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Use of alternative additives for broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Uso de aditivos alternativos para frangos de corte desafiados com *Eimeria* vacinal e *Clostridium perfringens*

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Highlights

Replacement of antibiotics to avoid microbial resistance.
Plant-based products and prebiotics as alternatives to antibiotics for poultry.
Improve intestinal health and performance with the use of prebiotic.

Abstract

The objective of this study was to evaluate the use of alternative additives in diets for broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens.* A total of 600 broiler chicks were allocated to five treatments in a completely randomized design with six replicates and 20 birds per experimental unit. The treatments were as follows: negative control - basal diet without a growth promoter; positive control - basal diet with the inclusion of 50 g t^{-1} of 20% avilamycin; diet A - basal diet plus the inclusion of 100 g t^{-1} of a product based on *Macleaya cordata* extract; diet B - basal diet plus the inclusion of 1000 g t^{-1} of a product based on sweet chestnut (*Castanea sativa*) and red quebracho (*Schinopsis lorentzii*); and diet C - basal diet plus the inclusion of 100 g t^{-1} of a product based on prebiotics from Pichia yeast, glutamine, and aluminosilicate. All birds were individually challenged at four days of age with 0.6 mL of the vaccine for *Eimeria* spp., and at seven and ten days of age with 0.5 mL of *Clostridium perfringens.* The evaluated parameters included performance, intestinal health, blood parameters, litter

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quality, and carcass and cut yields. The use of prebiotics (product C) improved weight gain (P<0.05) in the periods from one to 21 days and one to 42 days of age. There was a statistical difference (P<0.05) in the intestinal health index, with the use of product C leading to a lower total lesion score compared to other treatments at 28 days of age. The negative control treatment demonstrated lower intestinal permeability compared to the other treatments (P<0.05). Regarding serum parameters at 14 days of age, uric acid concentrations were higher (P<0.05) in the birds in the negative control group compared to those in the positive control group. The concentration of lactate dehydrogenase was higher (P<0.05) in birds fed products B and C compared to those fed the positive and negative control treatments. Moreover, the concentration of total proteins was higher (P<0.05) in the blood of birds fed product C compared to product A. Cholesterol concentrations at 42 days were lowest (P<0.05) in the treatment with product B. Uric acid concentrations were highest (P>0.05) in the birds receiving additive B and lowest in the group of birds fed product A. Products B and C may serve as substitutes for antibiotics in birds challenged with *Eimeria* vaccine and *Clostridium perfringens.*
Key words: Necrotic enteritis. Yeasts. Intestinal permeability.

Resumo

O objetivo do trabalho foi avaliar o uso de aditivos alternativos em dietas para frangos de corte desafiados com Eimeria vacinal e *Clostridium perfringens.* Ao todo 600 pintos de corte, distribuídos em delineamento inteiramente casualizado, com cinco tratamentos, seis repetições e 20 aves por unidade experimental. Os tratamentos foram constituídos por: controle negativo, ração basal sem promotor de crescimento; controle positivo, ração basal com inclusão de 50 g ton^{-1} de avilamicina 20%, ração A, ração basal mais inclusão de 100 g ton^{-1} de um produto a base de extrato de *Macleaya cordata,* ração B, ração basal mais inclusão de 1000 g ton^{-1} de um produto a base de castanha vermelha (*Castanea sativa*) e quebracho colorado (*Schinopsis lorentzii*) e ração C, ração basal mais inclusão de 100 g ton^{-1} de um produto a base de prebióticos de levedura de pichia, glutamina e aluminosilicato. Todas as aves foram desafiadas individualmente aos quatro dias de idade com 0,6 ml da vacina para Eimeira spp., e aos sete e 10 dias de idade com 0,5 ml de *Clostridium perfringens.* Foram avaliados os parâmetros de desempenho, saúde intestinal, parâmetros sanguíneos, qualidade de cama e rendimento de carcaça e cortes. O uso de prebióticos (produto C) melhorou o ganho de peso (P<0,05) no período de um a 21 e um a 42 dias de idade. Houve diferença estatística (P<0,05) para o índice de saúde intestinal, em que o uso do produto C proporcionou menor escore total de lesões quando comparado aos demais tratamentos aos 28 dias de idade das aves. O tratamento controle negativo apresentou menor permeabilidade intestinal em relação aos outros tratamentos (P<0,05). Quanto aos parâmetros séricos aos 14 dias de idade, as concentrações de ácido úrico foram maiores (P<0,05) nas aves do grupo controle negativo comparado as do grupo controle positivo. A concentração da enzima lactato desidrogenase foi maior (P<0,05) para as aves alimentadas com o produto B e C em comparação as alimentadas com os tratamentos controle positivo e negativo. Além disso, a concentração das proteínas totais foi maior (P<0,05) no sangue das aves alimentadas com produto C em comparação ao produto A. As concentrações de colesterol aos 42 dias foram menores (P<0,05) para o tratamento com produto B quando comparadas as aves dos demais tratamentos. As concentrações de ácido úrico foram maiores (P>0,05) nas aves do tratamento recebendo aditivo B e menores no grupo de aves alimentadas com o produto A. O uso do produto B e

C, podem atuar como substituto ao antibiótico em aves desafiadas com *Eimeria* vacinal e *Clostridium perfringens*.

Palavras-chave: Enterite necrótica. Leveduras. Permeabilidade intestinal.

Introduction

With the intensification of broiler production, concerns regarding biological issues potentially harmful to the poultry sector are increasing (Soutter et al., 2020; Attree et al., 2021). Protozoa from the genus *Eimeria* spp. and bacteria from the genus *Clostridium perfringens* are among the most detrimental microorganisms in poultry farming, causing remarkable production losses and high mortality (Ariza et al., 2018; Leite et al., 2021).

The proliferation of *Eimeria* spp. protozoa in the intestinal epithelium of birds leads to tissue damage, disrupts digestive processes, reduces performance, and facilitates lesions caused by *Clostridium perfringens* bacteria (Muro et al., 2015).

These health issues are most commonly addressed through the use of antibiotics (Diaz et al., 2018; Genova et al., 2020). They are added to commercial poultry feeds to enhance performance, aiming to maintain and ensure the health of the gastrointestinal tract and maximize performance (Reis & Vieites, 2019). However, the use of antibiotics is increasingly scrutinized and restricted due to consumer demands for products free of chemical residues and concerns over bacterial resistance (Zhai et al., 2018).

In response to these challenges, there is a growing interest in alternatives that can enhance bird performance without leaving residues in the meat and ensure consumer food safety. Options include probiotics, prebiotics, synbiotics, and phytogenic additives, among others (Leite et al., 2021; Almeida et al., 2019).

Phytogenic additives, derived from plant or herbal extracts, possess antimicrobial, antioxidant, and immunomodulatory properties. They can inhibit the growth of pathogenic microorganisms in the intestines of birds, thereby improving intestinal health and the digestibility of ingredients (Fernandes et al., 2017).

Prebiotics are indigestible additives that beneficial intestinal bacteria can utilize. These compounds positively affect the host by promoting the selective growth of one or more beneficial strains, thereby contributing to the intestinal health of birds (Reis & Vieites, 2019).

Given this background, the objective of this study was to evaluate the impact of incorporating various commercial products derived from herbal extracts and prebiotics on performance, intestinal health, blood parameters, litter quality, and carcass and cut yields in broilers challenged with *Eimeria* vaccine and *Clostridium perfringens*.

Materials and Methods

The experiment was conducted at the Poultry Research Center, situated at the Professor Antônio Carlos dos Santos

Pessoa Experimental Station, which is part of the State University of Western Paraná (UNIOESTE) at the Marechal Cândido Rondon Campus/Paraná.

All handling procedures with the birds followed Normative Resolution No. 27, dated October 23, 2015, issued by the National Council for the Control of Animal Experimentation (CONCEA). Furthermore, the experimental procedures received approval from the Ethics Committee for the Use of Animals of the University, under approval number 12/2022.

The birds were housed in 1.76-m^2 enclosures with concrete floors covered by fifth-use pine shavings. Each enclosure was equipped with individual trough feeders and nipple drinkers. The temperature and humidity were monitored to maintain thermal comfort according to the strain-specific recommendations for each age group. This was achieved using brooders with 250 W electric resistance, two exhaust fans, and evaporative cooling plates.

Six hundred male one-day-old Cobb 500® broiler chicks, with an average initial weight of 43.88±0.48 g, were allocated in a completely randomized design. The setup included five treatments, six replicates, and 20 birds per experimental unit.

Treatments were as follows:

Negative control (NC) - Basal diet.

Positive control (PC) - Basal diet with the inclusion of 50 g t^{-1} of 20% avilamycin.

A - Basal diet with inclusion of 100 g t^{-1} of product A (composed of quaternary benzophenanthridine alkaloids (QBA), protopine alkaloids (PA), sodium chloride, *Macleaya cordata* extract, and dried *Macleaya cordata*).

B - NC diet with inclusion of 1000 g t^{-1} of product B (composed of water-soluble and condensed polyphenols, simple sugars, lignin, cellulose, hemicellulose, and mineral salts, extracted from sweet chestnut [*Castanea sativa*] and red quebracho [*Schinopsis lorentzii*] wood).

C - Basal diet with inclusion of 1000 g t^{-1} of product C (composed of a mixture of pichia yeast, glutamine, and aluminosilicate).

The additives were included on top of the diet. The experimental diets (Table 1) are isoenergetic and isonutritive, formulated based on corn and soybean meal to meet the birds' nutritional needs as per Rostagno et al. (2017) for the pre-starter (1 to 10 days), starter (11 to 21 days), grower (22 to 35 days), and finisher (36 to 42 days) phases.

Table 1
Ingredients and nutritional composition of the basal diet for the pre-starter phase (1-10 days), starter phase (11-21 days), growth phase (22-35 days), and finisher phase (36-42 days)

Ingredients (kg)	Pre-starter	Starter	Growth	Finisher
Corn (7.88%)	572.90	634.25	639.50	696.81
Soybean meal (46%)	367.04	311.29	299.45	246.84
Meat and bone meal	30.97	19.71	15.38	15.00
Soybean oil	5.00	10.02	24.77	20.77
Limestone	7.39	7.21	7.10	7.15
Salt	3.58	3.75	3.48	3.61
Sodium bicarbonate	1.00	1.00	1.50	2.00
Lysine sulfate (60%)	2.58	3.39	2.32	2.76
DL-Methionine (99%)	3.39	1.00	2.84	2.18
L-Threonine (99%)	0.98	0.95	0.34	0.35
L-Valine (98%)	0.21	0.45	-	0.17
Choline chloride (60%)	0.40	0.40	0.40	0.30
[1]Vitamin premix	1.30	1.00	1.00	0.80
[2]Mineral premix	0.50	0.50	0.50	0.50
[3]Adsorbent	1.00	1.00	-	-
[4]Coccidiostat	0.55	0.55	0.20	-
[5]Antioxidant	0.12	0.12	0.12	0.12
[6]Phytase	0.10	0.10	0.10	0.10
[7]Inert (kaolin)	1.00	0.10	1.00	0.10
Total	1000	1000	1000	1000
Nutritional composition	**Pre-Starter**	**Starter**	**Growth**	**Finish**
Metabolizable energy (kcal kg^{-1})	2950	3050	3204	3180
Crude protein (g kg^{-1})	234.36	209.09	200.04	180.12
Dig. lysine (g kg^{-1})	12.60	11.60	10.00	9.60
Dig. methionine + cysteine (g kg^{-1})	9.40	8.80	8.00	7.10
Dig. threonine (g kg^{-1})	8.60	7.80	7.00	6.34
Dig. valine (g kg^{-1})	9.60	8.80	8.10	7.39
Dig. tryptophan (g kg^{-1})	2.54	2.24	2.17	1.89
Dig. arginine (g kg^{-1})	14.32	12.50	12.04	10.56
Dig. Isoleucine (g kg^{-1})	8.65	7.65	7.41	6.53
Calcium (g kg^{-1})	9.60	8.00	7.40	7.20
Available phosphorus (g kg^{-1})	4.80	4.00	3.70	3.60
Total phosphorus (g kg^{-1})	7.24	6.33	6.00	5.80
Sodium (g kg^{-1})	2.00	2.00	2.00	2.00
Potassium (g kg^{-1})	8.72	7.84	7.61	6.83
Chlorine (g kg^{-1})	3.02	3.09	2.90	2.72

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[1]Vitamin supplement, composition per kg of diet: 1 to 10 days of age: Vitamin A (min) 14.300 IU; Vitamin D_3 (min) 5.200 IU; Vitamin E (min) 71.50 IU; Vitamin K_3 (min) 3.90 mg; Vitamin B1 (min) 2.99 mg; Vitamin B_2 (min) 9.10 mg; Pantothenic acid (min) 15.60 mg; Vitamin B_6 (min) 5.20 mg; Vitamin B_{12} (min) 32.50 mg; Niacin (min) 78.00 mg; Folic acid (min) 2.60 mg; Biotin (min) 0.33 mg; Selenium (min) 0.39 mg. 11 to 35 days of age: Vitamin A (min) 11.000 IU; Vitamin D_3 (min) 4.000 IU; Vitamin E (min) 55 IU; Vitamin K_3 (min) 3.00 mg; Vitamin B_1 (min) 2.30 mg; Vitamin B_2 (min) 7.00 mg; Pantothenic acid (min) 12.00 mg; Vitamin B_6 (min) 4.00 mg; Vitamin B_{12} (min) 25.00 mg; Niacin (min) 60.00 mg; Folic acid (min) 2.00 mg; Biotin (min) 0.25 mg; Selenium (min) 0.30 mg. 36 to 42 days of age: Vitamin A (min) 8.800 IU; Vitamin D_3 (min) 3.200 IU; Vitamin E (min) 44.00 IU; Vitamin K3 (min) 2.40 mg; Vitamin B_1 (min) 1.84 mg; Vitamin B_2 (min) 5.60 mg; Pantothenic acid (min) 9.60 mg; Vitamin B_6 (min) 3.20 mg; Vitamin B_{12} (min) 20.00 mg; Niacin (min) 48.00 mg; Folic acid (min) 1.60 mg; Biotin (min) 0.20 mg; Selenium (min) 0.24 mg. [2]Mineral supplement, composition per kg of diet: Iron (min) 50 mg; Copper (min) 10 mg; Manganese (min) 65 mg; Zinc (min) 65 mg; Iodine (min) 1 mg. [3]Adsorbent based on bentonite. [4]Coccidiostat: From 1 to 21 days of age, salinomycin 12% is used. and from 22 to 36 days of age, salinomycin 24% is used. [5]Antioxidant based on butylated hydroxytoluene. [6]Phytase: Ronozyme hyphos 100g. 20.000 FYT g^{-1}. [7]Inert: Kaolin.

Experimental challenge

At four days old, all birds were challenged with 0.6 mL or 20 times the commercial dose of the Biococcivet R® vaccine, containing a concentrated suspension of sporulated oocysts from five species of *Eimeria* spp. (*E. acervulina, E. praecox, E. maxima, E. tenella,* and *E. mitis*). Additional challenges occurred at seven and 10 days of age; all birds received 0.5 mL of a culture inoculum containing 10^8 CFU mL^{-1} of *Clostridium perfringens* from a necrotic enteritis outbreak in broilers. All challenges were administered via gavage directly to the crop region.

Slaughter and collections

On the 14th day of the experiment, one bird per experimental unit was randomly selected and positioned in lateral decubitus for blood collection. Subsequently, this same bird was slaughtered by cervical dislocation, eviscerated, and the organs were exposed for collection of biological material. Samples of the cecum were collected for analysis of short-chain fatty acids, and approximately two cm sections of the jejunum were taken for analysis of the intestinal health index.

On the 28th day of the experiment, one bird per experimental unit was randomly selected, slaughtered by cervical dislocation, eviscerated, and the organs were prepared for collection of biological material. Samples of the cecum for analysis of short-chain fatty acids and approximately two cm sections of the jejunum for intestinal health index analysis were collected.

On the 35th day of the experiment, litter samples were collected from three different points for analysis of litter quality.

At 42 days of age, two broilers were randomly selected; one underwent blood collection before both were sacrificed and processed to evaluate carcass and cut yields.

Growth performance

Bird and feed weights were recorded at one, 21, and 42 days of age per experimental unit to calculate average feed intake (AFI), body weight gain (BWG), and feed conversion

(FC). Animal mortality was noted along with the weight of the remaining feed in the feeder for correction of AFC and consequently FC, following the methodology of Sakomura and Rostagno (2016).

Short-chain fatty acids

The cecum of each bird was removed, placed in a labeled plastic bag, and stored at -20 ºC for later analyses.

To extract short-chain fatty acids, approximately 200 mg of cecal content was collected in 2 mL microtubes (CRAL, Cotia, São Paulo, Brazil) and weighed on an analytical balance (Bel 0.0001g, M214AI, CapLab, São Paulo, Brazil). Then, 1800 µL of a 1% NaOH solution was added and the mixture was homogenized by vortexing to ensure complete dissociation of the cecal content. The samples were immediately centrifuged (Kasvi K14-4000 Centrifuge, Kasvi, São Paulo, BR) at 1000 G for five minutes to sediment the solid fraction completely. Afterward, 900 µL of the supernatant was transferred to a new 2-mL microtube, acidified with 50 µL of a 50% orthophosphoric acid solution, and homogenized again.

The concentrations of short-chain fatty acids were determined using a gas chromatograph (Shimadzu© GC-2010 Plus) equipped with an automatic injector (AOC-20i), a Stabilwax-DATM capillary column (30 m, 0.25 mm ID, 0.25 µm df, Restek©), and a flame ionization detector (FID). Before injection, the samples were acidified with 1 mL of analytical-grade orthophosphoric acid (Ref. 100573, Merck©) and fortified with a mixture of free volatile acids (Ref. 46975, Supelco©).

A 1-µL aliquot of each sample was injected with a split ratio of 40:1, using helium as the carrier gas at a linear velocity of 42 cm s-1. The separation of the analytes was achieved in a chromatographic run of 11.5 min. The injector and detector temperatures were set at 250 ºC and 300 ºC, respectively, with the initial column temperature at 40 ºC.

The temperature ramp of the column began with a gradient from 40 ºC to 120 ºC at a rate of 40 ºC min^{-1}, followed by a gradient from 120 ºC to 180 ºC at a rate of 10 ºC min^{-1}, and from 180 ºC to 240 ºC at a rate of 120ºC min^{-1}. The temperature was then held at 240 ºC for an additional three minutes. Quantification of the analytes was conducted using a calibration curve prepared with dilutions of the WSFA-2 standard (Ref. 47056, Supelco©) and glacial acetic acid (Ref. 33209, Sigma-Aldrich©) analyzed under the described conditions. Peak determination and integration were performed using GCsolution software v. 2.42.00 (Shimadzu©).

Intestinal health

Segments of the jejunum (2 cm) were collected, immersed in 10% formalin, and then processed to assess the intestinal health index using the I See Inside (ISI) methodology developed by Kraieski et al. (2017).

This methodology utilizes an impact factor (IF) to quantify each macroscopic and microscopic alteration. The IF ranges from 1 to 3, reflecting the severity or frequency of each lesion depending on the organ evaluated, as documented in the literature. Lesion frequency (S) is also assessed and scored from 0 (no lesion) to 3 (lesions affecting more than 50% of the tissue).

Evaluated lesions included the thickness of the lamina propria, epithelial thickness, enterocyte proliferation, infiltration of inflammatory cells into the epithelium, infiltration of inflammatory cells into the lamina propria, increased goblet cells, congestion, and presence of oocysts. To calculate the total ISI score, each alteration was multiplied by its respective score and included in the formula: Σ(IF*S), in which IF = impact factor and S = score.

At 18 days of age, one random bird per experimental unit was orally administered a dose of 8.32 mg kg^{-1} body weight of fluorescein isothiocyanate dextran (FITC-d) (MW 4,000; Sigma-Aldrich, Canada). One hour post-ingestion, blood was collected via brachial puncture and centrifuged (Kasvi K14-4000 Centrifuge, Kasvi, São Paulo, BR) at 1000 g for 15 min to separate the serum, which was then diluted (1:5) in a 9% saline solution. Serum from birds that did not receive FITC-d was used as a blank and to establish the standard curve.

FITC-d levels in the samples were measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Flex Station 3 Molecular Devices). The results were compared against the standard curve of known FITC-d concentrations. Intestinal permeability results were expressed in ng mL^{-1} (Baxter et al., 2019).

Blood biochemical parameters

At 14 and 42 days of age, following a 6-h fast, one bird per experimental unit was randomly selected for blood collection via brachial puncture. The birds were positioned in lateral decubitus, and blood was collected using 25x0.8 mm needles into 4-mL microtubes containing a clot activator. The tubes were left to stand horizontally for 15 min at room temperature.

The tubes were then centrifuged (Centrifuge Kasvi K14-4000, Kasvi, São Paulo, BR) at 1000 g for 10 min to separate the serum, which was subsequently transferred to 2-mL microtubes and stored at -20 ºC. Prior to analysis, the serum samples were thawed at 2 to 8 ºC and centrifuged in a microcentrifuge (Centrifuge 5425 R K14-4000, Eppendorf, São Paulo, Brazil) at 1000 g for five minutes to remove any potential hemolysis.

The evaluated blood parameters included cholesterol, uric acid, aspartate aminotransferase, glucose, lactate dehydrogenase, and total proteins. Measurements were conducted using an automatic biochemical analyzer (Flexor EL200, equipped with spectrophotometry) using reagents, calibrators, and standards from Elitech®.

Litter quality

At 35 days of age, litter samples were collected from five distinct points within each experimental unit, avoiding areas near feeders and drinkers. The samples were homogenized and individually packaged for subsequent analysis of dry matter, pH, and volatilized ammonia.

For dry matter evaluation, 200 g of each sample were weighed on a precision scale (Bel 0.0001g, M214AI, CapLab, São Paulo, Brazil), then dried in an oven at 80 ºC for 48 h. The samples were weighed again post-drying to determine dry matter content by weight difference.

The pH was assessed using 10 g of each sample diluted in 100 mL of deionized water. The mixture was homogenized on a shaker for five minutes, allowed to settle for 30 min, and then the pH was measured with a digital pH meter (Edge® Hi2002-02 Hanna).

Volatilized ammonia was determined by placing 100 g of each sample in a sealed plastic container with a 50-mL universal collector containing 10 mL of 2% boric acid to capture the volatilized ammonia. The container was kept at 25 °C for 24 h. Afterward, the collector was removed and the boric acid was titrated with 0.05N sulfuric acid (H_2SO_4). The amount of volatilized ammonia was calculated using the formula A = ((0.05×1.7×V) / SW) ×100, in which A represents volatilized ammonia (mg 100 g g^{-1} sample), V = volume of H_2SO_4 used in the titration (ml), and SW = sample weight.

Carcass and cut yield

At 42 days of age, two broilers per experimental unit were randomly selected, individually weighed (Cbr1046, Commerce Brasil, São Paulo, Brazil), euthanized, and processed. The birds were stunned using electronarcosis, followed by exsanguination through a ventral neck incision. The carcasses were scalded at 60 °C for 30 s and the feathers mechanically removed. Following the removal of feathers, viscera, feet, and neck, the carcasses were chilled in a static ice-water mixture for 60 min and then drained for 10 min. Next, the carcasses were reweighed (Marte AD 3300, TopLab, São Paulo, Brazil) to determine the carcass weight. Carcass yield was calculated by dividing the carcass weight by the live weight of the bird.

The processed carcass was then divided into breast fillets (*pectoralis major,* skinless and boneless), legs (drumstick and thigh), wings, and tender (*pectoralis minor*). The yield for each cut was determined by dividing the weight of each part by the weight of the cold eviscerated carcass. The relative weights of the liver and abdominal fat were calculated based on the live weight of the chicken.

Statistical analysis

The data were initially assessed for normality (Shapiro-Wilk) and homogeneity (Levene) using PROC UNIVARIATE; outliers were identified and removed. Data following a normal distribution were subjected to analysis of variance, and if a significant effect was observed, means were compared using the Student-Newman-Keuls test, utilizing the PROC GLM procedure.

For variables not exhibiting a normal distribution, the Kruskal-Wallis analysis was employed. If a significant effect was detected, means were compared using the Dunnett test via the PROC NPAR1WAY procedure.

All statistical procedures were performed at a 5% significance level using the Statistical Analysis System - SAS Software (Statistical Analysis System Institute [SAS Institute], 2022).

Results and Discussion

There was a significant effect ($P<0.05$) on average feed intake at 21 days of age (Table 2), with birds fed diet C displaying a higher mean compared to all other treatments. Additionally, birds that received

the PC diet consumed more feed than those on the B diet. There was also a significant difference in weight gain (P<0.05), with birds on the C diet showing greater weight gain compared to those on the NC, A, and B diets. Regarding feed conversion, the C, B, and PC treatments exhibited better feed conversion (P<0.05) compared to the A diet group.

At 42 days of age (Table 2), birds fed the C diet consumed more feed (P<0.05) than those on the NC, A, and B diets. Birds on the C diet also showed greater weight gain compared to those on the NC, A, and B diets. There was no significant effect (P>0.05) on feed conversion at 42 days of age.

Coccidiosis impacts bird growth by reducing feed and nutrient intake due to the imbalance caused by the proliferation of *Eimeria* spp. and the destruction of the intestinal epithelial surface, leading to low nutrient absorption by the intestine (Wajiha & Afridi, 2018).

Table 2
Performance from 1 to 21 days and 1 to 42 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	1 to 21 days of age			1 to 42 days of age		
	FE	WG	FCR	FE	WG	FCR
NC	1020^{bc}	751^{b}	1.359^{ab}	4562^{b}	2895^{b}	1.576
PC	1055^{b}	783^{ab}	1.348^{b}	4647^{ab}	2979^{ab}	1.560
A	1026^{bc}	741^{b}	1.386^{a}	4485^{b}	2858^{b}	1.570
B	968^{c}	720^{b}	1.344^{b}	4458^{b}	2890^{b}	1.542
C	1110^{a}	803^{a}	1.348^{b}	4831^{a}	3084^{a}	1.567
SEM	28.64	30.72	0.02	151.24	92.00	0.04
P Value	0.0001	0.0034	0.0385	0.0039	0.0022	0.8989

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. FE: Feed intake (g); WG: Weight gain (g); FCR: Feed conversion ratio (g g^{-1}); SEM: Standard error of the mean. ^{a,b} Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

Studies indicate promising results regarding the effectiveness of yeasts in reducing pathogen contamination in broilers, enhancing productive performance, especially in challenged birds (Hofacre et al., 2018; Bosetti et al., 2020).

The improvement in bird performance may be attributed to the beneficial mechanisms of yeasts and glutamine present in the prebiotic tested, which include increased mucus production, enzyme production, and intestinal villi growth that

enhance nutrient absorption (Wang et al., 2016; Leite et al., 2021; Li et al., 2023).

J. Liu et al. (2018) evaluated glutamine associated with yeast and found that these products can positively influence the performance and intestinal morphometry of birds subjected to vaccine stress, aiding in the recovery of epithelial losses in the intestinal mucosa.

There was no significant difference (P>0.05) in the concentrations of acetic, propionic, and butyric acids at 14 and 28 days of age (Table 3). Another factor that may correlate with the improvement in bird performance is the production of short-chain fatty acids (Alexandrino et al., 2020). The intestinal microbiota and the proportion of bacteria present in the cecal content play a fundamental role in intestinal health and may influence animal performance (Dittoe et al., 2018; Liao et al., 2020).

Table 3
Concentrations of short-chain fatty acids (mmol kg^{-1}) in cecal contents at 14 and 28 days of age in broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	14 days of age			28 days of age		
	Acetic	Propionic	Butyric	Acetic	Propionic	Butyric
NC	32.78	4.23	5.90	40.92	5.08	7.32
PC	25.78	3.41	4.76	39.99	6.24	6.65
A	25.12	4.18	5.34	37.13	7.18	6.28
B	26.32	4.32	6.02	37.46	8.49	8.91
C	26.78	4.79	6.49	35.35	8.62	8.45
SEM	9.57	1.03	2.52	13.56	3.70	2.80
P Value	0.7200	0.5552	0.8404	0.9883	0.5444	0.1497

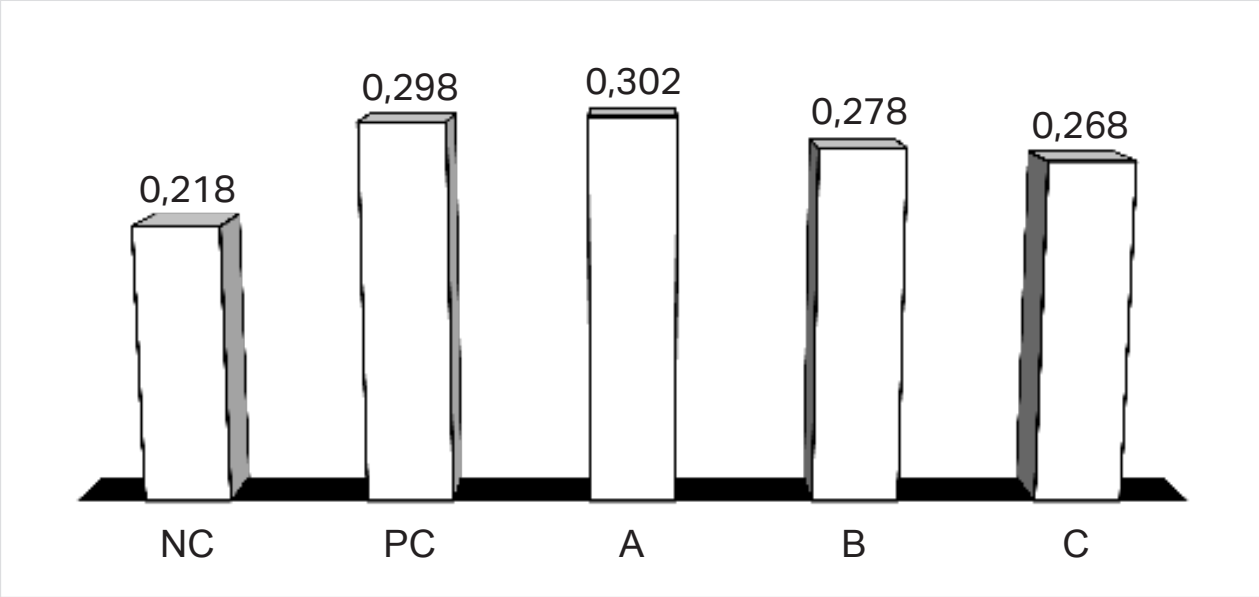
Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. SEM: Standard error of the mean. ^{a,b}Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

The production of short-chain fatty acids occurs through the fermentation of indigestible carbohydrates such as cellulose, starch, and non-starch polysaccharides, and these acids are used as an energy source by birds (Clavijo & Flórez, 2018). This modulation of the cecal microbiota in relation to short-chain fatty acids enhances the utilization of energy by birds, as they can act both as an energy source and as a barrier in the intestine to prevent diseases, in addition to their immunological function, among other

benefits (Kouhounde et al., 2022; Mátis et al., 2022).

Birds fed the NC diet had the lowest intestinal permeability (P=0,0486), while the other treatments showed similar results to the PC diet (Figure 1). Intestinal permeability is an indicator of intestinal quality. The FITC-d assay quantifies and compares intestinal permeability in birds fed with or without additives that alter this parameter (Kuttappan et al., 2015; Morales-Mena et al., 2020). When the intestinal barrier is disrupted, permeability increases, leading to greater translocation of pathogenic bacteria, which can induce an increased immune response or susceptibility to diseases (Volynets et al., 2016; González-González et al., 2019).

Figure 1. Intestinal permeability (FITC-d) at 18 days in broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens* (P=0,0486).



Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. ^{a,b}Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

For the intestinal health index (ISI) at 14 days of age (Table 4), birds on diet A exhibited a thicker lamina propria (P>0.05) compared to those on the PC diet. Enterocyte proliferation was greater (P<0.05) in birds on diets NC and product A than in those on diet B. Inflammatory infiltration of the epithelium was greater (P<0.05) only in birds on the PC diet, while the lamina propria showed higher (P<0.05) infiltration in birds on diet product C compared to those on diets B and NC. Congestion was lower (P<0.05) in birds on diet C compared to other treatments. The presence of oocysts was lower (P<0.05) in birds on diet B than in those on diets C, PC, and NC. The total score for epithelial lesions was lower (P<0.05) in birds on diet B than in those on diets A, PC, and NC. There were no significant differences (P>0.05) in epithelial thickness or goblet cells.

Table 4
Intestinal health index (IHI) at 28 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Intestinal health index	NC	PC	A	B	C	*P Value*
Thickness of lamina propria	1.64^{ab}	1.38^{b}	1.82^{a}	1.56^{ab}	1.56^{ab}	0.0201
Epithelial thickness	1.20	1.06	1.17	1.09	1.14	0.0611
Enterocyte proliferation	1.02^{a}	0.94^{ab}	1.01^{a}	0.90^{b}	0.92^{ab}	0.0029
Inflammatory infiltration in the epithelium	0.00^{b}	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.0170
Inflammatory infiltration in the lamina propria	0.02^{b}	0.12^{ab}	0.12^{ab}	0.02^{b}	0.20^{a}	0.0121
Caliciform cells	1.80	1.86	2.00	1.92	1.91	0.1456
Congestion	1.70^{a}	1.76^{a}	1.44^{a}	1.32^{a}	0.78^{b}	0.0001
Presence of oocysts	0.75^{a}	0.72^{a}	0.48^{ab}	0.09^{b}	0.63^{a}	0.0002
Total	8.13^{a}	7.87^{ab}	8.04^{a}	6.90^{c}	7.15^{bc}	0.0001

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. ^{a,b}Means with different letters in the columns differ statistically by the Dunn test at 5%.

At 28 days of age, the thickness of the lamina propria was lower (P<0.05) in birds that received diet C (Table 5), compared to those that received diets NC, PC, A, and B. Enterocyte proliferation was higher (P<0.05) in birds fed diet C compared to birds fed the PC diet, which showed the lowest enterocyte proliferation. Greater (P<0.05) inflammatory infiltration of the lamina propria was observed in birds fed NC compared to birds in treatments PC, A, B, and C. The lowest (P<0.05) congestion values were observed in birds fed NC. The total number of epithelial lesions was higher in birds fed diet A compared to birds fed diet C. No differences (P>0.05) were found in epithelial thickness, inflammatory infiltration in the epithelium, goblet cells, and enterocyte proliferation.

Table 5
Intestinal health index (IHI) at 28 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Intestinal health index	NC	PC	A	B	C	*P Value*
Thickness of lamina propria	1.68^{a}	1.64^{a}	1.76^{a}	1.58^{a}	1.14^{b}	0.0004
Epithelial thickness	1.15	1.14	1.16	1.17	1.14	0.6166
Enterocyte proliferation	0.96^{ab}	0.92^{b}	1.02^{ab}	1.00^{ab}	1.05^{a}	0.0004
Inflammatory infiltration in the epithelium	0.00	0.00	0.00	0.00	0.00	1.0000
Inflammatory infiltration in the lamina propria	0.22^{a}	0.02^{b}	0.08^{ab}	0.02^{b}	0.06^{ab}	0.0433
Caliciform cells	1.90	2.00	2.00	1.88	1.84	0.0987
Congestion	0.28^{b}	0.74^{a}	0.80^{a}	0.74^{a}	0.52^{ab}	0.0204
Presence of oocysts	0.72	0.24	0.54	0.39	0.48	0.0819
Total	6.91^{ab}	6.91^{ab}	7.36^{a}	6.78^{ab}	6.23^{b}	0.0279

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. a.b Means with different letters in the columns differ statistically by the Dunn test at 5%.

The gastrointestinal tract is the body's first line of defense and is considered the largest immunological organ in birds (Galipeau & Verdu, 2016; Robert et al., 2017). The intestinal epithelium acts as a barrier and, together with the mucin layer, allows the selective passage of water and nutrients while preventing the penetration of pathogens (Liu et al., 2021a).

The altered and higher ISI scores may be correlated with inflammatory processes, indicating that the challenge imposed on the birds was effective. These changes in histological parameters were expected, as they are associated with inflammation and the presence of enteric problems (Schmidt & Silva, 2018).

The higher total ISI was observed due to the high presence of oocysts. This may have occurred due to a possible correlation with macroscopic lesions resulting from coccidiosis, which causes intestinal damage, resulting in greater plasma flow to the lumen, subsequently providing a substrate for the proliferation of Clostridium perfringens (Guardabassi & Prescott, 2015; Ritzi et al., 2016).

Yeasts present in the prebiotic may have adhered to the intestinal mucosa, stimulating the beneficial intestinal microbiota and blocking the proliferation of bacteria that could cause greater damage to the gastrointestinal tract, thus contributing to a balanced microbiota (Al-Khalaifah, 2018; Jacquier et al., 2019).

Blood parameters at 14 days of age (Table 6) showed higher uric acid concentrations ($P<0.05$) in birds fed NC compared to birds fed PC. The enzymatic activity of lactate dehydrogenase was

higher (P<0.05) in birds fed diets B and C compared to birds that received diets PC and NC. Birds fed diet C exhibited the highest concentration of total proteins (P<0.05) compared to the other treatments PC, NC, A, and B. No significant differences (P>0.05) were observed in the levels of cholesterol, glucose, or the enzymatic activity of aspartate aminotransferase.

Table 6
Blood biochemical parameters and enzymatic activity at 14 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	CHO	UA	AST	GLU	LDH	TP
NC	151.52	8.70a	147.64	288.57	726.67b	25.01ab
PC	137.50	6.88b	150.33	290.28	747.33b	24.59ab
A	149.27	8.08ab	174.36	286.50	870.80ab	22.90b
B	154.36	7.28ab	161.49	303.07	961.20a	25.72ab
C	153.21	7.69ab	162.12	292.11	917.17a	26.32a
SEM	12.89	0.95	16.88	10.67	144.39	1.82
P Value	0.1887	0.0322	0.1335	0.1019	0.0439	0.0342

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. CHO: cholesterol (mg/dl); UA: uric acid (mg/dl); AST: aspartate aminotransferase activity (IU/dl); GLU: glucose (mg/dl); LDH: lactate dehydrogenase activity (mg/dl); TP: total proteins (g/l); SEM - standard error of the mean. [a.b]Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

At 42 days of the experiment (Table 7), cholesterol concentrations were lower (P<0.05) in birds fed diet B compared to those fed diets PC, NC, A, and C. Uric acid concentrations were higher, (P>0.05) in birds fed diet B and lower in those fed diet A. There were differences (P>0.05) in the enzymatic activity of aspartate aminotransferase, lactate dehydrogenase, glucose concentrations, and total proteins.

Table 7
Blood biochemical parameters and enzymatic activity at 42 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	CHO	UA	AST	GLU	LDH	TP
NC	161.70[a]	5.82[ab]	331.25	350.28	1503.75	34.98
PC	159.92[a]	4.51[bc]	398.86	330.73	1683.33	32.56
A	163.95[a]	3.94[c]	450.18	338.57	2290.00	30.75
B	135.46[b]	6.56[a]	311.27	320.70	1319.50	30.29
C	159.41[a]	5.38[abc]	358.35	309.14	1550.40	31.07
SEM	11.97	0.92	93.82	28.36	609.50	2.61
P Value	0.0459	0.0045	0.3172	0.2423	0.2473	0.1114

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicateCHO: cholesterol (mg/dl); UA: uric acid (mg/dl); AST: aspartate aminotransferase activity (IU/dl); GLU: glucose (mg/dl); LDH: lactate dehydrogenase activity (mg/dl); TP: total proteins (g/l); SEM - standard error of the mean. [a,b]Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

Serum biochemical parameters are critical for assessing the clinical health of animals as they provide insights into nutritional and pathological statuses and the potential impacts, both positive and negative, of dietary additives (Liu et al., 2021b; Morais et al., 2023).

Uric acid, the primary nitrogenous waste excreted by birds, is synthesized in the liver and to some extent in the kidneys. Variations in uric acid levels can indicate issues in protein catabolism or kidney function. Considering that normal uric acid levels for young birds range from 1 to 2 mg dL^{-1}, the observed results are within acceptable limits (Nunes et al., 2018).

Cholesterol is synthesized in the intestinal mucosa and liver from the components of fat digestion (Siyal et al., 2017). An increase in cholesterol may be linked to liver damage caused by the challenges faced by the birds (Abudabos et al., 2016), although no increase in relative liver weight was noted.

The activity of lactate dehydrogenase in healthy birds is typically less than 1,000 IU L-1; elevated levels at 42 days could suggest potential hematocellular issues (Silva et al., 2022). Michalska et al. (2021) studied the effects of herbal extract-based additives on quails under a health challenge by *Clostridium perfringens* and noted that the additives reduced lesions, demonstrating their anti-inflammatory and hematoprotective effects.

The rapid growth of birds during the starter phase is directly linked to their intestinal health, enhancing nutrient absorption and muscle deposition (Cruz et al., 2022). The observed increase in total protein concentrations in birds fed diet

C may be related to improved intestinal health, as evidenced by their greater weight gain and more efficient nutrient utilization. Studies suggest a relationship between diet composition and serum total protein levels; however, since all treatments had the same nutritional composition aside from additives, this factor did not influence the results (Rezende et al., 2019).

Regarding litter quality (Table 8), no significant differences (P>0.05) were found between treatments. The average pH was 9.09; the DM content averaged 58.08%, and volatilized ammonia averaged 1.50 mg kg^{-1}.

Table 8
Bedding quality at 35 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	pH	Dry Matter (%)	Ammonia (mg kg^{-1})
NC	9.01	56.25	1.34
PC	9.09	60.45	1.48
A	9.11	58.39	1.22
B	9.08	57.03	1.20
C	9.16	58.30	2.20
SEM	0.14	7.3	0.98
P Value	0.4812	0.8767	0.4749

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. SEM: standard error of the mean.

De Cesare et al. (2017) assessed the impact of prebiotics on broiler chicken litter quality and observed no significant changes, highlighting that good litter quality considering moisture, pH, and ammonia levels is paramount for optimal bird performance, growth, disease prevention, and reducing injuries to feet and carcasses.

The pH, moisture, and ammonia levels in the litter are interconnected; higher moisture content in the litter leads to greater ammonia release, which decreases when the pH is below 7.0 (Toledo et al., 2019). Ideal litter moisture levels range from 20 to 35%; deviations from this range can lead to respiratory issues or an increase in the incidence of foot and carcass injuries, resulting in economic losses. Studies have shown that fecal matter accumulation raises pH, consequently increasing volatilized ammonia levels (Gonçalves et al., 2019).

The yield of carcass, legs, breast fillet, tender, wings, percentage of abdominal fat, and relative liver weight showed no differences (P>0.05) among the natural additives studied (Table 9).

Table 8
Carcass and cuts yield, and relative organ weight (%) at 42 days of age of broiler chickens challenged with *Eimeria* vaccine and *Clostridium perfringens*

Treatments	CCY	LY	BFY	TY	WY	RAFW	RLW
NC	68.26	30.75	27.26	5.86	9.44	1.24	1.74
PC	68.58	30.68	27.18	5.50	9.45	1.36	1.81
A	69.57	30.77	28.49	5.80	9.05	1.27	1.81
B	68.64	30.21	27.82	5.63	9.12	1.16	1.84
C	68.58	31.58	27.22	5.84	9.14	1.10	1.72
SEM	1.96	1.55	2.05	0.43	0.71	0.34	0.19
P Value	0.5479	0.3436	0.4588	0.2262	0.5197	0.4202	0.4923

Treatments: NC: Negative control - no growth promoter; PC: Positive control - basal diet with inclusion of 50 g ton^{-1} of avilamycin 20%; Diet A: Basal diet plus inclusion of 100 g ton^{-1} of a product based on *Macleaya cordata* extract; Diet B: Basal diet plus inclusion of 1000 g ton^{-1} of a product based on red chestnut (*Castanea sativa*) and quebracho colorado (*Schinopsis lorentzii*); Diet C: Basal diet plus inclusion of 100 g ton^{-1} of a product based on yeast prebiotics, glutamine, and aluminosilicate. CCY: cold carcass yield; LY: leg yield; BFY: breast fillet yield; TY: tender yield; WY: wing yield; RAFW: relative abdominal fat weight; RLW: relative liver weight; SEM: standard error of the mean.

Carcass and cut yields are primarily influenced by the nutritional content of the diet. Some studies indicate that natural additives do not affect the yield of carcass and cuts. The natural additives used in this study did not alter the relative weight of the liver, suggesting that they can be used within the recommended guidelines without affecting liver metabolism.

Research by Ramos et al. (2014) on natural additives as alternatives to antibiotics in broilers raised on reused commercial litter showed no impact on carcass and cut yields. Similar findings were reported by Pascual et al. (2020), who evaluated antibiotic alternatives.

Conclusions

Product A, containing *Macleaya cordata* extract; product B, which includes water-soluble and condensed polyphenols, simple sugars, lignin, cellulose, hemicellulose, and mineral salts; and product C, formulated with pichia yeast, glutamine, and aluminosilicate, can serve as replacements for antibiotics as growth promoters without compromising bird performance, short-chain fatty acids levels, intestinal permeability, litter quality, or the yields of carcass and cuts.

Products B and C are particularly notable for their superior performance in improving the intestinal health index at both 14 and 28 days of age.

Given these findings, products B and C can replace growth promoters in broilers challenged with Eimeria vaccine and *Clostridium perfringens,* enhancing both bird performance and intestinal health.

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