.121 ^{c}	1.125 ^{c}	6.956 ^{c}	0.894 ^{c}	0.897 ^{c}	5.732 ^{c}
PC + prob	2.075 ^{a}	0.194 ^{ab}	10.991 ^{b}	1.022 ^{bc}	1.027 ^{b}	6.027 ^{bc}	0.842 ^{abc}	0.821 ^{bc}	5.277 ^{c}	2.515 ^{ab}	2.497 ^{a}	14.971 ^{b}	1.262 ^{b}	1.270 ^{b}	6.959 ^{c}	0.988 ^{ab}	0.990 ^{ab}	5.840 ^{bc}
PC + β-man + prob	1.930 ^{b}	0.192 ^{ab}	10.354 ^{b}	1.039 ^{b}	1.050 ^{b}	5.666 ^{c}	0.868 ^{a}	0.847 ^{b}	6.073 ^{b}	2.563 ^{a}	2.560 ^{a}	17.394 ^{a}	1.256 ^{b}	1.216 ^{b}	8.481 ^{a}	1.040 ^{b}	1.040 ^{a}	6.399 ^{abc}
SEM	0.009	0.002	0.097	0.006	0.005	0.072	0.004	0.004	0.066	0.012	0.013	0.189	0.008	0.008	0.101	0.006	0.006	0.081
***p*-value**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Villus height; [2] Crypt depth; [3] Villus/crypt Ratio; Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic. SEM: Standard error of the mean ^{abcd} Different letters in the same column represent statistical difference by the Tukey test (p-value < 0.05 was considered statistically different). Means that do not follow a letter are significantly different.

At 42 days of the test, there was an increase in the VH variable in the duodenum in the NC, PC and PC + β-man + prob treatments, differing from the birds that received PC + β-mannanase. In the jejunum and ileum segments, VH was higher in the NC and PC treatments. The CD at 42 days in the duodenum, jejunum and ileum was lower with the PC + B-mannanase treatment compared to the other treatments. The villus/crypt ratio in the duodenum increased in the PC + β-man + prob treatment, differing from the NC, PC and PC + prob groups. In the jejunum, this relationship was greater in the NC and PC + β-man + prob groups of birds, and in the NC ileum it was greater, differing from the PC + β-mannanase and PC + probiotic groups.

3.4. Cecal Microbiota Diversity

The cecal contents microbiome was assessed for alpha and beta diversity, taxonomic composition and differential taxon abundance.

3.4.1. Alpha Diversity

Alpha diversity analysis, encompassing Chao1, Observed OTUs, Fisher, Simpson, Shannon and Evenness Pielou metrics, unveiled statistically significant distinctions ($p < 0.05$) among treatments on their respective sampling dates (Figure 2A–F).

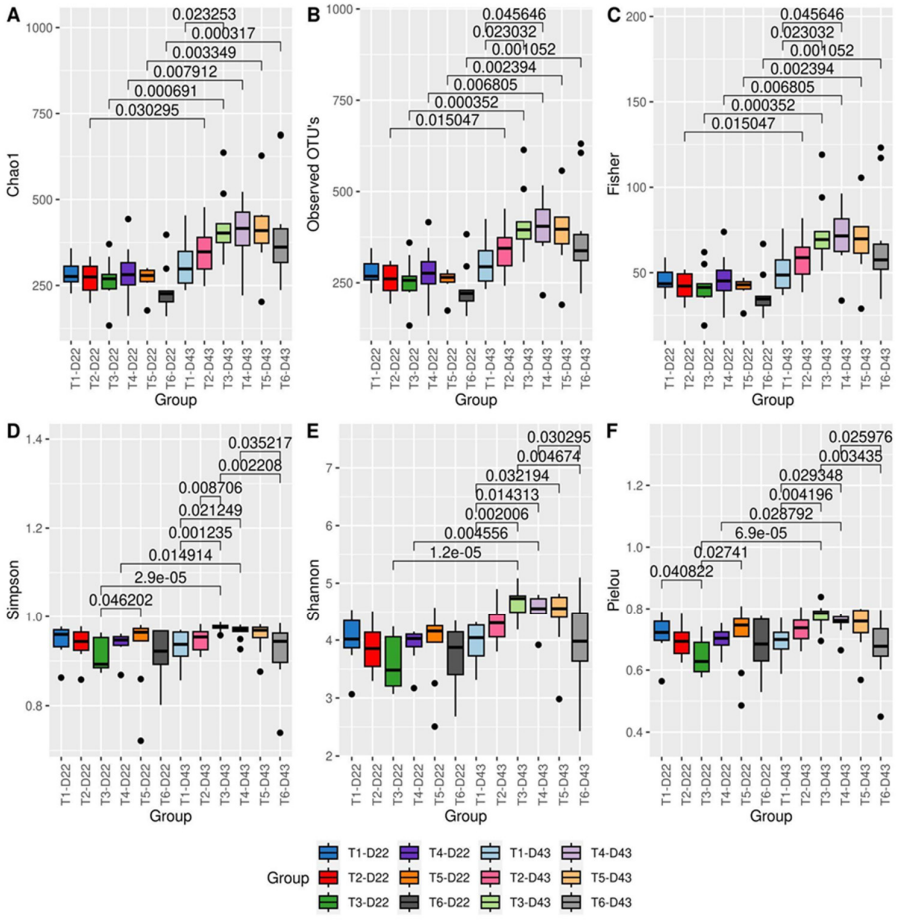

Figure 2. Alpha diversity estimated by Chao1 parameters (**A**), observed OTUs (**B**), Fisher test (**C**), Simpson index (**D**), Shannon Entropy (**E**) and Evenness Pielou (**F**). Statistical comparison between groups was performed using the non-parametric Kruskal–Wallis and Post hoc Dunn tests. Statistical results below 0.05 were accepted as statistically significant. The treatments were: T1—Negative control (NC) (birds without challenge); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); T3—PC + Antibiotic; T4—PC + β-mannanase; T5—PC + probiotic; T6—PC + β-mannanase + probiotic.

At 22 days, discernible differences manifested between treatments in birds subjected to challenges. Specifically, birds from the PC and PC + probiotic exhibited higher diversity compared to the PC + Antibiotic treatment, as evidenced by the Simpson (p = 0.04) and Pielou (p = 0.02) metrics (Figure 2D,F). Moreover, distinctions in alpha diversity were evident between the NC and PC + Antibiotic groups, particularly in the Pielou metric (p = 0.04) at this age.

Upon reaching 43 days, variations across all metrics were observed between the NC and PC + Antibiotic treatment, with the latter displaying greater alpha diversity. The NC treatment exhibited lower alpha diversity than the PC + β-mannanase treatment in all metrics except for Chao1 (Figure 2B–F). Furthermore, the group of challenged birds receiving diets supplemented with the association of PC + β-man + prob showed reduced diversity (p = 0.002) compared to the PC + Antibiotic treatment. However, the PC + β-mannanase treatment group demonstrated heightened diversity (p = 0.03) regarding the Simpson, Shannon and Pielou metrics (Figure 2D–F). Significant distinctions were also apparent between the PC treatment (p = 0.008) and the PC + Antibiotic treatment in Simpson's metric (Figure 2D). Additionally, differences surfaced between the NC treatment, exhibiting lower alpha diversity compared to the PC + Antibiotic (p = 0.02) or PC + β-mannanase (p = 0.04) treatment in the Fisher metric (Figure 2C).

3.4.2. Beta Diversity

Beta diversity analysis, as illustrated in Figure 3, was conducted using parameters with statistical significance, including Bray–Curtis (p = 0.0001), Jaccard (p = 0.0001), UniFrac (p = 0.0001) and Weighted UniFrac (p = 0.0001). Principal component analyses (PCoAs) based on Bray–Curtis distances were carried out to elucidate cluster formation according to the treatments.

At 22 days, the PC + β-mannanase dietary treatment exhibited dissimilarities compared to the NC treatment, as well as to the PC treatment without supplementation and the PC + probiotic treatment, as indicated by both the Bray–Curtis and Jaccard metrics (Figure 3A,B). Furthermore, the group of birds fed the combination diet of PC + β-man + probiotic showed distinctions compared to the NC and PC treatments, as reflected in the Jaccard and UniFrac metrics (Figure 3B,C). Just the Jaccard metric revealed differences between the NC treatment and the PC, PC + Antibiotic, and PC + probiotic treatments. Additional disparities were observed in the PC + β-mannanase treatment compared to the PC + Antibiotic and PC + β-mannanase + probiotic treatments, as well as between the PC and PC + probiotic treatments (Figure 3B).

At 43 days of age, the NC treatment group exhibited differences compared to the PC + Antibiotic and PC + probiotic treatments across all metrics (Figure 3A–D). Specifically, the NC treatment group displayed dissimilarities compared to the PC, PC + β-mannanase and PC + β-man + prob groups. Additionally, antibiotic supplementation in challenged birds differed from the PC + probiotic treatment in terms of the Bray–Curtis, Jaccard and UniFrac metrics (Figure 3A–C). When considering the Jaccard, UniFrac and Weighted UniFrac metrics, the PC + Antibiotics group exhibited distinctions when compared to the PC + β-man + prob treatment (Figure 3B–D). Furthermore, the PC group differed from the PC + probiotic treatment in terms of the Jaccard and UniFrac metrics (Figure 3B,C). Unique differences were observed in the PC + β-mannanase treatment compared to the PC + probiotic and PC + β-man + prob groups. In a complementary way, significant differences were found between the PC + Antibiotics and PC + β-mannanase dietary treatments based on the Jaccard metric (Figure 3B). All comparisons considered the abundance and phylogenetic relationships among taxa.

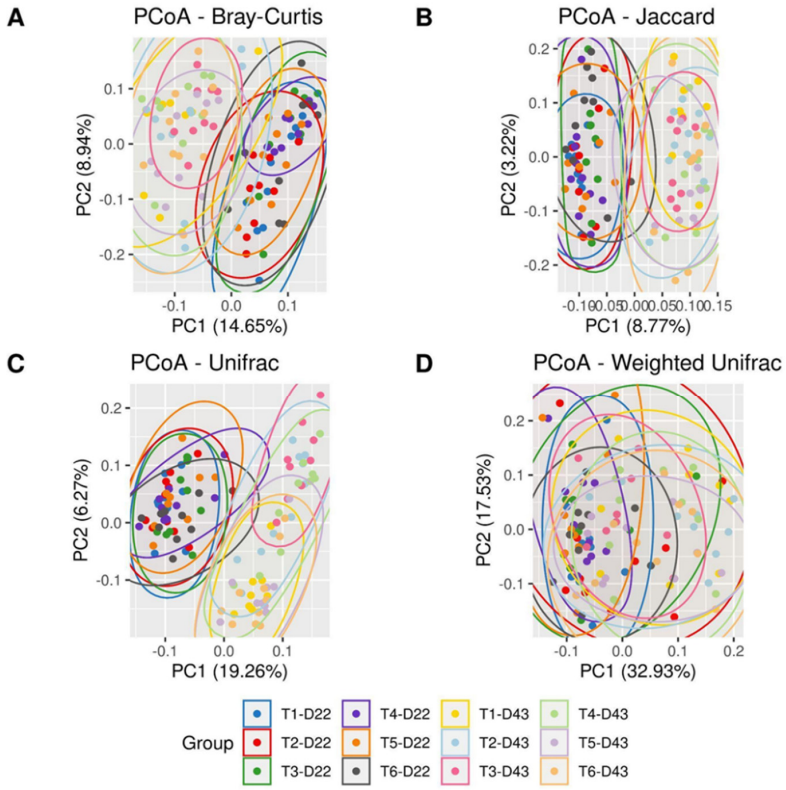

Figure 3. Beta diversity estimated by Bray–Curtis parameters (**A**). Jaccard (**B**). UniFrac (**C**) and weighted Unifrac (**D**). Colored ellipses were automatically added via the ggforce library in R. The treatments were as follows: T1—Negative control (NC) (birds without challenge); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); T3—PC + Antibiotic); T4—PC + β-mannanase; T5—PC + probiotic; T6—PC + β-mannanase + probiotic.

3.4.3. Composition of the Bacterial Community

The Firmicutes/Bacteroidota ratio was computed for each analyzed sample (Figure 4). Significant distinctions were observed among all treatments at their respective sampling ages. Furthermore, at 22 days, the group of birds of the PC treatment exhibited a statistically significant difference compared to the PC + probiotic and PC + β-man + prob treatments.

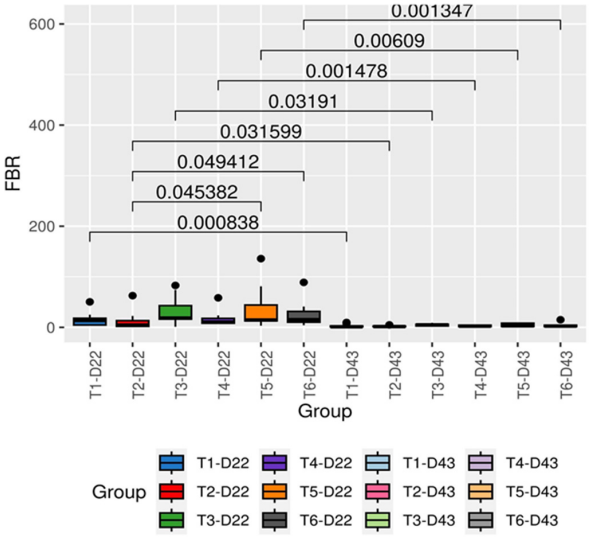

Figure 4. The bar graph shows the ratio between Firmicutes and Bacteroidota taxa in the tested groups. Differences with *p* value below 0.05 were considered statistically significant. The treatments

were as follows: T1—Negative control (NC) (birds without challenge); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); T3—PC + Antibiotic; T4—PC + β-mannanase; T5—PC + probiotic; T6—PC + β-mannanase + probiotic.

3.4.4. Differences in the Abundance of Taxa

Only taxa with statistically significant differences in relative abundance (Kruskal–Wallis $p < 0.05$) were evaluated.

In the Acutalibacteraceae family, statistically significant differences were observed between the NC and PC + β-mannanase treatments at the corresponding sample ages. At 43 days, there were differences between the NC treatment and the PC + Antibiotic treatment, with a greater quantity of this family being found in the PC + Antibiotic treatment (Figure 5A). In the Bacteroidaceae family, except for NC, there were significant differences between treatments at both bird ages (22 and 43 days old). At 22 days, the PC + Antibiotic treatment differed from the NC and PC treatments (Figure 5B). From the Lactobacillaceae family, the PC + Antibiotic, PC + probiotic and PC + β-man + prob treatments differed at the two ages of the birds (22 and 43 days old). At the 43-day age, there were differences between the PC + β-mannanase and PC + probiotic treatments and the PC + β-man + prob treatment (Figure 5C). In the Oscillospiraceae family, the undisputed treatment differed between the two ages of the birds. At 22 days of age, the NC and PC + β-mannanase treatments differed, and there was a statistical difference between the PC + probiotic and PC + β-man + prob treatments. At 43 days, there were statistical differences between the PC + Antibiotic and NC treatments compared to the PC and PC + β-man + prob treatments (Figure 5D). In the Butyricicoccaceae family, the PC treatment showed differences between the two corresponding sampling ages (22 and 43 days). At 22 days of age, there were differences between the PC + β-mannanase treatment and the PC + probiotic treatment. At 43 days, there were differences in relation to the PC + probiotic treatment (Figure 5E). In the Lachnospiraceae family, the PC + β-mannanase and PC + β-man + prob treatments differed at the respective sampling ages. At 22 days, the PC treatment differed from the PC + β-mannanase, PC + probiotic and PC + β-man + prob treatments. Furthermore, at the same age, the NC treatment showed differences in relation to the PC + β-mannanase treatment (Figure 5F). In the Rikenellaceae family, differences occurred in all groups, except in the PC + Antibiotic treatment, which showed differences only between the respective sampling ages of the birds (Figure 5G). At 43 days, the Ruminococcaceae family showed a significant difference between the PC treatment and the PC + probiotic treatment (Figure 5H).

In the genus *Agathobaculum*, the PC birds showed differences in their corresponding sampling ages. At 43 days, the PC treatment differed from the PC + probiotic treatment (Figure 6A). In *Alistipes*, the PC + β-mannanase, PC + probiotic and PC + β-man + prob treatments showed differences between sampling ages. At 22 days, the NC treatment differed from the PC + β-mannanase treatment (Figure 6B). In the genus *Lactobacillus*, the PC + Antibiotic and PC + β-man + prob treatments showed differences between the corresponding sampling ages. At 43 days, the PC + β-mannanase treatment differed from the PC + probiotic and PC + β-man + prob treatments (Figure 6C). In terms of *Mediterraneibacter*, the NC, PC and PC + β-mannanase treatments differed between bird ages. At 22 days, the treatment of PC + β-mannanase differed from the PC + β-man + prob treatment (Figure 6D). In *Faecalibacterium*, the NC and PC + β-man + prob treatments showed differences between the two ages. At 22 days, the NC treatment differed from the PC + Antibiotic supplemented treatment (Figure 6E). In terms of *Gemmiger*, there were differences between the PC + Antibiotics and PC + β-mannanase treatments at both corresponding sampling ages (Figure 6F). In terms of *Prevotellamassilia*, the PC + Antibiotic treatment differed between bird ages. At 22 days of age, there were differences between treatments for NC, PC and PC + β-mannanase (Figure 6G). In terms of *Tidjanibacter*, the NC, PC + β-mannanase and PC treatments differed in their respective ages. At 22 days, there were differences between the NC and PC treatments (Figure 6H).

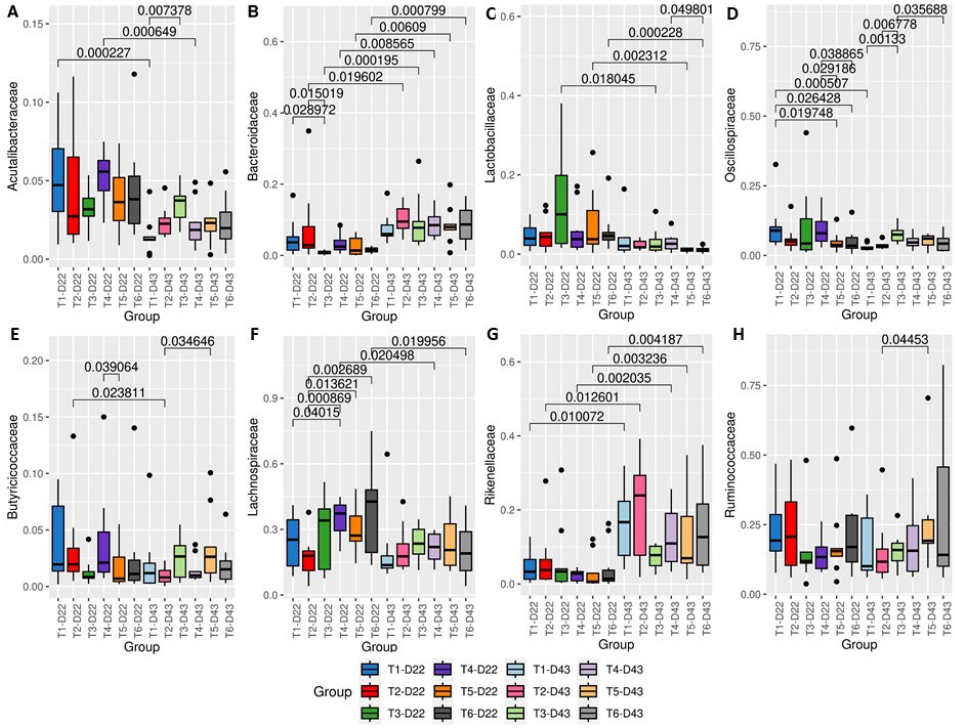

Figure 5. Differential abundance of the Acutalibacteraceae family (**A**), Bacteroidaceae (**B**), Lactobacillaceae (**C**), Oscillospiraceae (**D**), Butyricicoccaceae (**E**), Lachnospiraceae (**F**), Rikenellaceae (**G**) and Ruminococcaceae (**H**). Statistical comparison between groups was performed using the non-parametric Kruskal–Wallis and Post hoc Dunn tests. Statistical results below 0.05 were accepted as statistically significant. The treatments were: T1—Negative control (NC) (birds without challenge); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); T3—PC + Antibiotic; T4—PC + β-mannanase; T5—PC + probiotic; T6—PC + β-mannanase + probiotic.

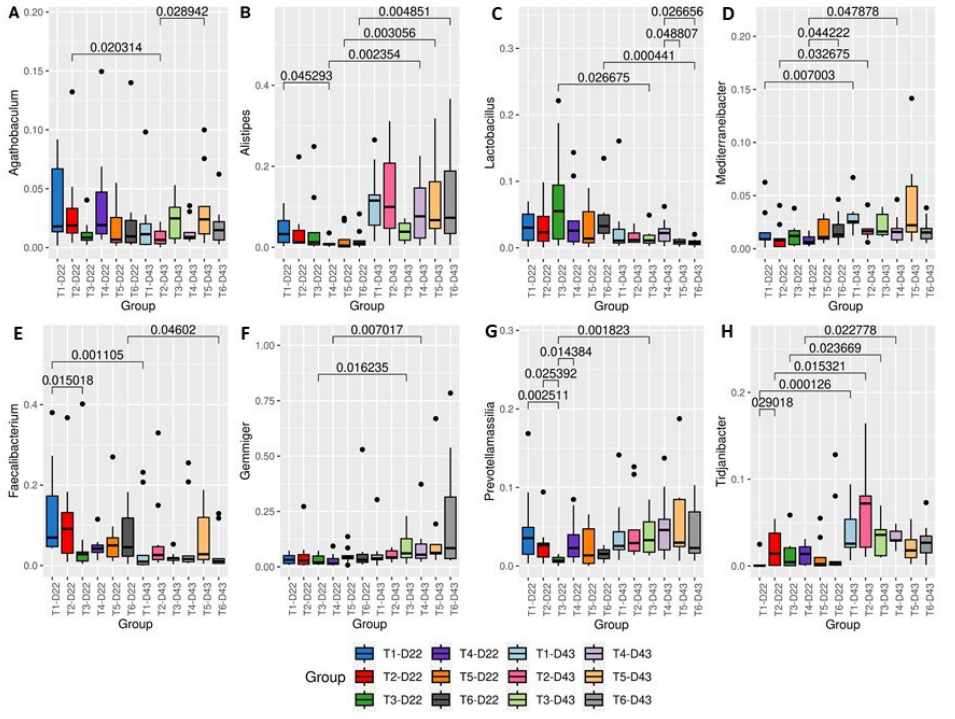

Figure 6. Differential abundance of *Agathobaculum* generum (**A**), *Alistipes* (**B**), *Lactobacillus* (**C**), *Mediterraneibacter* (**D**), *Faecalibacterium* (**E**), *Gemmiger* (**F**), *Prevotellamassilia* (**G**) and *Tidjanibacter* (**H**).

Statistical comparison between groups was performed using the non-parametric Kruskal–Wallis and Post hoc Dunn tests. Statistical results below 0.05 were accepted as statistically significant. The treatments were: T1—Negative control (NC) (birds without challenge); T2—Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); T3—PC + Antibiotic; T4—PC + β-mannanase; T5—PC + probiotic; T6—PC + β-mannanase + probiotic.

3.5. HTSi (Health Tracking System)

There was a significant influence of treatments ($p < 0.05$) on the HTSi analysis at 22 days of age (Figure 7). The birds in the PC + probiotic group demonstrated a value closer to 100%, that is, indiciating smaller intestinal lesions. The NC treatment showed a lower percentage of HTSi, indicating greater intestinal lesions in the birds in this group.

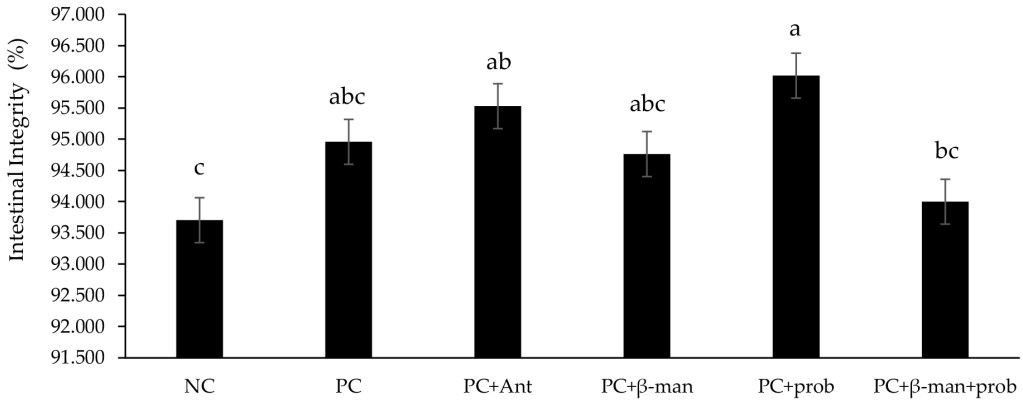

Figure 7. The graph shows that through the analysis of intestinal integrity (HTSi), there was a significant difference between treatments in broilers challenged or not by *Eimeria maxima* and *Clostridium perfringens*. ^{abc} Different letters in the same column represent statistical difference by the Tukey test (p-value < 0.05 was considered statistically different). Means that do not follow a letter are significantly different. Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic.

3.6. Intestinal Permeability (FITC-Dextran)

The results for intestinal permeability were not significant ($p > 0.05$), as demonstrated in Table 5.

Table 5. Hematological parameters of intestinal permeability of 42-day-old broiler chickens.

Treatments	FITC-Dextran (µg/mL)
NC	0.476
PC	0.404
PC + Ant	0.418
PC + β-man	0.438
PC + prob	0.402
PC + β-man + prob	0.400
SEM	0.010
***p*-value**	0.190

Negative control (NC) (birds without challenge); Positive control (PC) (birds challenged with *Eimeria maxima* + *Clostridium perfringens*); PC + Antibiotic; PC + β-mannanase; PC + probiotic; PC + β-mannanase + probiotic. SEM: Standard error of the mean. Means that do not follow a letter are significantly different.

4. Discussion

4.1. Performance Response

Coccidiosis, caused by protozoa of the genus *Eimeria* spp., damages the barrier of the intestinal epithelium, leading to severe inflammatory responses and the appearance of

lesions in the villi, interfering with the performance responses and health of birds [41]. To evaluate the effectiveness of the inoculation, *E. maxima* oocyst counts were performed at 20 days. The inoculation protocol was demonstrated to be effective since all challenged treatments showed the presence of oocysts six days post-inoculation, corresponding to the incubation cycle of E. *maxima*, and the performance responses are due to the challenge depending on the treatments. We believe that the greater abundance of oocysts in the excreta of birds in the PC + Antibiotic treatment is due to the antibiotic's non-selective mechanism of action, which reduced or eliminated an entire healthy microbial population [42], promoting an increase in *E. maxima* in the gastrointestinal tract of these birds.

For the productive performance responses before the challenge, in the period from 1 to 14 days of age, the results demonstrate that the birds that received the PC + Antibiotic treatment obtained higher BW, BWG and FI values than the PC treatment, indicating that the previous supplementation of antibiotics from the beginning of the birds' life benefits productive performance responses.

On the other hand, in relation to productive performance in the age group of 15 to 28 days, which corresponds to the challenged period, the challenge negatively affected the reduction in the FI and consequently the BWG values. A decrease of about 10% in FI was previously observed in [43], who used a similar challenge model. Furthermore, the reduced FI directly reflects the lower BWG, due to the damage caused sub clinically by *E. maxima* and *C. perfringens* in the small intestine of challenged birds that influenced nutrient utilization [43,44]. A reduction in FI and BWG due to the association of *E. maxima* and *C. perfringens* challenge models has been demonstrated laterally in broiler chickens [44, 45]. Previous studies [43–46] have shown that the most drastic reduction in FI occurs about three to seven days after inoculation. A study developed in [21] reports that broiler chickens immunologically challenged with *E. acervulina, E. maxima* and *C. perfringens*, from 8 to 21 days of age, that received a diet containing β-mannanase showed a reduction in FI compared to birds that received the same diet without enzyme supplementation. Our study corroborates this finding, since birds that received β-mannanase also had reduced FI, due to the proposed challenge.

In the period from 29 to 42 days, the challenge was less intense, and the results demonstrated that an increase in FI and BW was achieved in challenged birds fed with PC + Antibiotic, PC + β-mannanase and β-man + prob. The authors of [47,48] demonstrated that for broiler chickens aged four to six weeks, diets supplemented with β-mannanase in diets based on soybean meal and corn increased the daily weight gain of the animals, evidencing a partial recovery in the performances after three weeks of the challenge. The authors of [48], evaluating probiotic supplementation in broiler chicken diets, reported that probiotics significantly increased average daily weight gain during the first three weeks of growth but not during the next few weeks of growth (4–6). In a study conducted by [49], it was demonstrated that broiler chickens fed diets supplemented with 10^8 CFU of *Bacillus subtilis*/kg had greater live weight gain, while other studies did not report the same positive effect [50,51].

The response (from 1 to 42 years) demonstrates that unchallenged birds had a greater productive performance in relation to the PC and PC + probiotic treatments, except in the FCR variable. This is related to the damage that coccidiosis, through *Eimeria*, causes to the epithelial tissue [52], causing desquamation of the epithelium, resulting in damage to the intestinal villi, which impairs the absorption of nutrients and energy expenditure for the repair of injured intestinal cells. Regarding the use of probiotics in broiler chickens, although several studies demonstrate their efficiency and viability, there is some conflicting literature on productive performance responses [53,54]. Several factors can alter the effectiveness of the probiotic, such as the route of administration, strains used, handling conditions when preparing the product, animal category and physiological state, in addition to the environment, which can jointly influence the response to the use of probiotics [55–57].

4.2. Gut Health

The advances in broiler genetics have made it possible to maximize weight gain and protein deposition. However, for broilers to reach their full genetic potential, we must pay attention to intestinal health. Intestinal health is of fundamental importance for the good performance of animals in the production chain, and there appears to be a direct relationship between a healthy intestine and productive performance, although there is still no clear definition that encompasses all physiological functions of the intestinal tract, such as digestion and absorption, host metabolism and energy production.

Variations in diet composition, as well as physical characteristics, can affect intestinal integrity. For example, the internal epithelium of the intestine is constantly changing (turn over) and some characteristics of the diet, such as a higher fiber content, can increase the turnover rate, reducing the physical barrier and resulting in the intestine becoming susceptible to the actions of pathogenic microorganisms [58]. Furthermore, depending on the remaining substrate in the feed, it can be a fuel for the proliferation of microorganisms, altering the composition of the microbiota [59]. Moreover, intestinal integrity is strongly correlated with microbiome diversity because intestinal functions are positively regulated by the microbiome, which has adequate mucosal wall development, functional intestinal barrier and mucosal immune response [59]. The intestine regulates physiological homeostasis, allowing the animal to resist harmful and nonmalignant stressors [60]. Therefore, a healthy intestine has intact villi and crypts, a healthy microbiome and decreased intestinal permeability.

4.2.1. Intestinal Morphometry

Villus height and crypt depth constitute the morphology of intestinal tissue and are indicators for measuring intestinal health, injury and recovery [61]. Crypts are also measures of cell multiplication, and shallower crypts indicate better intestinal health [62]. The size and density of villi are related to cell loss and renewal by the intestinal mucosa [63]. When there is a balance between these processes, a constant turnover occurs, that is, the maintenance of the size of the villi, also generating maintenance in the digestive and absorptive capacity of the intestine. In this sense, when we talk about the morphometric variables of villus height and crypt depth in general, our results demonstrated that supplementation with β-mannanase, probiotics or both together positively affect the morpho functional integrity of the digestive system.

At 21 days, supplementation with probiotics stimulated greater villi height in the duodenum due to the action of probiotics on microbiological development, in which the intestinal epithelium inhibits the colonization of pathogens, causing changes in the barrier against microorganisms, triggering benefits to the intestinal mucosa, thus favoring the structure of the villi [64,65]. It is hypothesized that the probiotic reduced the inflammatory process, resulting in greater height of the duodenal villi. Furthermore, CD in the jejunum was lower in PC + β-mannanase birds, a beneficial effect also demonstrated in [66,67].

At 42 days, in the three segments of the intestine, the CD variable of the birds that received β-mannanase was lower compared to the other treatments. The villus/crypt ratio (V:C) in the duodenum and jejunum demonstrated that challenged birds receiving β-man + prob had a higher V:C value, that is, recovery in intestinal integrity. The beneficial effect of enzymatic and probiotic supplementation was due to the interaction of both additives, where the probiotic positively influenced the establishment of beneficial microbiota, protecting the integrity of the intestine, and the enzyme acts in the improvisation of nutrients from the feed [68–70]. The beneficial action of probiotic supplementation, such as *Lactobacillus acidophilus* and *C. butyricum*, was evidenced by the increase in the renewal of intestinal morphology, observed through the increase in the height of the villi and the reduction in the depth of the crypts [49,71,72].

In general, the joint use of additives provided broiler chickens with greater resistance to damage to the intestinal epithelium caused by *E. maxima* and *C. perfringens*. This is due to the modulation of intestinal health to maximize the defense of the immune system

in terms of protection of epithelial tissues, demonstrating an immunomodulatory action that modifies the morphological structure of the intestine to reduce the harmful effects of immunological challenges.

4.2.2. Cecum Microbiome

It was previously demonstrated that *Eimeria* spp. can cause major changes in microbiota composition in birds affected by necrotic enteritis [73–75]. Studies claim that dietary use of antibiotics and other performance enhancers affects the amounts and the set of bacterial populations in the intestinal tract [76]. Incidentally, a stable and healthy intestinal ecosystem weakens the colonization of harmful microbial populations, enhancing intestinal barrier function and increasing growth performance [60].

Alpha diversity depicts a synopsis of diversity in a unique population. It has also been reported that alpha diversity can be related to baseline inferences about the mechanisms and functionalities of the microbiome [77]. Diversity richness refers to the amount of rare operational taxonomic units (OTUs) present in the samples. Therefore, the higher the index, the greater the number of rare OTUs. The diversity index is calculated, considering the abundance and number of OTUs present in a fragment. Additionally, the increase in the Shannon index concerns the increase in species richness and uniformity and, thus, diversity [78]. The authors of [79] identified that greater richness is linked to good health, but species richness and diversity reduction may be directly related to pathologies.

In our study, the analysis of alpha diversity (Figure 2) reveals that at 43 days of age, the animals that received antibiotics, β-mannanase or probiotics through the diet showed greater richness and microbial diversity in the cecum. It is believed that this greater richness is related to the action of the antibiotics, enzymes and probiotics in modulating the microbiome of these birds, even in a situation of immunological challenge, to minimize the effects of harmful bacteria on the host. This result corroborates the findings of [10,21,22], which also reported in their studies that β-mannanase had a modulating action on the microbiota of broilers fed corn–soy diets and challenged with *C. perfringens* and *E. maxima*. Studies have shown that probiotics are effective in optimizing intestinal health and performance in birds, establishing a healthy microbiome, as they regulate the microbial community through enabling competitive exclusion, producing antibodies, and allowing intestinal development of beneficial microorganisms and intestinal immunity [80–82]. The author of [83] observed at 42 days the balance between the bacteria that make up the microbiome, regardless of the enzyme supplementation or the challenge that occurred four weeks ago. And this study indicates that the proper balance of microorganisms is probably not seen in high health challenges.

β-diversity is used when comparing the microbiome to determine the number of OTUs or the shared rate, thus understanding how the actions of microbial species vary in numerous microbiomes. A dissimilarity index is provided as a response, for example, Bray–Curtis, which considers additional information such as the extent to which functions change in diverse microbiomes and shared memberships between microbiome profiles of numerous communities [84]. Furthermore, derivation interpretations of βeta diversity can be quantitative (weighted UniFrac) or qualitative (unweighted UniFrac) [77]. In our study, it was observed that the βeta diversity is very expressive in all treatments, showing that it changes with the advancing age of the animal and possibly with the presence of undesirable microorganisms, such as the challenge proposed in this study. In a healthy intestine, bacteria act synergistically, where each bacterium is an integral link in the generation of metabolites that the host uses. However, during an imbalance of the gut microbiome, the result of inflammation by the host targets commensal microorganisms, causing a decrease in bacterial diversity and essential functions, resulting in a decrease in metabolic activity, inhibiting the host from numerous end products that limit intestinal health and bird performance [85,86]. In other words, in the present study, at 22 days, a homogeneous population of bacteria according to Bray–Curtis and Jaccard parameters was observed in the cecum microbiome of birds that received β-mannanase in the diet compared to unchallenged birds or challenged without supplementation. This reflects less dissimilarity

in the birds that received β-mannanase, that is, that the microbial communities have similar functions, in which they modulate the microorganisms in the cecum to bring benefits to the host.

It has been determined that a higher Firmicutes/Bacteroidetes (F/B) ratio favors chicken growth [87–89]. Consequently, the relationship between the amount of Firmicutes and Bacteroidetes has been used as evidence of a bird's efficiency in synthesizing dietary nutrients. When comparing the Firmicutes/Bacteroidota ratio between the treatments and the respective ages of the birds in our study, in all treatments, this ratio was higher at 22 days of age when compared to 43 days. It was also observed that at 22 days, this relationship was higher in treatments that received probiotics or β-man + prob, when compared to birds challenged without any supplementation. Therefore, we observe that even during a challenge by *E. maxima* and *C. perfringens* at 22 days, the birds tried to modulate their microbiota to minimize the losses related to the immunological challenge, and the animals that received the probiotic or β-mannanase associated with the probiotic managed to achieve this beneficial modulation more effectively (Figure 4).

Studies report a significant continuous change in the taxonomic composition, which is more abundant and taxonomically varied as the bird ages [90]. Taken together, part of the modulations that occur in birds come from families, genera and species that play a crucial role in the homeostasis of the gastrointestinal tract. In our study, the main families that showed a significant difference between the treatments that were challenged without supplementation and the treatments supplemented with antibiotics, probiotics or β-mannanase were the families described in Figure 5. This corroborates the findings of authors who describe that the families *Bacteroidaceae*, *Lactobacillaceae*, *Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Enterobacteriaceae* and *Prevotellaceae* are common members of the cecal microbiota of chickens [91–93].

The *Bacteroidaceae* family belonging to the phylum Bacteroidota was present in greater quantity in the microbiome of birds challenged without supplementation at 22 days of age. Some bacteria of this family, in situations of dysbiosis, as caused by *Eimeria*, can multiply and become pathogenic, consequently reducing the feed efficiency of the birds [94]. In our study, at 22 days of age, there was a significant decrease in the *Lachnospiraceae* family, belonging to the phylum *Firmicutes* in negative control birds or challenged without supplementation, and an increase in those that received β-mannanase, probiotics and β-man + prob. This is one of the main families whose function is to ferment non-digestible polysaccharides in the cecum, producing short-chain fatty acids that lead to the growth of epithelial cells [95], being the main generators of butyrate [96], considered an anti-inflammatory metabolite. Studies in pigs reported that the *Lachnospiraceae* family was abundant in the microbiota of dietary treatments containing B-mannanase [97]. Another study with broiler chickens using the enzyme reported that at 21 days of age, the cecal microbiome was compromised mainly by members of the *Ruminococcaceae* and *Lachnospiraceae* families [10].

However, when we analyze the *Butyricicoccaceae* family at 43 days of age, the treatment that received probiotics had a higher concentration of this family in the microbiome, compared to birds challenged without supplementation. The genus *Butyricoccus* has been described as beneficial for the intestinal microbiome [98]. Therefore, its greater predominance shows that the use of the probiotic was effective in the intestinal modulation of the cecum even after the immunological challenge. In terms of the *Lactobacillaceae* family, there was an increase in their proportion in the microbiome of birds that were supplemented with β-mannanase. In a study where birds received β-mannanase, they also recorded an increase in the microbiota of the *Lactobacillaceae* and *Ruminococcaceae* families and a reduction in bacteria associated with low feed efficiency [10]. It has already been seen that exogenous enzymes, including β-mannanase, have been supplemented in broiler diets to improve the digestibility of feed ingredients and modulate the intestinal microbiota of birds [99]. At 22 days of age, the *Lactobacillus* family was abundant in animals that received antibiotics. At 43 days of age, there was a reduction in this family in all treatments,

compared to 22-day-old birds. Members of this family, such as *Lactobacillus* spp., play a beneficial role in intestinal health, immunological parameters and zootechnical performance [100], as well as selectively exclude pathogens from adhering to the intestine, due to their rapid proliferation and acidifying characteristics in the GIT [101]. One study shows an increase in the abundance of the *Lactobacillales* family in the microbiota in 28-day-old broilers challenged with coccidiosis receiving antibiotics (Enramycin and Tylosin); however, at 42 days, all treatments showed a reduction in the relative abundance of this family [102], corroborating our findings. The author of [103] also observed a higher proportion of *Lactobacillaceae* in broiler chickens challenged with *Eimeria.* The *Ruminococcaceae* family was in greater abundance at 43 days of age in the microbiome of birds that received probiotics in the diet compared to birds challenged without inclusion of any performance enhancer. It has been suggested that the increase in this family is related to broilers with a lower feed conversion ratio [104]. That is, the results of the present study show that the animals challenged with the probiotic treatment, even though their feed conversion was higher, had a lower weight gain compared to other treatments, and the probiotic had a positive action in the modulation of the microbiome, as *Ruminococcaceae* is known to produce butyrate and therefore helped in the regulation of inflammation [105]. In a study administering postbiotics in the feed, it was observed that the dominance of *Ruminococcaceae* in the birds of this treatment at 28 days of age decreased by 24.2%, and they had a lower weight and higher feed conversion rate [106].

Certain pathological states induce a loss of diversity, with an increase in the concentration of certain bacterial genera to the detriment of others [78]. The genera that predominate in the cecum are *Clostridium, Lactobacillus, Bacteroides* [107], *Ruminococcus* [108] and *Prevotella* [109]. The genus *Agathobaculum* at 43 days of age showed greater abundance in challenged birds that received probiotics in the diet, compared to challenged birds without supplementation. This genus is linked to butyrate-producing anaerobic bacteria [110]. This genus belongs to the *Firmicutes phylum,* so it is believed that its higher concentration in the birds that received probiotics in this study is positively linked to the modulation of the microbiome of the cecum, since in general bacteria of this phylum can inhibit the growth of opportunistic pathogens and some are known to be involved in the breakdown of complex carbohydrates [111]. This may also be correlated to the benefits of probiotics, as they reduce and prevent colonization by enteric pathogens through competitive exclusion and the formation of bacteriostatic and bactericidal substances [108,109].

In our study, at 22 days, the genus *Faecalibacterum* was higher in the negative control birds compared to birds that received the PC + Antibiotic treatment. This genus has been described as having anti-inflammatory properties [112,113]. A decrease in the richness of *Faecalibacterium,* like butyrate-producing bacteria, may impede the development of the immune response and reduce the synthesis of butyrate used as an energy source [71]. A study using bulk sequencing techniques established the composition of the microbiota, and on days 21 and 42, the genus *Faecalibacterium* apparently predominated [114]. The genus *Alistipes* at 22 days of age was more abundant in negative control birds when compared to challenged birds receiving β-mannanase in the diet. This genus, like its species, is known to have anti-inflammatory properties [115]. However, these bacteria produce butyrate and short-chain fatty acids through two main pathways, the butyryl pathway (CoA: acetate CoA transferase) and the butyrate kinase pathway, in which these substances are related to the physiological processes and energy homeostasis of the host [116]. A study in [10] found an inverse response when birds challenged at 21 days received β-mannanase, obtained through the microbiota of the cecal content, the enzyme or with increase in *Alistipes.* In general, with the aim of replacing antibiotics, the use of β-mannanase or probiotics can be an effective alternative in beneficially modulating the intestinal microbiota and mitigating the effects of the challenge.

4.2.3. HTSi (Health Tracking System)

The presence of visual intestinal lesions at necropsy can be used as a good measure to evaluate intestinal health disorders and can be related to the results of morphometric and microbiological analyses, enabling the classification of intestinal health status. In the study, the incidence of abnormal characteristics in NC treatment birds was notably high compared to the group that received PC + probiotics in the feed at 22 days of age (Figure 7). In other words, through the HTSI score, birds supplemented with probiotics in their feed had a lower incidence of intestinal lesions at the time of the challenge. Therefore, the probiotic promoted an increase in intestinal protection and barrier functions, demonstrating improved intestinal health.

4.2.4. Intestinal Permeability (FITC-Dextran)

According to [59], the action of the intestinal barrier is necessary to maintain the functionality of the intestine's cells. Consisting of a single layer of epithelial cells trapped in columns, they perform the first line of protection of the organism against pathogens and harmful products present in the lumen [117]. Lymphoid tissue provides immune cells to help against pathogenic microorganisms [118]. The outer layer, which has a beneficial microbiota, and the inner layer, which defends the intestine, consisting of mucus rich in mucin and IgA, help preserve and control health, thus inhibiting the entry of pathogens. Furthermore, they protect the epithelium from anti-nutritional factors and the effects of toxins [119]. Multiple conditions regulate intestinal permeability and determine which molecules can cross this barrier and enter the bloodstream [120]. Damage to the epithelial barrier has the potential to cause increased intestinal permeability that directly transports intraluminal macromolecules and pathogens into the blood [121]. Coccidiosis afflicts intestinal barrier function, which is critical to host health and defense, as demonstrated. Thus, an ideal food additive can promote effective gut barrier action [122]. The permeability test can influence the experimental conditions, which can be considered an index of the gut integrity and the absorption capacity of the enterocytes. Also, the induced lesions can provide a rapid response in terms of a permeability test and depending on what moment post-infection they occur and the degree of challenge [123]. Thus, cell breakdown allows greater intestinal permeability, which was observed in a study in [43] and was corroborated in [123], which showed greater passage of FITC-dextran in the blood of birds challenged by *E. maxima.*

Another study observed in [124] reported that diets containing probiotics with 1×10^6 CFU of *Bacillus amyloliquefaciens* CECT 5940 per g of feed, through FITC-dextran analysis, did not have a significant effect, so there was no significant influence on intestinal permeability. Similarly, the study in [125] obtained the same effect in the birds using a *B. amyloliquefaciens* strain at 42 days of age. This result may be related to the fact that after the period of infection by *E. maxima* and *C. perfringens,* birds regain intestinal health at the level of more serious injuries that affect the intestine. In an assay with a dose of 50,000 sporulated oocysts per bird, the study in [123] reported that if the birds in the experiment survived severe infection between five and seven dpi, gastrointestinal permeability would return to normal levels within nine dpi, regardless of the challenge dose administered. In our study, the challenge dose did not cause mortality in the birds, so we can note that they did recover after the challenge. Through this, it is believed that the broilers in the present study recovered their intestinal health, and the non-significant concentration of FITC-dextran shows that intestinal permeability was not affected at this age (42 days).

5. Conclusions

The challenge with *E. maxima* and *C. perfringens* reduces feed consumption and the weight gain of birds, negatively affecting production performance responses. However, based on our findings, supplementation with β-mannanase, probiotics or β-mannanase + probiotics can prevent damage to intestinal integrity, modulating intestinal morphometry

and the microbiome of the cecum, beneficially impacting the health of the gastrointestinal tract of these birds.

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