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Bacillus-based probiotics on broiler chicken performance under coccidiosis and *Clostridium perfringens challenge*

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Abstract

The study analyzed the impacts of two probiotics (Ecobiol® and GutCare®) on growth performance, carcass and cut yields, intestinal morphometry and lesion score, biochemical parameters, and short-chain fatty acid concentration in chickens using a challenge model combining coccidiosis vaccine and *Clostridium perfringens.* A total of 880 one-day-old male broilers (Cobb 500) were randomly assigned to four treatments with 10 replicates and 22 birds per experimental unit (EU). The treatments consisted of: PC: positive control with the inclusion of 80 g ton^{-1} of Enramax® (8% enramycin) until 35 d of age; NC: negative control without the inclusion of growth-promoting probiotic; BA: NC with the inclusion of 1000 g ton^{-1} of Ecobiol® (*Bacillus amyloliquefaciens* - CECT 5940); BS: NC with the inclusion of 500 g ton^{-1} of GutCare® (*Bacillus subtilis* - DSM 32315). Diets used maize–soybean meal in a three-phase plan. Broiler chickens on the BA and BS diets had higher feed consumption, body weight gain, and improved feed conversion efficiency at 28 and 42 d when compared to the birds in the negative control. Broiler chickens fed PC, BA, and BS diets had a higher villus height and absorption area in the jejunum at 28 d, compared to the birds in the negative control. There was more butyric acid production by the intestinal microbiota at 28 d of age in broilers on the BA diet. Supplementing with 1000 g/ton of BA and 500 g/ton of BS effectively substituted the 8% enramycin antibiotic, enhancing broiler growth during an induced intestinal challenge.

Keywords: Antibiotic alternatives, intestinal health, poultry nutrition
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Introduction

Enteric diseases pose a considerable concern in the poultry industry, with necrotic enteritis being one of the most important, causing billions in annual losses (Fathima *et al*., 2022; Ningsih *et al*., 2023). The primary causative agent is *Clostridium perfringens*, which can produce toxins harmful to the gastrointestinal tract of birds (Hofacre *et al*., 2018; Ma *et al*., 2019; Ayalew *et al*., 2022; Rodrigues *et al*., 2024). The presence of this pathogen is exacerbated by factors such as changes in diet, imbalances in the intestinal microbiota, stress, and the occurrence of underlying diseases, such as coccidiosis caused by *Eimeria* spp. (Ahmed *et al*., 2014; Mwangi *et al*., 2019; Cirilo *et al*., 2023). High adaptability

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to various environments, tolerance to oxygen presence, the ability to survive in a wide pH range, and the ability to form spores make disease control challenging (Zhang *et al*., 2018; Liao *et al*., 2020; Ayalew *et al*., 2022; Lee *et al*., 2023; Zhang *et al*., 2023).

The use of antibiotics is a common alternative to control the spread of these enteric diseases in poultry farming (Gadde *et al*., 2017; Cai *et al*., 2023; Cirilo *et al*., 2023). However, indiscriminate antibiotic use can lead to the development of microbial resistance, making these treatments less effective over time (Bortoluzzi *et al*., 2019; Aljumaah *et al*., 2020; Madlala *et al*., 2021; Bao *et al*., 2022; Wang *et al*., 2023). Given this scenario, it has become essential to seek sustainable and effective alternatives for controlling enteric diseases in birds (Hernandez-Patlan *et al*., 2019; Gharib-Naseri *et al*., 2021; Memon *et al*., 2021; Zhu La *et al*., 2024). In this context, probiotics emerge as a promising alternative (Wang *et al*., 2021; Rahman *et al*., 2022; Chen *et al*., 2024). Specifically, *Bacillus* spp.-based probiotics have proven to be a viable solution, as they modulate the intestinal microbiota, optimize nutrient digestion, strengthen the immune system of broiler chickens, and reduce intestinal lesions caused by pathogens, such as *Clostridium perfringens* and *Eimeria* spp. (Cao *et al*., 2018; Adhikari *et al*., 2020; Ahmat *et al*., 2021; Goo *et al*., 2023).

Based on this, the hypothesis of this study is that probiotics derived from *Bacillus* may replace antibiotics in the diet of broiler chickens, resulting in improvements in performance and reduction of lesions caused by intestinal pathogens. Therefore, two probiotics (Ecobiol® with *Bacillus amyloliquefaciens* - CECT 5940 and GutCare® with *Bacillus subtilis* - DSM 32315) were used as alternatives to enramycin to evaluate the growth performance, carcass yield and cuts, intestinal morphometry, lesion score, biochemical parameters, and short-chain fatty acid concentration in broiler chickens challenged with *Clostridium perfringens* and *Eimeria* vaccine.

Materials and methods

The use of animals and approval for all experimental protocols were granted by the Ethics Committee for Animal Use of Western Paraná State University (Protocol number: 20/2020). The study was conducted at the Poultry Research Center of Western Paraná State University, Marechal Cândido Rondon, Paraná, Brazil.

A total of 880 one-day-old male broiler chickens (Cobb 500) with a mean weight of 43.48 ± 0.55 g were distributed in a completely randomized design with four treatments and 10 replicates of 22 broilers per pen. The treatments were composed of: PC: positive control with the inclusion of 80 g ton^{-1} of Enramax® (8% enramycin) until 35 d of age; NC: Negative control without the inclusion of growth-promoting probiotic; BA: NC with the inclusion of 1000 g ton^{-1} of Ecobiol® (*Bacillus amyloliquefaciens* - CECT 5940); BS: NC with the inclusion of 500 g ton^{-1} of GutCare® (*Bacillus subtilis* - DSM 32315). The dosage of the probiotics was adjusted to meet the specific criteria of the experiment, ensuring their effectiveness in the study conditions.

The Enramax® used in this study is a formulation containing 8% enramycin, recognized for its specific efficacy in the digestive tract microbiota *against Clostridium perfringens, Staphylococcus*, and *Streptococcus*, contributing to the preservation of intestinal integrity. The Ecobiol®, also used in the research, consists of a natural strain of *Bacillus amyloliquefaciens* - CECT 5940, acknowledged for its ability to stabilize the intestinal flora and promote intestinal microbial balance. GutCare® used in the study is also recognized for maintaining this intestinal microbial balance with a natural strain of *Bacillus subtilis* - DSM 32315.

The diets were formulated based on maize and soybean meals according to the nutritional requirements established by Rostagno *et al*. (2017), for the respective age phases: 1–21 d (starter), 22–35 d (grower), and 36–42 d (finisher). Broilers received feed, in mash form, and water *ad libitum* throughout the experimental period. The use of the test products (Enramax® – enramicina 8%, Ecobiol® and GutCare®) was done on weight-for-weight (g g^{-1}) replacement for the inert feed material (kaolin).

Table 1. Ingredient and nutrient composition (g/kg) of the experimental diets

Item (g/kg)	1 to 21 days	22 to 35 days	36 to 42 days
Maize (7.88% CP)	579.567	645.698	646.790
Soybean meal (46% CP)	336.108	262.165	264.818
Soybean oil	16.233	28.088	36.063
Meat and bone meal	50.988	48.993	35.618
Limestone (fine)	2.557	0.990	3.879
Sodium bicarbonate	4.176	3.734	3.688
Lysine sulphate (54.7%)	1.078	1.444	1.036
DL- methionine (99%)	2.674	2.294	2.169
L-threonine	0.428	0.450	0.392
Choline chloride (60%)	0.837	0.790	0.445
Vitamin Premix[1]	1.300	1.300	1.300
Mineral premix[2]	0.500	0.500	0.500
Adsorbent [3]	1.000	1.000	1.000
Anticoccidian[4]	0.500	0.500	0.250
Phytase [5]	0.050	0.050	0.050
Inert[6]	2.000	2.000	2.000
Nutritional composition			
Met. Energy (kcal kg^{-1})	3.000	3.150	3.200
Crude protein (g kg^{-1})	225.20	196.30	191.30
Dig. Lysine (g kg^{-1})	11.10	9.60	9.20
Dig. Methionine + Cystine (g kg^{-1})	8.20	7.60	7.10
Dig. Threonine (g kg^{-1})	7.10	6.20	6.10
Dig. Valine (g kg^{-1})	8.80	7.70	7.50
Dig. Tryptophan (g kg^{-1})	2.20	1.80	1.80
Dig. Arginine (g kg^{-1})	13.60	11.60	11.30
Isoleucine (g kg^{-1})	7.90	6.70	6.70
Calcium (g kg^{-1})	9.50	8.50	8.00
Phosphorus (g kg^{-1})	4.50	4.30	3.80
Sodium (g kg^{-1})	2.10	1.90	1.80
Potassium (g kg^{-1})	9.20	7.90	7.80
Chlorine (g kg^{-1})	3.50	3.20	3.00

[1]Vitamin supplement, composition per kg of diet: Vitamin A (min) 14,300 I.U.; Vitamin D_3 (min) 5,200 I.U.; Vitamin E (min) 71.50 I.U. ; Vitamin K3 (min) 3.90 mg; Vitamin B_1 (min) 2.99 mg; Vitamin B2 (min) 9.10 mg; Pantothenic acid (min) 15.60 mg; Vitamin B_6 (min) 5.20 mg; Vitamin B12 (min) 32.50 mg; Nicotinic acid (min) 78.00 mg; Folic acid (min) 2.60 mg; Biotin (min) 0.33 mg; Selenium (min) 0.39 mg
[2]Mineral supplement, composition per kg of diet: Iron (min) 50g; Copper (min) 10g; Manganese (min) 65g; Zinc (min) 65g; Iodine (min) 1000 mg
[3]Bentonite-based adsorbent
[4]Anticoccidial: from 1–35 d of age used salinomycin 12% and from 36–42 d of age salinomycin 24%
[5]Phytase: quantum blue 10 g, 10,000 FTU g^{-1}
[6]Inert kaolin

At day 4 of age, all broilers were challenged with 20 times the dose of the Biococcivet R® vaccine (concentrated suspension of sporulated oocysts of five species of *Eimeria*: *E. acervulina, E. praecox, E. maxima, E. tenella,* and *E. mitis*), i.e., they received 0.6 mL orally to damage the intestine to facilitate colonization of *Clostridium perfringens.* At 7 and 10 d of age, all broilers received a 0.5 mL solution of

culture inoculum with *Clostridium perfringens* (10E8 CFU mL^{-1}) directly into the oesophagus in the region near the crop. This *Clostridium perfringens* strain was obtained from a field sample isolated from an outbreak of necrotic enteritis in broilers.

The poultry were housed in a 25-m-long by 8-m-wide concrete-floor barn, divided into pens of 1.76 m² each, equipped with nipple-type drinkers and tubular feeders and 250-watt heating lamps. The environment featured negative pressure ventilation and evaporative cooling for climate control. Before the experiment, the concrete floor of these pens was covered with pine shavings. The lighting program followed the recommendations of the breeder manual (COBB 500, 2019). Temperatures and relative humidity were monitored daily.

During the experimental period, mortality was recorded daily to correct feed intake and feed conversion, following the methodology described by Sakomura & Rostagno (2016). At 28 and 42 d of age, both feeders and birds were weighed to determine feed consumption, body weight gain, and feed conversion rate of the birds.

At 42 d of age, two broilers per experimental unit were randomly selected, weighed, marked, and slaughtered. The carcasses were weighed and stripped, obtaining legs (thigh and drumstick), breast fillet and inner breast fillet, which were weighed individually. Carcass yield was determined by the eviscerated carcass weight in relation to the live weight of the poultry. The cut yield was determined by the ratio between the weight of the cuts and the weight of the eviscerated carcass. The liver and abdominal fat (consisting of the adipose tissue around the cloaca, gizzard, proventriculus and adjacent abdominal muscles) were separated and weighed to determine their weight relative to the live weight of broilers.

At 28 d of age, two broilers per experimental unit were randomly selected for blood sample collection via brachial puncture on the ulnar vein while in lateral recumbency. After the collection, the birds were returned to their respective experimental units. Specific vacuum blood collection tubes made of 13 × 75 mm glass with clot activator and 5 ml capacity (CRAL, São Paulo, Brazil), specific adapters and 25 × 0.8 mm needles (Labor Import, Shandong Weigao, China) were used to collect 4 ml of blood per bird. After collection, the samples remained horizontal for 15 min and then were centrifuged (Kasvi brand, K14-4000, Paraná, Brazil) at 2500 rpm (1,050 × *g*) for 10 min at room temperature (20–25 °C). After serum separation, they were identified and packed in 2 ml microtubes (CRAL, São Paulo, Brazil) and stored in a freezer at -20 ºC until the analysis was performed (Nunes *et al*., 2018). To perform the analyses, the samples were thawed under refrigeration (4 ºC), remaining in the refrigerator for 24 h. Before performing the analyses, the samples were centrifuged in a microcentrifuge (Eppendorf® brand, Minispin®, Hamburg, Germany) at 1,050× *g* for 10 min at room temperature to remove the possible presence of fibrin. The measurements of the biochemical parameters were performed using an automatic biochemical analyser with spectrophotometry using the Flexor EL200 (Elitech® brand, Flexor EL200 model, Puteaux, France), using reagents, calibrators (Elical II) and calibration standards (Elitrol I) from Elitech®.

The parameters evaluated were: uric acid, performed based on the Trinder enzymatic colorimetric endpoint method (Trinder, 1969); glucose, using the Trinder enzymatic colorimetric kinetic method (Trinder, 1969); cholesterol using the Trinder enzymatic colorimetric endpoint method (Allain *et al*., 1974); triglycerides using the enzymatic colorimetric endpoint method (Fossati and Prencipe, 1982); total protein, using the biuret endpoint method (Rifai *et al*., 2018); albumin using bromocresol green (BCG) colorimetric method (Doumas and Biggs, 1972; Wu, 2006); and creatinine using the Jaffe colorimetric kinetic method (Rifai *et al*., 2018). The determinations of the enzymatic activities for aspartate aminotransferase (AST) using the IFCC method without pyridoxal phosphate were performed using a kinetic assay and were measured with an ultraviolet spectrophotometer to monitor the absorption changes during the reaction (Schuman *et al*., 2002a); alanine aminotransferase (ALT) activity was determined using the IFCC method without pyridoxal phosphate, using a kinetic assay and measured with an ultraviolet spectrophotometer (Schuman *et al*., 2002b); gamma glutamyltransferase (GGT) using the glupa C substrate method, kinetic (Schuman *et al*., 2002c); lactate dehydrogenase (LDH) using the IFCC method, kinetic (Schumann *et al*., 2002d); creatine phosphokinase (CPK) using the IFCC method, kinetic (Schumann *et al*., 2002e).

At 28 d of age, one bird per experimental unit was randomly selected and euthanized for intestinal morphometry analysis. Crypt depth, villus height, absorptive area, and the ratio of villus height to crypt depth were evaluated. The small intestine was exposed, and both the duodenum and the jejunum were separated for sampling. In the duodenum, the segment considered ranged from the pylorus to the distal portion of the intestinal loop, whereas in the jejunum, it extended from the distal portion of the duodenal loop to the Meckel's diverticulum. A 2-cm fragment was collected from the duodenum in the ascending portion of the duodenal loop, and a 2-cm fragment was collected from the jejunum, 5 cm before the Meckel's diverticulum.

This fragment was fixed in 10% buffered formalin solution, dehydrated in increasing ethanol concentrations, and embedded in paraffin. Subsequently, semi-serial sections with a thickness of 5 µm were made from each segment, mounted on glass slides, and stained using the haematoxylin-eosin technique as described by Luna (1968). Following slide preparation, the length and width of 10 villi, as well as the depth and width of 10 crypts, were analysed using the PROPLUS IMAGE 5.0 imaging system. These morphometric measurements were used to calculate the absorptive surface area of the intestinal mucosa, using the formula proposed by Kisielinski *et al*. (2002). Additionally, the ratio of villus height to crypt depth was calculated by dividing the value of villus height by the value of crypt depth.

At 28 d of age, macroscopic lesions of *Clostridium perfringens* and *Eimeria* spp. were evaluated in broilers that were sacrificed to perform the intestinal morphometry analysis. Lesions caused by *Eimeria* spp. and *Clostridium perfringens* were assessed following the lesion scoring system established by Johnson & Reid (1970): Grade 0 - no lesions, Grade 1 - mild lesions, Grade 2 - moderate lesions, Grade 3 - severe lesions, and Grade 4 - very severe lesions.

One broiler per experimental unit was slaughtered at 28 d of age for collection of the cecal contents according to the methodology of Souza *et al*. (2022). The cecal contents were removed and 200 mg were weighed and transferred to 2-ml individually marked microtubes (CRAL, Cotia, São Paulo, Brazil). A solution of 1800 µl of 1% (w/v) NaOH was added, which was vigorously homogenized in a multifunctional vortex (Kasvi Brand, K40-1010, São José dos Pinhais, Paraná, Brazil) for 2 min at 3,000 rpm.

After homogenization, the microtubes were centrifuged (Kasvi Brand, K14-4000, Paraná, Brazil) at 1,050 × *g* for 5 min for complete sedimentation of the solid fraction of the sample. A total volume of 900 µl of the supernatant was transferred (Single-channel Micropipette Plus 100–1000 µl, Kasvi, K1 - P1000, Paraná, Brazil) to new microtubes (CRAL, Cotia, São Paulo, Brazil) and was acidified with 50 µl (Single-channel Micropipette Plus 10–100 µl, Kasvi, K1 - P100, Paraná, Brazil) of 50% (*w/v*) ortho-phosphoric acid solution. The acidified samples were homogenized in a vortex (Kasvi Brand, K40-1010, São José dos Pinhais, Paraná, Brazil) for 30 s at 3,000 rpm and stored in a freezer at -20 ºC until the readings were taken.

The concentrations of acetic, propanoic, butyric, valeric, and isovaleric acids in the samples were determined using gas chromatography using a Shimadzu® GC-2010 Plus chromatograph equipped with AOC-20i automatic injector, Stabilwax-DA™ capillary column (30m, 0.25mm DI, 0.25µm df, Restek®), and flame ionization detector (FID), after acidification with 1 M o-phosphoric acid p.a. (Ref. 100573, Merck®) and fortification with a mixture of free volatile acids (Ref. 46975, Supelco®). An aliquot of 1 µL of each sample was injected with a split ratio of 40:1, using helium as carrier gas with a linear velocity of 42 cm s^{-1}, obtaining the separation of the analytes in a chromatographic run of 11.5 min.

The injector and detector temperatures were 250 ºC and 300 ºC, respectively, and the initial column temperature was 40 ºC. The temperature ramp of the column started with a gradient from 40 to 120 °C at a rate of 40 °C min^{-1}, followed by a gradient from 120 to 180 °C at a rate of 10 °C min^{-1}, and from 180 to 240 °C at a rate of 120 °C min^{-1}, keeping the temperature at 240 °C for another 3 min at the end. For the quantification of the analytes, a calibration method was performed with dilutions of the standard WSFA^{-2} (Ref. 47056, Supelco®) and glacial acetic acid (Ref. 33209, Sigma-Aldrich®) analysed under the conditions described above. Peak determination and integration were performed using GCsolution v. 2.42.00 software (Shimadzu®). The results were expressed in mmol kg^{-1}.

Upon completion of the experiment, normality was assessed, followed by an analysis of variance. In cases of significant effects, the Tukey test was used with a 5% probability for comparing means. For data that did not meet the normal distribution criteria, a non-parametric Kruskal–Wallis analysis was conducted, and if a significant effect was observed (P <0.05), treatments were compared using Dunn's test at 5%. These analyses were conducted using SAS software (SAS, 2014).

Results and Discussion

Broilers fed the treatments BA (*Bacillus amyloliquefaciens* - CECT 5940), BS (*Bacillus subtilis* - DSM 32315) and PC (enramycin antibiotic) improved (P = 0.001) weight gain and feed conversion ratio in 28-d (Table 2) and 42-d (Table 3) broilers, compared to broilers fed the NC diet.

Table 2. Growth performance of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and antibiotic, enramycin 8%

Treatments[1]	Feed intake (g)	Body weight gain (g)	Feed conversion ratio (g g^{-1})	Viability (%)
PC	2290[b]	1470[b]	1.558[b]	95.87[a]
NC	983[c]	626[c]	1.792[a]	76.26[b]
BA	2353[a]	1514[a]	1.544[b]	92.05[a]
BS	2317[ab]	1476[b]	1.553[b]	96.59[a]
SEM[2]	44.63	34.95	0.04	2.12
P value[3]	0.001	0.001	0.001	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%

Table 3. Growth performance of 42-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and antibiotic, enramycin 8%

Treatments[1]	Feed intake (g)	Body weight gain (g)	Feed conversion ratio (g g^{-1})	Viability (%)
PC	4631[b]	2875[b]	1.611[b]	94.09[a]
NC	2602[c]	1731[c]	1.825[a]	75.76[b]
BA	4770[a]	2972[a]	1.580[b]	91.82[a]
BS	4736[a]	2951[a]	1.585[b]	93.39[a]
SEM[2]	74.82	59.82	0.04	4.54
P value[3]	0.001	0.001	0.001	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%

The lower feed conversion ratio indicated that a reduced amount of feed was required to reach 1 kg of live weight (de Oliveira *et al*., 2019). These results highlight a greater efficiency of broiler chickens in utilizing nutrients for muscle growth, contributing to an improvement in carcass yield in the groups of birds that received probiotics, which showed similar results to the PC group (enramycin). The increase in weight gain of broiler chickens fed with the probiotics BA (*Bacillus amyloliquefaciens* - CECT 5940) and BS (*Bacillus subtilis* - DSM 32315) can be attributed to the ability of these bacterial strains to promote better digestion and absorption of nutrients in the gastrointestinal tract of the birds (Ma *et al*., 2018; Ningsih *et al*., 2024). Additionally, these strains may have contributed to a greater balance of the intestinal microbiota, favouring beneficial bacteria, and inhibiting the growth of pathogens (Menconi *et al*., 2020; Sun *et al*., 2022), which reduces competition for nutrients, decreases gastrointestinal disorders, and increases the efficiency of feed conversion into body mass. These factors result in faster and healthier weight gain of broiler chickens during the growth period (Wang *et al*., 2023; Rodrigues *et al*., 2024).

Other studies have also observed favourable outcomes in performance when evaluating the use of probiotics based on *Bacillus subtilis* - DSM 32315 and *Bacillus amyloliquefaciens* - CECT 5940 in the diets of broilers challenged with necrotic enteritis. These studies suggest improved nutrient absorption, enhanced energy utilization by the birds, and greater antioxidant capacity, humoral immunity, and increased activity of endogenous gastrointestinal enzymes (Hernandez-Patlan *et al*.,

2019; Whelan *et al*., 2019; de Oliveira *et al*., 2019; Menconi *et al*., 2020; Gharib-Naseri *et al*., 2021; Sun *et al*., 2022; Larsberg *et al*., 2023).

Broilers fed with the PC, BA, and BS treatments exhibited higher carcass yields (P = 0.001), fillet yield (P = 0.001), yield of sasami (P = 0.001), and relative weight of abdominal fat (P = 0.001) in comparison to chickens fed with the NC diet. However, the birds fed with NC demonstrated higher breast fillet yield (P = 0.001), wing yield (P = 0.016), and relative liver weight (P = 0.001) when compared to the other treatments (Table 4).

Table 4. Carcass and cut yield, abdominal fat percentage, and relative liver weight of 42-day-old broilers challenged with *Eimeria* and *Clostridium perfringens* vaccine and fed diets supplemented with probiotics *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and the antibiotic, enramycin 8%

Treatments[1]	CY[4]	FY[5]	BFY[6]	WY[7]	YS[8]	RWAF[9]	RLW[10]
PC	69.57[a]	27.39[a]	31.94[b]	9.57[b]	5.55[a]	1.54[a]	1.87[b]
NC	65.11[b]	23.35[b]	34.66[a]	10.37[a]	4.44[b]	1.10[b]	2.35[a]
BA	70.18[a]	27.84[a]	32.34[b]	9.39[b]	5.42[a]	1.62[a]	1.87[b]
BS	69.67[a]	27.57[a]	32.28[b]	9.53[b]	5.65[a]	1.59[a]	1.90[b]
SEM[2]	1.59	1.56	0.99	0.66	0.36	0.28	0.17
P value[3]	0.001	0.001	0.001	0.016	0.001	0.001	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%.
CY[4]: Carcass yield (%); FY[5]: Fillet yield; BFY[6]: Breast fillet yield; WY[7]: Wing yield; YS[8]: Yield of sasami; RWAF[9]: Relative weight of abdominal fat; RLW[10]: Relative liver weight

The relative liver weight was lower in the poultry supplemented with probiotics, suggesting that there was a reduction in inflammation in the organ caused by necrotic enteritis, improving the resistance of birds to the disease. Corroborating with this study, Ramlucken *et al*. (2020) when evaluating the effect of a multi-strain *bacillus (*CPB 011, CPB 029, HP 1.6, and D014) probiotic on broilers challenged with *Clostridium perfringens*, observed that treatment without the probiotic promoted an increase in relative liver weight, suggesting that subclinical *Clostridium perfringens* infection caused inflammation of the liver.

According to Immerseel *et al*. (2004), it is possible that a high number of *Clostridium perfringens* bacteria in the small intestine of poultry pass into the bile ducts and through the portal circulation to reach the liver. Therefore, the current study suggests that supplementation with *B. subtilis* DSM 32315 and *Bacillus amyloliquefaciens* CECT 5940 in poultry diet may have a positive impact on reducing liver inflammation caused by necrotic enteritis, particularly following the challenge. This is likely achieved through the regulation of the intestinal microbiome and prevention of pathogen dissemination to other organs (Ma *et al*., 2018; Larsberg *et al*., 2023). This approach contributes to promoting overall health and disease resistance in the birds (Zhang *et al*., 2023; Zhu La *et al*., 2024).

The different responses of breast fillet, wing yield, and relative weight of abdominal fat may be associated with the metabolic changes caused by using the probiotics and the antibiotic, providing changes in the poultry caecal contents and amino acid metabolism, and reducing protein deposition in the muscle (Cao *et al*. 2018). The higher fat position in broilers supplemented with probiotics may be related to increased feed intake and weight gain (Abdel-Raheem and Abd-Allah *et al*., 2011; Wang *et al*., 2021; Ningsih *et al*., 2023).

The probiotic (BA and BS) did not affect any of the blood parameters (P >0.05), such as albumin, cholesterol, creatinine, glucose, total protein, triglycerides, and uric acid (Table 5). Probiotic (BA and BS), PC, and NC did not affect (P >0.05) alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, gamma glutamyltransferase, and creatinine phosphokinase content (Table 6).

Table 5. Biochemical parameters of the blood of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%.

Treatments[1]	GLU[4] (mg/dL)	CHOL[5] (mg/dL)	TRIG[6] (mg/dL)	ALB[7] (g/L)	TP[8] (g/L)	UA[9] (mg/dL)	CRE[10] (mg/dL)
PC	280.26	167.94	145.85	13.56	29.11	3.96	0.21
NC	271.62	153.67	156.75	12.47	28.31	4.05	0.21
BA	282.06	165.94	147.05	13.51	30.15	4.39	0.22
BS	275.89	163.46	152.82	13.63	30.03	3.78	0.22
SEM[2]	10.98	15.26	31.19	1.22	2.30	0.82	0.03
P value[3]	0.200	0.206	0.846	0.144	0.250	0.356	0.920

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
GLU[4]: glucose (mg/dL); CHOL[5]: cholesterol (mg/dL); TRI[6]: triglycerides (mg/dL); ALB[7]: albumin (g/L); TP[8]: total proteins (g/L); UA[9]: uric acid (mg/dL); CRE[10]: creatinine (mg/dL)

Table 6. Enzyme profile (IU/L) of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%

Treatments[1]	ALT[4]	AST[5]	LDH[6]	GGT[7]	CPK[8]
PC	6.63	305.22	1135.44	21.58	5802.22
NC	6.87	253.61	925.89	21.18	5338.89
BA	7.45	306.75	969.36	23.57	3602.50
BS	6.36	277.53	959.25	21.88	4270.00
SEM[2]	1.69	77.67	268.71	3.91	3165.09
P value[3]	0.473	0.379	0.353	0.509	0.390

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
ALT[4]: Alanine aminotrasferase; AST[5]: Aspartate aminotrasferase; LDH[6]: Lactate dehydrogenase; GGT[7]: Gamma glutamyltrasferase; CPK[8]: Creatine phosphokinase

In the analysis of duodenal intestinal morphometry in 28-day-old chickens (Table 7), the PC, NC, and diets containing probiotics (BA and BS) showed no effect (P >0.05) on crypt depth and the ratio between villus height and crypt depth. Villus height was altered in birds that received diets with probiotics (BA and BS) and with the antibiotic (PC) (P = 0.001). Additionally, it was observed that crypt width was greater in birds fed BA (P = 0.001), whereas villus width was greater in birds that received the probiotic, BS (P = 0.001).

Table 7. Intestinal morphometry of the duodenum (µm) of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%

Treatments[1]	VH[4]	VW[5]	CD[6]	CW[7]	VH:CD[8]	AA[9]
PC	1898[a]	208[b]	201	80b[c]	9.84	19.86[a]
NC	1508[b]	201[b]	162	87[b]	9.77	15.14[c]
BA	1866[a]	229[b]	193	99[a]	9.92	16.44[bc]
BS	1857[a]	261[a]	209	74[c]	8.98	17.68[b]
SEM[2]	123.69	31.42	38.78	11.02	1.80	1.97
P value[3]	0.001	0.001	0.068	0.001	0.640	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%
VH[4]: villus height; VW[5]: villus width; CD[6]: Crypt depth; CW[7]: Crypt width; VH:CD[8]: Ratio of villus height to crypt dept; AA[9]: absorption area

In the analysis of jejunal intestinal morphometry in 28-day-old chickens (Table 8), the probiotic (BA and BS) and PC diets influenced villus height (P = 0.001), villus height to crypt depth ratio (P = 0.040), and absorption area (P = 0.001). Probiotic (BA) provided greater change in villus width (P = 0.003), crypt depth (P = 0.001), and crypt width (P = 0.023) when compared to probiotic BS and PC diets.

Table 8. Intestinal morphometry of the jejunum (µm) of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%

Treatments[1]	VH[4]	VW[5]	CD[6]	CW[7]	V:C[8]	AA[9]
PC	950[a]	211[b]	124[b]	87[a]	7.84[a]	9.50[a]
NC	615[b]	187[b]	95[c]	88[a]	6.3[b]	6.60[b]
BA	1052[a]	239[a]	182[a]	94[a]	6.74[ab]	9.54[a]
BS	916[a]	198[b]	132[b]	71[b]	7.03[ab]	10.55[a]
SEM[2]	121.95	29.08	23.04	12.22	1.16	1.17
P value[3]	0.001	0.003	0.001	0.023	0.040	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%
VH: villus height; VW: villus width; CD: Crypt depth; CW: Crypt width; V:C: Ratio between villus height and Crypt depth; AA: Absorption area

The intestinal morphometry of the duodenum and jejunum in 28-day-old birds suggests that probiotics derived from *Bacillus* hold promise as a substitute for antibiotics in broiler diets (Ningsih *et al*., 2023; Rodrigues *et al*., 2024). This substitution not only enhances performance but also reduces lesions caused by intestinal pathogens (Wang *et al*., 2023; Zhang *et al*., 2023). The observed changes in intestinal morphometry parameters suggest that probiotics can trigger structural modifications in the intestine, potentially boosting nutrient absorption, immune function, and overall intestinal health (Memon *et al*., 2021; Osho *et al*., 2023).

These findings deepen our comprehension of the mechanisms behind the beneficial impacts of probiotics in poultry farming and advocate for their adoption as antibiotic alternatives to bolster intestinal health and performance in broilers (Poudel *et al*., 2022; Rodrigues *et al*., 2024). This metabolic

improvement likely occurred through various mechanisms, including competition with pathogenic bacteria for nutrients and space, production of antimicrobial substances such as organic acids and bacteriocins, and modulation of the immune response in the intestine, ultimately resulting in improved intestinal health and performance (Xu *et al*., 2021; Zhu La *et al*., 2024).

Other studies corroborate these findings, reinforcing the potential of probiotics as an alternative to antibiotics in poultry production (Mazanko *et al*., 2022; Cirilo *et al*., 2023; Wang *et al*., 2023). They consistently demonstrate improvements in intestinal morphology, nutrient utilization, and immune responses in birds (Cirilo *et al*., 2023; Zhang *et al*., 2023). Additionally, they highlight a substantial reduction in the occurrence of intestinal diseases (Lee *et al*., 2023; Osho *et al*., 2023; Chen *et al*., 2024).

Lesion score in broilers fed the NC diet and infected at 28 d *with Eimeria* spp. increased (P = 0.001) to 1.30 (presence of streaks or petechiae on the mucosa) (Table 9). This condition was recovered with supplementation of the probiotics (BA and BS) and the antibiotic enramycin, substantially improving the gut lesion score (score 0). For the treatments with feed additives, no differences were detected between enramycin and the probiotics.

Table 9. Mean intestinal lesion scores of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%

Treatments[1]	*Clostridium perfringens*	*Eimeria* spp.
PC	0.20	0.20[b]
NC	0.10	1.30[a]
BA	0.00	0.00[b]
BS	0.00	0.00[b]
P value[2]	0.276	0.001

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)

P value[2]: probability of significance

[a,b,c]: Means followed by different letters in the column differed by the Dunn test at the 5% level

The reduction in intestinal lesions was possibly related to the decrease in coccidiosis oocysts in the presence of probiotics (BA and BS), reducing damage to the epithelium and the leakage of nutrients into the lumen. Typically, enteric infections are caused by pathogens that dominate the mucosal surface in the animal's intestine, disrupting the balance of the microbiota (Abd El-Hack *et al*., 2022; Larsberg *et al*., 2023).

The use of probiotics (BA and BS) enabled the exclusion of pathogens through competition and reduced the severity of intestinal lesions on the mucosa, as reflected in the intestinal morphometry of the duodenum and jejunum, where greater villus height and absorption area were observed compared to the negative control, maintaining the integrity and functionality of the epithelial barrier (Liao *et al*., 2020). These findings emphasise the efficacy of probiotics in promoting a healthier and infection-resistant intestinal environment, resulting in substantial benefits for the health and performance of poultry (Cirilo *et al*., 2023; Rodrigues *et al*., 2024).

Other studies support the findings of the present study, emphasising the importance of probiotics, particularly *Bacillus* strains, in playing a crucial role in reducing intestinal damage and enhancing the overall health and performance of poultry (Wang *et al*., 2023; Zhang *et al*., 2023; Ningsih *et al*., 2024). In the study conducted by Farhat-Khemakhem *et al*. (2018), it was observed that the *Bacillus amyloliquefaciens* US573 strain exhibited high adherence to the intestinal mucosa and the capability to form biofilms. These characteristics provide protection against pathogens, possibly forming a barrier that prevents the infective form *of Eimeria* spp. from invading enterocytes and causing damage to the intestinal mucosa. Additionally, Wang *et al*. (2021) investigated the benefits of *Bacillus subtilis* in minimizing the negative effects of necrotic enteritis in broiler chickens, noting a reduction in intestinal lesions and an increase in the ratio between villus height and crypt depth. The probiotic also induced a marked decrease in the expression of the *Muc-2* gene, potentially reducing mucus secretion and, consequently, diminishing the availability of nutrients for *Clostridium perfringens* and its adhesion to the mucosa, providing a preventive effect against necrotic enteritis.

Broilers supplemented with the diet containing the probiotic, BA (Table 10) exhibited a higher concentration of butyric acid in the cecum (P = 0.004). There was no difference observed between the treatments for the other tested short-chain fatty acids (P >0.05).

Table 10. Short chain fatty acids (mmol kg^{-1}) of 28-day-old broilers challenged with vaccine *Eimeria* and *Clostridium perfringens* and fed diets supplemented with probiotics from *Bacillus amyloliquefaciens*, *Bacillus subtilis,* and the antibiotic, enramycin 8%

Treatments[1]	AA	PA	IBA	BA	IVA	VA
PC	29.219	2.702	0.049	3.882[b]	0.069	0.255
NC	34.298	2.610	0.052	4.267[b]	0.192	0.393
BA	31.348	2.302	0.227	6.918[a]	0.247	0.400
BS	32.348	2.565	0.085	4.562[b]	0.125	0.316
SEM[2]	8.41	0.72	0.11	1.51	0.15	0.16
P value[3]	0.814	0.894	0.064	0.004	0.297	0.439

Treatments[1]: PC: positive control with 8% enramycin (Enramax®); NC: negative control (no feed additive included); BA: *Bacillus amyloliquefaciens* - CECT 5940 (Ecobiol®); BS: *Bacillus subtilis* - DSM 32315 (GutCare®)
SEM[2]: The standard error of the mean
P value[3] (0.05%)
[a,b,c]: Means followed by different letters are statistically different according to Tukey's test at 5%
AA: acetic acid; PA: propanoic acid; IBA: isobutyric acid; BA: butyric acid; IVA: isovaleric acid; VA: valeric acid

The BA probiotic modulates the intestinal microbiota, favouring the proliferation of beneficial bacteria and increasing butyric acid production, known for its positive effects on intestinal health, including the suppression of inflammatory diseases and improvement of immune function (Ayalew *et al*. 2022; Kouhounde *et al*., 2022). Previous studies highlight the crucial role of probiotics containing *Bacillus* strains in regulating the intestinal microbiota, promoting an environment conducive to the growth and development of birds (Zhang *et al*., 2018; Liao *et al*., 2020; Xu *et al*., 2021; Mátis *et al*., 2022).

The presence of *Bacillus amyloliquefaciens* in the BA probiotic may favour the colonization of *Lactobacilli* and *Bifidobacteria*, which in turn contribute to butyric acid production (Qaisrani *et al*., 2015). These findings are consistent with the observation of a reduction in intestinal lesions and an improvement in intestinal morphology in birds supplemented with the BA probiotic (Ma *et al*., 2019; Hong *et al*., 2019; Whelan *et al*., 2019; Cirilo *et al*., 2023). Furthermore, the increased presence of butyric acid may confer resistance to intestinal pathogens, such as *Clostridium perfringens* and *Eimeria* spp., contributing to the prevention of gastrointestinal diseases in broiler chickens (Timbermont *et al*., 2011; Li *et al*., 2018; Bao *et al*., 2020; Sun *et al*., 2022; Larsberg *et al*., 2023).

Therefore, the results of this study indicate that probiotics BA (*Bacillus amyloliquefaciens* - CECT 5940) and BS (*Bacillus subtilis* - DSM 32315) outperform the NC diet (without probiotics or antibiotics) and yield comparable results to enramycin (PC). This superiority of probiotics over the NC diet suggests that these bacterial strains play a crucial role in optimizing bird performance (Cai *et al*., 2023; Wang *et al*., 2023; Rodrigues *et al*., 2024). Probiotics enhance nutrient digestion and absorption, as demonstrated by the studies of Ma *et al*. (2018) and Ningsih *et al*. (2024), while balancing the intestinal microbiota by promoting beneficial bacteria and inhibiting pathogens, as evidenced by Menconi *et al*. (2020) and Sun *et al*. (2022). The similarity between probiotic results and the PC diet (enramycin) suggests that probiotics can effectively replace antibiotics as growth promoters, reducing the risk of antimicrobial resistance and ensuring bird performance and intestinal health (Osho *et al*., 2023; Chen *et al*., 2024; Zhu La *et al*., 2024).

Conclusion

The use of Ecobiol® (*Bacillus amyloliquefaciens* - CECT 5940) at a rate of 1000 g ton^{-1} and GutCare® (*Bacillus subtilis* - DSM 32315) at a rate of 500 g ton^{-1} in the diets of broiler chickens has proven to be an effective strategy to replace the antibiotic, Enramax® (8% enramycin). This administration resulted in performance and carcass yields similar to those obtained with the use of

Enramax®. Moreover, these *Bacillus-based* probiotics promoted improvements in the intestinal health of broiler chickens, without affecting serum metabolites.

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Author contributions
Rafaela Berto, Nilton Rohloff Junior, Cristine Kaufmann, Heloisa Sartor, Ana Paula Guimarães Cruz Costa, Gabriel Natã Comin: investigation, methodology, data curation, formal analysis, software, and project administration; Vinicius de Queiroz Teixeira, Victor Daniel Naranjo, Cinthia Eyng, Ricardo Vianna Nunes: conceptualization, methodology, and project administration, supervision, validation, and visualization; Thiago dos Santos Andrade: writing - review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest
The authors declare no competing interests.

Ethics approval
The protocol of this research was in accordance with the Brazilian Normative Act No. 37, from February 15, 2018, by the national animal experimentation control board.

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