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Essential oil blends in broiler chicken diets on performance, intestinal health and antioxidant enzyme activity

Mistura de óleos essenciais na dieta de frangos de corte sobre o desempenho, saúde intestinal e atividade de enzimas antioxidantes

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Highlights

Essential oils to replace antibiotics as growth promoters.
Phytobiotics as alternatives to antibiotics.
Activity of antioxidant enzymes.

Abstract

The objective was to evaluate the inclusion of different commercial products based on essential oils in the diet of broiler chickens challenged by *Eimeria* vaccine and *Clostridium perfringens* as replacements for growth-promoting antibiotics. A total of 720 one-day-old male broiler chicks from the Cobb 500® strain were distributed in a completely randomized design, with six treatments, six replications and 20 birds per experimental unit. The treatments consisted of: NC: negative control – basal diet without growth-promoting antibiotics; PC: positive control – basal diet with inclusion of growth-promoting antibiotic; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle and NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential

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oils, turmeric, tannins, vitamin E and zinc. At four days of age, all birds received orally 20 times the dose of *Eimeria* spp. vaccine, and seven and 10 days into the experiment, all birds were challenged with culture *Clostridium perfringens.* At one, 21 and 42 days of age, birds and feed were weighed to determine zootechnical performance. At 14 days of age, blood was collected from one bird per experimental unit to evaluate the enzymes superoxide dismutase, catalase, lactate dehydrogenase and aspartate aminotrasferase. At 14 and 28 days of the experiment, one bird per experimental unit was sacrificed for cecum collection for analysis of short-chain fatty acids and intestine (jejunum) samples for analysis of the intestinal health index and histomorphometry of the jejunum. At 18 days of experiment, one bird per experimental unit was orally administered a dose of fluorexin dextran isothicionate (FTC-d) for intestinal permeability analysis. The data were subjected to analysis of variance followed by the SNK test. For those that did not present have normal distribution, the Kriskal-Walis test and the Dunn test were used. All statistical procedures were conducted at 5% probability. There was a difference (P≤0.05) in weight gain and feed conversion at 21 days of age of the birds. The use of product D can improve the feed conversion of birds when compared to the positive control treatment. There was a difference (P≤0.05) for the intestinal health index at 14 days of age of the birds, the birds from product D had a lower total injury score when compared to the negative control treatment, product A and product C. There was a difference (P≤0.05) for the activity of the enzyme superoxide dismutase and aspartate aminotransferase, the inclusion of product B decreased the production of superoxide dismutase at 14 days of age when compared to C. There was a difference in the production of aspartate aminotransferase at 14 days of bird age, the inclusion of products C and D increases production compared to the negative control treatment. The use of product D based on organic acids, a blend of essential oils, turmeric, tannins, vitamin E and zinc can replace antibiotics as a growth promoter, promoting better feed conversion and a lower intestinal injury score.
Key words: Antimicrobial action. Bioactive compounds. Superoxide dismutase.

Resumo

O objetivo foi avaliar a inclusão de diferentes produtos comerciais à base de óleos essenciais na dieta de frangos de corte desafiados por *Eimeria* vacinal e *Clostridium perfringens* como substituitos aos antibioticos promotores de crescimento. Um total de 720 pintos de corte, machos, com um dia de idade da linhagem Cobb 500® foram distribuídos em delineamento inteiramente casualizado, com seis tratamentos, seis repetições e 20 aves por unidade experimental. Os tratamentos foram constituídos por: CN: controle negativo – ração basal sem antibiótico promotor de crescimento; CP: controle positivo – ração basal com inclusão de antibiótico promotor de crescimento; CN+A: ração basal com inclusão de 100g ton^{-1} produto composto por cinamaldeído, carvacrol e timol; CN+B: ração basal com inclusão de 1000g ton^{-1} produto composto por óleo de caju (*Anacardium occidentale*) e óleo de mamona (*Ricinus communis*); CN+C: ração basal com inclusão de 150g ton^{-1} de produto composto por extrato de eucalipto (*Eucalyptus*), carvacrol, cinamaldeído, oleoresina de páprica e veículo e CN+D: ração basal com inclusão de 300g ton^{-1} de produto composto por ácidos orgânicos, blend de óleos essenciais, cúrcuma, taninos, vitamina E e zinco. Aos quatro dias de idade, todas as aves receberam via oral 20 vezes a dose de vacina de *Eimeria* spp. e com sete e 10 dias de experimento todas as aves foram desafiadas com cultura de *Clostridium perfringens.* Aos um, 21 e 42 dias de idade as aves e as rações foram pesadas

para determinar o desempenho zootécnico. Aos 14 dias de idade foi realizado colheita de sangue de uma ave por unidade experimental para avaliar as enzimas superóxido dismutase, catalase, lactato desidrogenase e aspartato aminotrasferase. Aos 14 e 28 dias de experimento, uma ave por unidade experimental foi sacrificada para coleta ceco para análise de ácidos graxos de cadeia curta e amostras de intestino (jejuno) para análise do índice de saúde intestinal e histomorfometria do jejuno. Aos 18 dias de experimento, uma ave por unidade experimental foi recebeu via oral uma dose de isoticionato fluorexina dextran (FTC-d) para análise de permeabilidade intestinal. Os dados foram submetidos à análise de variância seguido do teste de SNK. Para aqueles que não apresentarem distribuição normal, foi utilizado teste de Kriskal-Walis e teste de Dunn. Houve diferença (P≤0,05) para o ganho de peso e conversão alimentar aos 21 dias de idade das aves, a utilização do produto D pode melhorar a conversão alimentar das aves quando comparadas ao tratamento controle positivo. Houve diferença (P≤0,05) para o índice de saúde intestinal aos 14 dias de idade das aves, as aves do tratamento D apresentaram menor score total de lesões intestinais quando comparadas ao tratamento controle negativo, produto A e produto C. Houve diferença (P≤0,05) para a atividade da enzima superóxido dismutase e aspartato aminotransferase, a inclusão do produto B diminuiu a produção de superóxido dismutase aos 14 dias de idade quando comparadas com C. Houve diferença para produção de aspartato aminotransferase aos 14 dias de idade das aves, a inclusão dos produtos C e D aumentam a produção em comparação ao tratamento controle negativo. O uso do produto D a base de ácidos orgânicos, blend de óleos essenciais, cúrcuma, taninos, vitamina E e zinco pode substituir o antibiótico como promotor de crescimento promovendo melhor conversão alimentar e menor índice de score de lesões intestinais.
Palavras-chave: Ação antimicrobiana. Compostos bioativos. Superóxido dismutase.

Introduction

Avian coccidiosis, caused by the infectious protozoan *Eimeria* spp., invades the epithelial cells of intestinal tissue, damaging them and leading to intestinal inflammation and cellular deterioration. This condition reduces feed intake, impairs bird performance, and suppresses immune responses, resulting in significant economic losses for the poultry industry (Khukhodziinai et al., 2024).

Eimeria spp. is widely distributed and frequently detected in various environments. Its different species affect specific regions of the intestine, from the duodenum to the cecum, due to their site-specific infectivity. Once established, these species possess distinct mechanisms to evade the host's immune response (Abd El-Hack et al., 2022).

The subtherapeutic use of antibiotics as growth promoters can improve performance and intestinal health, reduce mortality in birds, and help control diseases such as necrotic enteritis (Oladokun et al., 2021). However, their use, even at subtherapeutic doses, has been widely prohibited in recent years, mainly due to concerns regarding food safety and microbial resistance, which poses a significant risk to public health (Jazi et al., 2018; Su et al., 2021).

In response to the growing global demand for antibiotic alternatives, nutritional strategies are constantly adopted to control avian coccidiosis. Among the available options, organic acids, probiotics, prebiotics, tannins, and essential oils have emerged as promising candidates (Elbaz et al., 2023).

Essential oils, extracted from herbs and spices, contain bioactive compounds that can enhance performance and promote intestinal health in birds (Lee et al., 2020; Oladokun et al., 2021). These spices are known for their specific bioactive components, such as thymol in thyme (Huang & Lee, 2018), carvacrol in oregano (Huang & Lee, 2018), cinnamaldehyde in cinnamon (El-Kholy et al., 2021), and carnosol in rosemary (Oladokun et al., 2021), among others.

Current studies aim to understand how essential oils and their bioactive components can act alone, synergistically, or in combination with other products to deliver benefits, including antibacterial, antiviral, antifungal, antimycotic, and other properties (Stevanović et al., 2018).

The objective of this study was to evaluate the inclusion of different commercial products based on essential oils in the diet of broiler chickens challenged with Eimeria (vaccine-derived) and Clostridium perfringens, as potential substitutes for growth-promoting antibiotics, and to assess their potential to protect against coccidiosis.

Materials and Methods

Ethics committee

The experiment was conducted at the Poultry Research Center (CPA) of the State University of Western Paraná (UNIOESTE), located in Marechal Cândido Rondon, PR, Brazil. All experimental procedures involving animals were approved by the Ethics Committee for the Use of Animals at UNIOESTE (approval no. 012/2022).

Animals and diets

A total of 720 one-day-old male Cobb 500® broiler chicks, with an average initial weight of 43.88 ± 0.48 g, were randomly assigned in a completely randomized design to six treatments, with six replicates of 20 birds per experimental unit.

The treatments were as follows:

NC: Negative control – basal diet without growth-promoting antibiotics;

PC: Positive control – basal diet with 550 g t^{-1} of avilamycin 20% in the pre-starter and starter phases, and 200 g t^{-1} in the grower and finisher phases;

NC+A: Basal diet with 100 g t^{-1} of a product composed of cinnamaldehyde, carvacrol, and thymol;

NC+B: Basal diet with 1000 g t^{-1} of a product composed of cashew (Anacardium occidentale) oil and castor (*Ricinus communis*) oil;

NC+C: Basal diet with 150 g t^{-1} of a product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin, and carrier;

NC+D: Basal diet with 300 g t^{-1} of a product composed of organic acids, a blend of essential oils, turmeric, tannins, vitamin E, and zinc.

The products were added to the diets. The experimental diets (Table 1) were formulated according to the recommendations of Rostagno et al. (2017), being isoenergetic and isonutritive, based on corn and soybean meal to meet the nutritional requirements of the birds across the following phases: pre-starter (1 to 10 days), starter (11 to 21 days), grower (22 to 35 days), and finisher (36 to 42 days).

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

[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 B_1 (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 K_3 (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 period

The birds were housed in 1.76 m^2 pens with concrete floors covered with pine shavings (5th reuse), and equipped with individual trough feeders and nipple drinkers. Temperature and humidity were monitored to ensure thermal comfort, using heat lamps with electric resistance (250 W), two exhaust fans, and evaporative cooling panels, in accordance with the recommended conditions for each age.

Feed and water were provided *ad libitum.* The lighting program followed a decreasing light/dark regimen: 24/0 h of light on the 1st day of life, 21/3 h of light from the 2nd to the 7th day of life, and 18/6 h of light from the 8th to the 42nd day of age. Light intensity was maintained at 20 lux throughout the experiment.

Bird challenge

On the 4th day of the experiment, all birds were challenged by oral gavage in the crop region with 0.6 mL (20 times the standard dose) of Biococcivet R®, a vaccine composed of a concentrated suspension of sporulated oocysts from five *Eimeria* spp. (*E. acervulina, E. praecox, E. maxima, E. tenella,* and *E. mitis*). On days 7 and 10, all birds received an additional challenge consisting of 0.5 mL of a *Clostridium perfringens* culture inoculum (10^8 CFU mL^{-1}), isolated from a field outbreak of necrotic enteritis in broilers.

Growth performance

On days 1, 21, and 42 of the experiment, all birds and feed were weighed by experimental unit to determine average feed intake (FI), body weight gain (WG), and feed conversion (FC). The remaining feed in each feeder was weighed and recorded, and the average feed intake was corrected accordingly, following the methodology described by Sakomura and Rostagno (2016).

Short-chain fatty acids

At 14 and 28 days of age, one bird per experimental unit was randomly selected and sacrificed by cervical dislocation for subsequent organ collection. The cecum of each bird was removed, placed in a labeled plastic bag, and stored in a freezer at -20 °C for later analyses.

Short-chain fatty acid extraction was performed by weighing 200 mg of cecal content into 2 mL microtubes. Then, 1800 µL of 1% NaOH solution was added, and the contents were homogenized by vortexing to ensure complete dissociation. The samples were then centrifuged (Kasvi K14-4000 Centrifuge, Kasvi, São Paulo, BR) at 1000 G for five minutes to sediment the solid fraction. A volume of 900 µL of the supernatant was transferred to a new 2 mL microtube and acidified with 50 µL of 50% orthophosphoric acid solution.

Short-chain fatty acid concentrations 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, samples were acidified with 1 mL of orthophosphoric acid (analytical grade; Ref. 100573, Merck©) and fortified with a mixture of free volatile acids (Ref. 46975, Supelco©).

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

The temperature program for the column consisted of 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 10 °C min^{-1}, and from 180 to 240 °C at 120 °C min^{-1}, with a final hold at 240 °C for three minutes. For quantification, a calibration curve was prepared using dilutions of the WSFA-2 standard (Ref. 47056, Supelco©) and glacial acetic acid (Ref. 33209, Sigma-Aldrich©), under the same analytical conditions. Peak detection and integration were carried out using GCsolution software v. 2.42.00 (Shimadzu©).

Jejunal histomorphometry

To assess intestinal histomorphometry (villus height, crypt depth, villus height:crypt depth ratio, and absorption area), one bird per experimental unit was sacrificed by cervical dislocation for jejunum collection on days 14 and 28 of the experiment. Jejunal segments (2 to 5 cm) were collected, fixed in 10% buffered formalin, dehydrated in a graded ethanol series, and embedded in paraffin. Semi-serial 5 µm sections of each segment were mounted on glass slides and stained using the hematoxylin-eosin technique, following Luna (1968).

Measurements were performed using the PROPLUS IMAGE 4.1 system. For each slide, the height and width of 10 villi and the depth and width of 10 crypts were measured. These morphometric parameters were used to calculate the intestinal mucosal absorption area (AA), using the formula proposed by Kisielinski et al. (2002). The villus height:crypt depth (V:C) ratio was calculated by dividing villus height (VH) by crypt depth (CD).

Intestinal permeability

At 18 days of age, one randomly selected bird per experimental unit received an oral dose of fluorescein isothiocyanate-dextran (FITC-d) (8.32 mg/kg body weight) (MW 4,000; Sigma-Aldrich© Canada). One

hour after administration, blood was collected via brachial puncture and centrifuged (Kasvi K14-4000 Centrifuge, Kasvi© São Paulo, Brazil) at 1000 G for 15 minutes to separate the serum, which was then diluted (1:5) in 9% saline solution. Serum samples were collected from birds (n=3) that had not received FITC-d to serve as baseline controls for the standard curve.

The FITC-d concentration in serum was determined using a spectrophotometer set at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (FlexStation 3, Molecular Devices©). Results were compared with a standard curve containing known FITC-d concentrations. The values of intestinal permeability were expressed in nanograms per milliliter (ng mL^{-1}), as described by Baxter et al. (2019).

Intestinal health index

On days 14 and 28 of the experiment, one bird per experimental unit was randomly selected, sacrificed, and eviscerated. The gastrointestinal tract was removed and exposed, the Meckel's diverticulum was located, and 2 cm segments of the jejunum were collected and preserved in 10% formalin.

The jejunal segments were processed for analysis of the intestinal health index using the I See Inside (ISI) methodology proposed by Kraieski et al. (2017). In this method, an impact factor is assigned to each macroscopic and microscopic lesion, ranging from 1 to 3 based on its extent or frequency and depending on the affected organ. The frequency (S, score) of each lesion is also scored from 0 (absent) to 3 (more than 50% affected). The intestinal health index is calculated as the sum of each lesion's score multiplied by its impact factor.

Blood enzyme activity

At 14 days of age, one bird per experimental unit was randomly selected for blood collection after a six-hour fast, following the protocol of Nunes et al. (2018). Blood samples were centrifuged (Kasvi K14-4000 Centrifuge, Kasvi©) to obtain the serum, which was stored in cryogenic tubes and kept in liquid nitrogen for later analysis of superoxide dismutase and catalase activity.

Superoxide dismutase and catalase were determined colorimetrically (enzymatic method) using a commercial kit (Cayman Chemical©) and analyzed with an ELISA microplate reader (FlexStation 3, Molecular Devices©).

For the lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) parameters, after blood collection, the tubes were left to rest in a horizontal position for 15 min at room temperature. The samples were then centrifuged (Kasvi K14-4000 Centrifuge, Kasvi©) at 1000 G for 10 min to separate the serum. The serum was transferred to 2 mL microtubes and stored in a freezer at -20 ºC. Before analysis, the samples were thawed at a temperature between 2 and 8 ºC and subjected to a second centrifugation in a microcentrifuge (Eppendorf©) at 1000 G for 5 min to remove any potential hemolysis. The analyses were carried out using an automatic biochemical analyzer with spectrophotometric detection (Flexor EL200©).

Statistical analysis

Data were initially tested for normality (Shapiro-Wilk) and homogeneity (Levene) using PROC UNIVARIATE, and outliers were identified and excluded. Data with a normal distribution were subjected to analysis of variance, and when significant effects were found, means were compared using the Student-Newman-Keuls test at a 5% significance level, employing the PROC GLM procedure.

For variables that did not meet normality assumptions, a Kruskal-Wallis test was conducted. If a significant effect was detected, means were compared using the Dunn test with the PROC NPAR1WAY procedure. All statistical procedures were performed at a 5% significance level using the Statistical Analysis System Institute [SAS Institute] (2022).

Results and Discussion

At 21 days of the experiment, no difference (P>0.05) was observed in average feed intake. However, birds that received the diet with product A showed lower weight gain (P=0.0243) and higher feed conversion (P=0.0050) at 21 days of age (Table 2). No significant differences (P>0.05) were observed for performance variables at 42 days.

Table 2
Performance from one to 21 days and one to 42 days of age of broilers challenged with Eimeria vaccine and Clostridium perfringens

Treatments	1 to 21 days of age			1 to 42 days of age		
	FI	WG	FCR	FI	WG	FCR
NC	1020	751ab	1.359ab	4558	2861	1.594
PC	1055	783a	1.348b	4647	2958	1.571
NC+A	1029	738b	1.395a	4589	2879	1.594
NC+B	1053	764ab	1.380ab	4618	2880	1.603
NC+C	1054	765ab	1.377ab	4731	3010	1.572
NC+D	1022	759ab	1.348b	4570	2929	1.560
SEM	28.66	21.46	0.02	171.20	101.43	0.03
P Value	0.0941	0.0243	0.0050	0.5577	0.1416	0.0586

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc. FI: 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%.

A healthy and functional intestine is essential for optimal animal performance. When intestinal health is compromised under challenge conditions, digestion and absorption processes are impaired, preventing birds from reaching their full performance potential (Abo Ghanima et al., 2021). The better results observed in the positive control treatment up to 21 days may be attributed to the efficacy of the antibiotic in combating the microorganisms used in the challenge (Dai et al., 2021; Liu et al., 2017).

The various bioactive compounds found in the tested essential oils have antimicrobial properties, capable of inhibiting the growth of pathogenic bacteria (Barbalho et al., 2023). Birds fed a commercial blend containing thyme essential oils, carvacrol, and organic acids demonstrated improved feed conversion, though without a corresponding increase in weight gain (Pham et al., 2020), a result similar to that observed in the present study.

According to findings by Pirgozliev et al. (2019), the addition of essential oils containing carvacrol, cinnamaldehyde, and capsicum improved bird performance, potentially through immune modulation and reduced intestinal inflammation. Similarly, Lee et al. (2020) observed that a blend of essential oils composed of carvacrol and thymol significantly mitigated the negative effects of coccidiosis on body weight gain and feed conversion.

However, the concentration of bioactive compounds in these products can influence their effectiveness in the animal's system. In the early stages of development, the gastrointestinal tract is still maturing, and enzymatic capacity is limited. In addition to the challenge imposed, this may explain the delayed effect of essential oils compared to the more immediate effect of antibiotic treatment (Oladokun et al., 2021; Qiao et al., 2022).

The production of short-chain fatty acids was not affected at either 14 or 28 days of age ($P>0.05$) (Table 3). Studies have shown that essential oils and their compounds, such as thymol, carvacrol, cinnamaldehyde, and eucalyptol, can influence cecal microbiota by reducing pathogenic bacteria and increasing beneficial bacteria (Mohebodini et al., 2021).

Table 3
Short-chain fatty acid concentrations (mmol kg^{-1}) in cecal contents at 14 and 28 days of age of broilers challenged with *Eimeria* vaccinal 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	39.99	6.24	6.24
PC	25.78	3.41	4.76	40.92	5.08	7.13
NC+A	30.12	3.83	5.98	37.13	7.18	7.10
NC+B	26.45	5.17	6.72	37.46	8.49	5.58
NC+C	20.56	3.44	4.49	38.87	5.47	6.97
NC+D	23.39	4.47	5.03	35.35	8.62	7.08
SEM	9.01	1.47	2.62	13.56	3.70	3.68
P Value	0.3515	0.4761	0.7899	0.9883	0.5444	0.9784

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc.

Their mechanism of action is based on supplying substrates for beneficial bacteria, such as butyric acid producers, which use these compounds as a carbon source, promoting cecal microbiome balance. Supplementation with essential oils in broiler diets can stabilize the intestinal microbiome, enhance enzyme secretion, and stimulate appetite (Ruff et al., 2021).

The cecum serves as the site of accumulation for all intestinal waste, and it is where most bacterial fermentation (beneficial or harmful) occurs. The concentrations of acetic, propionic, and butyric acids were unaffected, which may be attributed to the antibacterial properties of essential oils and their ability to support the homeostasis of intestinal microflora by inactivating *Clostridium perfringens* toxins (Stamilla et al., 2020).

No effect (P>0.05) was observed on intestinal histomorphometry at 14 and 28 days of age (Table 4). Likewise, there was no effect (P>0.05) of the treatments on intestinal permeability at 18 days of age (Table 5). A significant difference (P=0.0001) was observed in the total lesion score of the intestinal health index; birds receiving treatment with product D had fewer lesions compared to those in the NC, product A, and product C groups (Table 6). No difference (P>0.05) was observed in the total lesion score at 28 days of the experiment (Table 6).

Table 4
Histomorphometry of the jejunum (µm) at 14 and 28 days of age of broilers challenged with Eimeria vaccine and Clostridium perfringens

Treatments	VH	CD	V:CR	AA	VH	CD	V:CR	AA
NC	407.30	108.45	4.22	6.27	554.48	98.84	5.61	8.83
PC	395.53	94.87	4.88	6.86	556.10	111.02	5.49	8.77
NC+A	351.10	89.60	4.20	6.05	553.92	96.93	5.91	8.67
NC+B	378.65	102.96	4.09	6.04	506.79	87.57	6.32	9.08
NC+C	333.28	97.99	3.85	5.91	599.41	109.45	5.83	10.84
NC+D	365.94	79.26	5.00	6.34	501.42	105.08	5.11	8.56
SEM	66.07	15.51	0.94	1.10	76.10	14.29	1.05	1.18
P Value	0.5299	0.0881	0.3415	0.7889	0.3859	0.1328	0.5854	0.0865

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc. VH: villus height; CD: crypt depth; V:CR: villus:crypt ratio; AA: absorption area.

Table 5
Intestinal permeability (FITC-d) at 18 days in broiler chickens challenged with Eimeria vaccine and Clostridium perfringens

Treatments	Intestinal permeability
NC	0.218
PC	0.298
NC+A	0.279
NC+B	0.256
NC+C	0.256
NC+D	0.230
SEM	0.060
P Value	0.179

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc.

Table 6
Intestinal health index (IHI) at 14 and 28 days of age of broilers challenged with Eimeria vaccine and Clostridium perfringens

Treatments	14 days of age	28 days of age
NC	8.13[a]	6.91
PC	7.87[ab]	6.70
NC+A	8.09[a]	6.94
NC+B	7.36[ab]	7.41
NC+C	8.13[a]	7.20
NC+D	6.65[b]	6.98
P Value	0.0001	0.3779

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc. Total: Total. a.b Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

Villi and crypts are essential structures of the small intestine, and their integrity and morphometry serve as indicators of the organ's nutrient absorption capacity (Heydarian et al., 2020).

The renewal of the intestinal epithelium, or cell turnover, results from a dynamic balance between the production of enterocytes in the crypts and their shedding from the villi. Thus, the villus height-to-crypt depth ratio (VH:CD) is a criterion adopted for assessing intestinal health and function (Jazi et al., 2020).

The inclusion of essential oils as antibiotic substitutes has the potential to increase villus height and crypt depth in broilers (Amer et al., 2021). Hosseinzadeh et al. (2023) highlighted the protective role of essential oils in the intestine, favoring villus growth, expanding the absorptive surface area, and improving the microbial population in the gastrointestinal tract.

Choi et al. (2022) reported a higher villus:crypt ratio in the ileum of birds supplemented with essential oils compared to the control. Similarly, Abudabos et al. (2018) observed reduced intestinal lesions and improved intestinal morphology in chickens challenged with *Clostridium perfringens* and supplemented with oregano essential oil.

As the main barrier between the host and the external environment, the structural integrity of the intestine is essential for nutrient absorption and maintaining intestinal health. The intestinal barrier involves multiple protective mechanisms, including tight junctions, the mucus layer, the microbial community, and gut-associated lymphoid tissues (Sharma et al., 2020).

The present results corroborate those reported by Liu et al. (2017) and Yang et al. (2018), who found that broilers challenged with *Clostridium perfringens* and fed blends of organic acids and essential oils had lower lesion scores than birds in the positive control group.

Loss of intestinal integrity leads to a progressive increase in mucosal permeability, facilitating the invasion of pathogens (Jazi et al., 2020). One of the most important biological properties of phytogenic products is their antibacterial activity, which helps suppress harmful intestinal bacteria (Gilani et al., 2018). Pham et al. (2020) observed lower serum FITC-d concentrations in birds infected with *Clostridium perfringens* and fed essential oils.

A difference (P=0.0670) was observed in superoxide dismutase (SOD) activity, with birds fed product C showing lower concentrations compared to the other treatments (Table 7). A significant difference (P=0.0187) was also noted in aspartate aminotransferase activity, with birds receiving products C and D showing higher concentrations than those in the NC group. No difference (P≥0.05) was observed in catalase and lactate dehydrogenase levels after 14 days of the experiment.

Table 7
Blood enzyme activity at 14 days of age of broilers challenged with Eimeria vaccine and Clostridium perfringens

Treatments	SOD	CAT	LDH	AST
NC	1.0537^{ab}	0.0031	726.67	147.64^{b}
PC	1.0432^{ab}	0.0031	747.33	150.33^{ab}
NC+A	1.0467^{ab}	0.0525	815.25	165.63^{ab}
NC+B	1.0324^{b}	0.0031	891.83	154.54^{ab}
NC+C	1.0829^{a}	0.1235	854.83	168.49^{a}
NC+D	1.0579^{ab}	0.0123	832.40	168.12^{a}
P Value	0.0670	0.1310	0.4309	0.0187

Treatments: CN: negative control - without growth promoter; PC: positive control - basal diet with inclusion of 550g ton^{-1} avilamycin 20% in the pre-starter and starter phases and 200g ton^{-1} in the growth and finish phases; NC+A: basal diet with inclusion of 100g ton^{-1} product composed of cinnamaldehyde, carvacrol and thymol; NC+B: basal diet with inclusion of 1000g ton^{-1} product composed of cashew oil (*Anacardium occidentale*) and castor oil (*Ricinus communis*); NC+C: basal diet with inclusion of 150g ton^{-1} of product composed of eucalyptus extract (*Eucalyptus*), carvacrol, cinnamaldehyde, paprika oleoresin and vehicle; NC+D: basal diet with inclusion of 300g ton^{-1} of product composed of organic acids, blend of essential oils, turmeric, tannins, vitamin E and zinc. SOD: superoxide dismutase; CAT: catalase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase. ^{a,b}Means with different letters in the columns differ statistically by the Student-Newman-Keuls test at 5%.

Oxidative stress is a common biological process characterized by an imbalance between the production of free radicals and the antioxidant system's ability to neutralize them. Superoxide dismutase is one of the main antioxidant enzymes, protecting cells by preventing free radical formation and enhancing antioxidant defense (Mohebodini et al., 2021).

Essential oils from rosemary, oregano, thyme, and turmeric have been shown to enhance antioxidant responses in enterocytes under oxidative stress, suggesting a unique mode of action for these compounds (Coles et al., 2021). Oxidative damage can be mitigated by antioxidant defense mechanisms, which protect cells from oxidants and by repair systems that prevent the accumulation of oxidatively damaged molecules. An increase in SOD activity may indicate a response to oxidative stress and reduced efficiency of the body's defense system (Zhang et al., 2021).

Carvacrol and thymol, two major phenolic compounds found in product C, are believed to contribute to antioxidant activity by scavenging free radicals in the bloodstream. These compounds also stimulate the secretion of digestive and pancreatic enzymes, enhance intestinal morphology, and improve immune function (Chowdhury et al., 2018; Mohebodini et al., 2021).

Conclusions

The use of product D, composed of organic acids, a blend of essential oils, turmeric, tannins, vitamin E and zinc, provided improvements in performance associated with improvements in intestinal health

Acknowledgements

To the State University of Western Paraná - UNIOESTE.

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