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Effects of microencapsulated carvacrol and cinnamaldehyde on feed digestibility, intestinal mucosa, and biochemical and antioxidant parameters in broilers

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ABSTRACT - The objective of this study was to evaluate the effects of different levels of microencapsulated carvacrol and cinnamaldehyde on metabolizable energy, feed digestibility, intestinal morphometric analysis, and antioxidant parameters in broilers. A completely randomized design with five treatments and eight replications of eight broilers (Cobb male) was used, and collections were carried out at 20 and 42 days of age. Carvacrol and cinnamaldehyde concentrations (mg/kg) were: 0 (control), 50, 100, 150, and 200. Carvacrol and cinnamaldehyde improved apparent metabolizable energy but did not change protein and ether extract digestibility. Supplementation increased intestinal villus height and villi:crypt ratio; in which 100 mg/kg produced the greatest villus height. Serum uric acid levels were lower in birds receiving supplementation. Improvement in the activity of glutathione peroxidase and glutathione-S-transferase was observed, while lower uric acid, thiobarbituric acid reactive substances, and reactive oxygen species levels were observed. Microencapsulated carvacrol and cinnamaldehyde improve apparent metabolizable energy and can be administered in broiler feed without risk to the bird's health. These supplements may serve as alternative products to aid the performance of commercial poultry.

Keywords: cinnamon, intestinal health, metabolizable energy, oregano, serum biochemistry

1. Introduction

Severe restrictions and prohibition of the use of antibiotics as performance enhancers in animal feed have resulted in the development of a new generation of compounds designed to provide a healthy balance in animal gastrointestinal tract microbiota. Herbal extracts and essential oils as phytogenics additives are commonly used as feed additives (Zeng et al., 2015; Bozkurt et al., 2016; Zhai et al., 2018), aiming at improvement of animal performance, feed digestibility, and as replacements for antimicrobial

growth promoters. Some studies have evaluated the use of these additives (Petrolli et al., 2012; Fernandez-Alarcon et al., 2017; Reis et al., 2018), highlighting the improvements in broiler nutrient digestibility.

Carvacrol is a lipid compound present in oregano (*Origanum vulgare*) essential oil, and its antibiotic and antioxidant activity in broilers has been described over the last years, being a promising natural molecule to be used in growth-promoter replacement in broiler feed. Cinnamaldehyde is an aromatic molecule present in cinnamon (*Cinnamomum verum*), which antibacterial and antioxidant activity has been also proved to be used in broiler nutrition (Reis et al., 2018). This phytogenic has beneficial properties for the maintenance of villus integrity, providing better intestinal absorption capacity, and enhancing performance and feed efficiency (Petrolli et al., 2012). Also, phytogenic compounds have synergic effects on animal performance, and a mixture of these components usually has more beneficial effects than molecules used alone (Galli et al., 2020).

The microencapsulation process is used to modify and improve the appearance and properties of some substances, also having an important function in decreasing interactions of the encapsulated substance with environmental factors, preventing sensorial and nutritional losses (Pereira et al., 2018). Microencapsulated molecules can be better distributed along the broiler intestine, because these molecules are protected from early absorption, remaining available for the jejunum and ileum. Therefore, the objective was to evaluate the influence of a blend of microencapsulated carvacrol and cinnamaldehyde in broiler feed on digestibility, caloric feed utilization, intestinal morphometry, serum biochemical parameters, and antioxidant parameters.

2. Material and Methods

2.1. Additive, animals, experimental design, and diets

This study was conducted in Xanxerê, Santa Catarina, Brazil (Latitude: −26.8364531149; Longitude: −52.4079407059). The experimental protocol for evaluation and analysis was submitted to the local animal ethics commission (protocol number 41/2018) and after approval, research activities were initiated.

The additive used in the experiment consisted of a blend of compounds derived from essential oils of cinnamon and oregano. From cinnamon, the bioactive compound was cinnamaldehyde, from oregano, the bioactive molecule was carvacrol. The blend contained 300 g/kg of cinnamaldehyde, 200 g/kg of carvacrol, and 500 g/kg of calcium carbonate as the carrier (Enterosan, Phytobiotec, Canelinha, Brazil). The phytogenics were microencapsulated through coating by a thin lipid layer, through a spray chilling process (Pereira et al., 2018), aiming its dissolution only in the distal portion of broiler jejunum and ileum. Five treatments with varying levels of blend addition on broiler feed were evaluated: control (absence of carvacrol and cinnamaldehyde), 50, 100, 150, and 200 mg/kg of ration with 7% of active ingredients.

Broilers were previously raised (1 to 14 days) in a concrete-floor masonry shed, divided into boxes with wood shaving as litter material, receiving a standard diet without any phytogenic additive, to guarantee non-influence in the digestibility trial evaluation. Animals were raised according to the norms and management of the commercial farms and the strain manual. Diets were formulated according to Rostagno et al. (2017). Mashed feed (Table 1) and water were provided *ad libitum* throughout the experimental period. Three hundred and twenty males of the Cobb500 strain were allocated in an entirely randomized, ungraded design consisting of five treatments, and eight replicates (n = 8). At 14-24 days, the animals were placed in metabolic cages for digestibility testing. Birds were maintained in metallic cages, distributed on four floors, and equipped with manual feeders and nipple drinkers. Each cage had a tray for excreta collection, digestibility, and energy metabolism evaluation. After that, in the second phase, birds were allocated to wood shaving pens, and raised until 42 days old for intestinal morphometry evaluation and blood sample collections.

Table 1 - Feed composition and nutritional contents

	Starter phase (1-24 d)	Finishing phase (25-42 d)
Ingredient (g/kg as-fed)		
Corn	545.00	579.66
Soybean meal	361.65	309.00
Soybean oil	27.79	44.89
Dicalcium phosphate	18.30	18.64
Limestone	8.25	8.41
Salt	3.25	3.32
DL-Methionine (99%)	2.60	3.11
L-Lysine HCl	2.25	1.94
Choline chloride (60%)	1.00	1.00
Vitamin supplement[1]	15.00	15.00
Mineral supplement[2]	15.00	15.00
Calculated values (g/kg)		
Dry matter	884.51	886.00
Metabolizable energy (kcal/kg)	2950.00	3100.00
Crude protein	215.00	194.00
Digestible lysine	12.00	10.50
Digestible methionine	5.44	5.05
Digestible Met + Cys	8.39	7.75
Digestible threonine	7.55	6.84
Digestible tryptophan	2.46	2.13
Digestible arginine	14.14	12.27
Digestible valine	9.25	8.20
Calcium	9.02	8.24
Available phosphorus	4.51	4.10
Sodium	1.70	2.05
Potassium	8.49	7.46
Chloride	3.77	3.56
Ash	88.29	59.13
Neutral detergent fiber	113.37	109.84
Acid detergent fiber	46.77	43.86

[1] Vitamin supplement containing per kg of complete diet: vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 45 IU; vitamin B1, 3.0 mg; vitamin B2, 9.0 mg; vitamin B6, 6.0 mg; vitamin B12, 0.03 mg; pantothenic acid, 18.0 mg; biotin, 0.3 mg; vitamin K3, 4.5 mg; folic acid, 1.5 mg; nicotinic acid, 75.0 mg.
[2] Mineral supplement content per kg of complete diet: iron, 150.0 mg; copper, 30.0 mg; manganese, 240.0 mg; zinc, 150.0 mg; iodine, 3.0 mg; selenium, 0.40 mg.

2.2. Sample collection and energy calculation

The experimental period of digestibility was 10 days (14-24 age), five for adaptation of the bird to feed and management and five for the traditional total excreta collection method. The quantities of feed consumed and excreta produced per experimental unit were determined, and dry matter (DM; method 934.01; AOAC, 1995), nitrogen (method 990.03; AOAC, 1995), and crude fat (method 920.39; AOAC, 2012) contents were analyzed. Once the results of laboratory analyses of feed and excreta were obtained, retention values of diet protein and lipid content were calculated according to the following formula:

$$\text{Retention coefficient} = \frac{\text{g of nutrient in feces}}{\text{g of nutrient in diet}} \times 100 \quad \text{(Eq. 1)}$$

Feed and gross energy were determined in a bomb calorimeter, Leco AC500 (LECO Corporation, St. Joseph, MI, USA). Then, the calculation of apparent metabolizable energy (AME) and apparent metabolizable energy corrected for nitrogen retention (AMEn) was done using equations proposed by Matterson et al. (1965).

$$\text{Feed AME (kcal/kg)} = \frac{\text{GE ingested} - \text{GE excreted}}{\text{DM ingested}} \quad \text{(Eq. 2)}$$

$$\text{Feed AMEn (kcal/kg)} = \frac{\text{GE ingested} - (\text{GE excreted} - 8.22 \times \text{NB})}{\text{DM ingested}} \quad \text{(Eq. 3)}$$

in which GE = gross energy and NB = nitrogen balance (nitrogen ingested − nitrogen excreted).

At the end of the 14-24-day experimental period, birds were relocated to the concrete-floor masonry shed and kept in boxes until 42 days old for intestine and blood sample collection.

2.3. Intestinal morphometric analysis

After 42 days of the experiment, one animal per replicate was euthanized according to the guidelines and recommendations of CONCEA's Guide to Euthanasia Practices (Brasil/MCTI, 2013). After jejunum fragment collection for histological analysis, samples were processed and sectioned at 4 to 6 µm and stained with hematoxylin-eosin. Measurements of villus height and crypt depth were performed using the image analyzer Image pro Plus 1.3.2 (1994) (magnification 40X) under an optical microscope. From each slide (one per bird), 30 villi and 30 crypts were selected to obtain the average value of each section presented. To obtain the villus:crypt ratio, the value of the height of the intestinal villi was divided by the depth value of the adjacent crypt.

2.4. Serum biochemical, oxidant, and antioxidant parameters

For the evaluation of biochemical parameters, blood samples were collected from the brachial vein, 1 mL per animal, at 20 and 42 days of age. Serum was separated by centrifugation and stored at −20 °C until further analysis of serum concentrations of total protein, albumin, cholesterol uric acid, and aspartate aminotransferase (AST) activity. The biochemical tests were carried out using commercial kits (Analisa) specific for each of these parameters, measured using a Bioplus 2000 (Bioplus, Barueri, Brazil) semi-automatic analyzer. Globulin levels were calculated as the differences between total protein and albumin levels.

For oxidant evaluation, reactive oxygen species (ROS) production was assessed by determining dichloro-dihydro-fluorescein diacetate (DCFH-DA) oxidation (LeBel et al., 1992), which is hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity correlates to the amount of ROS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. The calibration curve was performed with standard DCF (0.1–1 µM), and data were calculated as micromoles DCF formed/mL of serum. Lipid peroxidation was determined as levels of thiobarbituric acid reactive substances (TBARS) in serum according to the method described by Jentzsch et al. (1996). Results were obtained by spectrophotometry at 535 nm and expressed in nmol of malondialdehyde (MDA)/mL of serum.

To evaluate antioxidant parameters, glutathione peroxidase (GPx) activity was measured using tert-butyl-hydroperoxide as a substrate (Wendel, 1981). The enzyme activity was determined by monitoring the nicotinamide-adenine-dinucleotide-phosphate (NADPH) disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH, and tissue supernatants. The results were calculated and expressed as U/mL of serum. The activity of glutathione S-transferase (GST) was measured according to Mannervik and Guthenberg (1981) with slight modifications; GST activity was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants (approximately 0.045 mg of protein); results were calculated and expressed as U/mL of serum. Superoxide dismutase (SOD) activity was determined according to the auto-oxidation principle of pyrogallol, inhibited in the presence of SOD. The optical density change

was determined kinetically for two minutes at 420 nm, at ten-second intervals according to the methodology described by Beutler (1984). The activity was expressed as U/mL of serum.

2.5. Statistical analysis

The results were subjected to the Shapiro-Wilk normality test. When normal, the analysis of variance used was parametric and if significant, subjected to linear and quadratic regression analysis and also to the Dunnett's test. The different levels of the exogenous enzymes were subjected to regression analysis using linear and quadratic functions. All analyzes were performed at a 5% significance level, using the R statistical program (R Core Team, 2013). The statistical model was as follows:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij}, \quad \text{(Eq. 4)}$$

in which Y_{ij} = dependent variable, μ = variable mean, β_i = effect of broilers, and ε_{ij} = experimental error associated with observation Y_{ij}.

3. Results

3.1. Feed energy values

There was quadratic effect (P<0.001) for values of AME expressed in DM ($Y = 3492 - 0.646x + 0.008x^2$) and as-fed basis ($Y = 3268 - 0.903x + 0.008x^2$) (Table 2). The feed supplemented with 200 mg/kg showed numerically higher values of AME than the control. There were differences (Table 2) in AMEn, expressed in natural matter basis ($Y = 3065 + 0.219x + 0.0005x^2$, P = 0.012) and DM ($Y = 3273 + 0.286x + 0.0005x^2$, P = 0.005), showing a quadratic effect (P = 0.005) and significance of samples subjected to the Dunnett's test (P<0.001) for AMEn in dry matter. Similar behavior was observed for the AMEn values with quadratic effect (P = 0.012) as a fed basis and averages with significance according to the Dunnett's test (P<0.001).

Table 2 - Metabolizable energy and digestibility of protein and ether extract in broilers fed various levels of microencapsulated carvacrol and cinnamaldehyde at 15-24 days of age

Item	Blend inclusion level[1] (mg/kg)					SEM	Linear effect	Quadratic effect	Dunnett
	0	50	100	150	200				
Feed intake (15-24 d, g)	325	323	323	340	319	18.23	0.312	0.214	0.323
DM AME (kcal/kg)	3473	3525	3491	3562	3706*	82.26	0.432	<0.001	<0.001
NM AME (kcal/kg)	3252	3281	3251	3313	3450*	76.62	0.142	<0.001	<0.001
DM AMEn (kcal/kg)	3274	3284	3297	3305	3310*	74.71	0.215	0.005	<0.001
NM AMEn (kcal/kg)	3306	3073*	3083*	3088*	3090*	69.58	0.215	0.012	<0.001
PD (g/100 g)	69.99	71.46	67.71	68.48	69.50	5.54	0.294	0.706	0.706
EED (g/100 g)	88.94	91.92	89.77	89.79	91.18	2.11	0.105	0.053	0.053

DM AME - dry matter apparent metabolizable energy; NM AME - natural matter apparent metabolizable energy; DM AMEn - dry matter apparent metabolizable energy corrected by nitrogen retention; NM AMEn - natural matter apparent metabolizable energy corrected by nitrogen retention; PD - protein digestibility; EED - ether extract digestibility; NS - non-significant; SEM - standard error of the mean.
[1] Blend containing 300 g/kg of cinnamaldehyde, 200 g/kg of carvacrol, and 500 g/kg of calcium carbonate as a carrier.
* Means differ from control treatment at 5% of significance level by Dunnett's test.

3.2. Digestibility of protein and ether extract

There was no effect (P>0.05) of the addition of carvacrol and cinnamaldehyde on the digestibility of protein and ether extract (Table 2) of feed according to the levels of phytogenics added.

3.3. Intestinal morphometric analysis

Inclusion of 100 mg/kg of carvacrol and cinnamaldehyde blend increased intestinal villus height according to the Dunnet's test (P = 0.033), crypt depth (P<0.001), as well as villus:crypt ratio (P<0.001). The inclusion of other levels did not cause changes (Table 3) in villus height, crypt depth, or villus:crypt ratios (P>0.05).

Table 3 - Intestinal histological analysis and feed intake of broilers fed various levels of microencapsulated carvacrol and cinnamaldehyde at 24-42 days of age

Item	Blend inclusion level[1] (mg/kg)					SEM	Linear effect	Quadratic effect	Dunnett
	0	50	100	150	200				
Feed intake (g)	3299	3315	3283	3304	3290	28.542	0.184	0.216	0.164
Villus height (µm)	637.24	687.59	787.63*	711.48	685.90	34.701	0.354	0.242	0.033
Crypt depth (µm)	59.00	52.92	53.54	76.48*	60.03	12.405	0.635	0.593	<0.001
Villus:crypt ratio	11.45	13.55	15.93*	9.96	12.03	0.436	0.790	0.774	<0.001

SEM - standard error of the mean.
[1] Blend containing 300 g/kg of cinnamaldehyde, 200 g/kg of carvacrol, and 500 g/kg of calcium carbonate as a carrier.
* Means differ from control treatment at 5% of significance level by Dunnett's test.

3.4. Biochemical indicators

Serum levels of AST did not change in both periods (20 and 42 days) (P>0.05) (Table 4). Levels of AST increased between the two collection periods evaluated in all groups, however, in a proportional way to all evaluated treatments, with no significant differences between them (P>0.05). Albumin levels significantly differed (Dunnett's test, P = 0.047) at 20 days of age in broilers; nevertheless, the same data did not show a significant quadratic effect (Table 4) (P>0.05). Total protein and globulin levels did not reflect a quadratic effect at the 20th day, but the same data were not significant when subjected to the Dunnett's test (P = 0.840 and P = 0.266, respectively). At 42 days of age, the means differed for total protein (P<0.001), albumin (P<0.001), and globulins (P<0.001) according to the Dunnett's test, and quadratic effect for total protein (P = 0.009).

Table 4 - Analysis of serum parameters in broilers fed various levels of microencapsulated carvacrol and cinnamaldehyde

Parameter	Blend inclusion level[1] (mg/kg)					SEM	Linear effect	Quadratic effect	Dunnett
	0	50	100	150	200				
				20 days					
AST (IU/L)	157.57	165.14	183.28	175.57	188.14	24.151	0.183	0.732	0.183
TP (g/dL)	2.62	2.75	2.95	2.83	2.77	0.562	0.164	0.840	0.840
Albumin (g/dL)	1.38	1.16	1.25	1.11*	1.40	0.213	0.256	0.070	0.047
Globulin (g/dL)	1.24	1.58	1.70	1.72	1.37	0.510	0.351	0.266	0.266
Cholesterol (mg/dL)	76.00	79.37	71.14	71.28	78.37	10.841	0.562	0.400	0.400
Uric acid (mg/dL)	6.21	6.38	5.35	4.02	6.78	2.151	0.260	0.169	0.168
				42 days					
AST (IU/L)	292.75	350.25	302.85	355.71	335.28	59.61	0.295	0.176	0.176
TP (g/dL)	2.98	3.01	3.64*	2.65	3.20	0.25	0.143	0.009	<0.001
Albumin (g/dL)	1.10	1.27	1.24	1.18*	1.40	0.08	0.264	0.154	<0.001
Globulin (g/dL)	1.88	1.73	2.40*	1.46*	1.80	0.25	0.255	0.175	<0.001
Cholesterol (mg/dL)	80.25	86.62	81.00	70.62	77.12	14.47	0.723	0.603	0.370
Uric acid (mg/dL)	6.68	5.25	5.14	4.46*	3.58*	0.86	0.092	0.219	0.040

AST - aspartate aminotransferase; TP - total protein; SEM - standard error of the mean.
[1] Blend containing 300 g/kg of cinnamaldehyde, 200 g/kg of carvacrol and 500 g/kg of calcium carbonate as a carrier.
* Means differ from control treatment at 5% of significance level by Dunnett's test.

Cholesterol values in broilers fed various levels of carvacrol and cinnamaldehyde microencapsulate showed no changes at 20 and 42 days of age (Dunnet's test, P = 0.400 and P = 0.370, respectively), with no linear or quadratic effects (Table 4). There were no changes in uric acid levels at 20 days of age (P = 0.168), but at 42 days, there was a tendency for a steady drop in uric acid due to increasing inclusion levels of carvacrol and cinnamaldehyde (P = 0.092), and lower uric acid levels (P = 0.040) in broilers fed 150 and 200 mg/kg of the blend than control (Table 4).

3.5. Lipid peroxidation and reactive oxygen species

There were no differences in TBARS levels at 20 days (P>0.05), but there was a quadratic effect (P = 0.007) at 42 days ($Y = 3.968 + 0.00005x + 0.003x^2$). Reactive oxygen species at 20 days showed higher levels at 50 mg/kg, and at 42 days, the same dose at 50 mg/kg presented the lowest values for this parameter (Dunnett's test, P<0.001).

3.6. Glutathione peroxidase, glutathione-S-transferase, and superoxide dismutase

At 20 days of age, there were increases in GPx (Dunnett's test, P = 0.007) and GST activities (Dunnett's test, P<0.001) at 50 mg/kg (Table 5). For SOD (P = 0.012), values were lower at 100, 150, and 200 mg/kg of the blend than in the control, with the tendency for quadratic effect (P = 0.060).

At 42 days, there were no differences among the doses for GPx, GST, and SOD activities (P>0.05). For GST, the dose of 100 mg/kg gave higher levels than the other doses studied (Dunnett's test, P = 0.023).

Table 5 - Analysis of serum oxidant and antioxidant parameters in broilers fed various levels of microencapsulated carvacrol and cinnamaldehyde

Parameter	Blend Inclusion levels[1] (mg/kg)					SEM	Linear effect	Quadratic effect	Dunnett
	0	50	100	150	200				
			20 days						
TBARS (nmol MDA/mL of serum)	4.045	6.187*	5.500	5.027	6.187*	0.7723	0.544	0.629	0.008
ROS (U DCF/mL of serum)	1.853	3.844*	1.827	1.749	2.299	1.2015	0.933	0.922	<0.001
GPx (U/mL of serum)	1.947	6.197*	3.917	2.818	4.617	0.7602	0.654	0.282	0.007
GST (U/mL of serum)	12.696	18.650*	14.739	12.289	12.372	2.3202	0.563	0.587	<0.001
SOD (U/mL of serum)	1.891	1.430	0.997*	1.181*	1.236*	0.1103	0.065	0.060	0.012
			42 days						
TBARS (nmol MDA/mL of serum)	3.958	4.067	3.783	3.474	2.775*	0.06	0.192	0.007	0.003
ROS (U DCF/mL of serum)	2.566	1.301*	2.549	1.904	2.515	0.69	0.781	0.598	<0.001
GPx (U/mL of serum)	3.485	2.369	6.414	2.917	3.881	1.41	0.892	0.258	0.051
GST (U/mL of serum)	12.248	11.407	18.139*	11.664	12.307	3.47	0.524	0.071	0.023
SOD (U/mL of serum)	1.361	1.099	3.450	2.033	1.217	0.46	0.401	0.326	0.264

TBARS - thiobarbituric acid reactive substances; MDA - malondialdehyde; ROS - reactive oxygen species; DCF - diclorofluorescin diacetate; GPx - glutathione peroxidase; GST - glutathione-S-transferase; SOD - superoxide dismutase; SEM - standard error of the mean.
[1] Blend containing 300 g/kg of cinnamaldehyde, 200 g/kg of carvacrol and 500 g/kg of calcium carbonate as a carrier.
* Means differ from control treatment at 5% of significance level by Dunnett test.

4. Discussion

Microencapsulated carvacrol and cinnamaldehyde have promising values regarding the improved feed energetic utilization, as well as beneficial effects on the intestinal mucosa and antioxidant effect in broilers. Improved AME and AMEn values were observed for 200 mg/kg supplementation level, suggesting an effect of carvacrol and cinnamaldehyde on broiler energy use. The data obtained for AME and AMEn were similar to results reported by Barreto et al. (2008), who did not find alterations in DM levels in diets supplemented with herbal extracts based on cinnamon, oregano, pepper, and clove.

Hernández et al. (2004) observed AME improvement, corroborating the results found in the present study. Jamroz et al. (2003) and Bravo et al. (2011) reported the stimulant effect of a mixture of carvacrol, cinnamaldehyde, and pepper essential oil, and suggested the ability of these phytogenics to increase biliary secretion, pancreatic enzyme activity, and brush border size.

No effect for CP and EE digestibility was found among broilers of different groups tested, agreeing with the findings of Barreto et al. (2008), Lee et al. (2003) and Petrolli et al. (2012), all of whom attribute the absence of response to the high feed digestibility of the diets and the low challenge in the housing. In addition, using highly digestible ingredients, the perception of increased digestibility of the phytogenic additives would be compromised.

Carvacrol and cinnamaldehyde cause morpho-histological changes in the gastrointestinal tract due to the attenuate mucosal damage and increased mucosal synthesis (Jamroz et al., 2006), improving intestinal health and influencing villus height. We also observed broilers fed 100 mg/kg of carvacrol and cinnamaldehyde had higher villous heights when compared with broilers fed the control diet, suggesting this level is appropriate to ensure better gut mucosal health. At 200 mg/kg of microencapsulated carvacrol and cinnamaldehyde, there was a reduction in villus height, suggesting a possible toxic effect occurred at higher supplementation doses.

Du et al. (2016) verified the supplementation of thymol and carvacrol in broiler feed decreases intestinal lesions caused by *C. perfringens* and increased villus:crypt ratio. Jerzsele et al. (2012) observed increased villus length in birds supplemented with carvacrol and ginger oil when compared with the control group. Hong et al. (2012), when supplementing broilers with oregano, anise, and citrus essential oils observed improved duodenal villus height, corroborating the findings observed in the present study. Morphological changes in gastrointestinal tissues caused by carvacrol and cinnamaldehyde inclusion in broiler diets may provide further information regarding the possible benefits to the digestive system, including perhaps synergistic effects of the use of various compounds, increasing their effectiveness of action as well as production efficiency. Higher villus:crypt ratio values were obtained in broilers supplemented with 100 mg/kg of carvacrol and cinnamaldehyde, which had a 15:1 ratio. Chickens with very deep crypts and low villus:crypt ratios might be experiencing enteric challenges, and the high villus:crypt ratio found in this study suggests a positive effect on the intestinal brush border, allowing the animal to achieve better intestinal health. This was also reported by Ahsan et al. (2018), but also suggests that higher levels of phytogenic compounds can cause some damage to the villi, reducing their height.

Broilers supplemented with microencapsulated carvacrol and cinnamaldehyde for up to 20 days did not show alterations in AST values, which remained within the normal range (275 IU/L, Campbell, 2006). At 42 days, AST levels in all groups increased to levels above the established reference values, suggesting the possibility of liver or muscle injury.

Cholesterol values in all groups were below the range of normal (100 to 250 mg/dL) (Campbell, 2006). These low values may also suggest hepatic injury, which would be interconnected with the AST values obtained in the present study. However, Koochaksaraie et al. (2011) reported that powdered cinnamon addition in broiler diets reduced serum levels of triglycerides, without affecting bird performance. Several active compounds, including thymol, carvacrol, and cinnamaldehyde can cause hypocholesterolemia by inhibiting the enzyme regulating cholesterol synthesis, 3-hydroxy-3-coenzyme A reductase (Lee et al., 2004), suggesting that it could have different effects on the metabolism of triglycerides in broilers. Nabiela et al. (2013) observed that the inclusion of cinnamon powder in diets resulted in lower cholesterol levels in birds than diets containing growth-promoting antibiotics, but it did not affect serum triglyceride levels.

The values of total protein and albumin (Table 4) were within the normal values reported by Campbell (2006): 2.5 to 4.5 g/dL and 0.8 to 2 g/dL, respectively. Santos-Buelga and Scalbert (2000) and Scalbert and Willianson (2000) stated the effect (or absence thereof) of herbal extracts can be attributed to the diverse biochemical transformations that affect the bioavailability of the polyphenols present in carvacrol and cinnamaldehyde, as well as to their effectiveness. The absorption of these compounds

varies because of molecular structure diversity, which determines the extent of their intestinal absorption and the nature of plasma circulating metabolites. Albumin is the main protein responsible for polyphenol compounds binding; its affinity for albumin varies according to the chemical structure of the compound. The degree of binding to albumin can affect the route of excretion of metabolites as well as their distribution to cells and tissues. Only half of the polyphenols from the diet are accessible to reaction with oxidizing agents (Santos-Buelga and Scalbert, 2000; Scalbert and Willianson, 2000).

Faix et al. (2009) demonstrated the antioxidant action of cinnamon essential oil in broiler chickens through activation of antioxidant enzymes, in addition to stimulating the phagocytic activity of macrophages, improving the immune response. According to Najafi and Taherpour (2014), powdered cinnamon in chicken diets may replace antibiotic growth promoters by maintaining performance and improving health parameters, such as serum lipid levels, red blood cell counts, and hemoglobin concentrations.

On serum uric acid (UA) levels measured in broilers at 42 days (Table 4), differences were found due to the increase of the levels of carvacrol and cinnamaldehyde, with doses of 150 and 200 mg/kg giving lower UA levels. Levels above 15 mg/dL suggest changes in renal function, possibly caused by several factors, including nephrotoxins, urinary obstruction, nephritis, and nephropathy associated with hypovitaminosis A (Campbell, 2006). Moreover, UA is used as an indicator of the efficiency of using aminoacids in broilers (Zhai et al., 2016). Therefore, UA increases are related to increased protein catabolism (Sahebi-Ala et al., 2021).

Digestion is accompanied by the formation of superoxide radicals, which can be released during the oxidation reactions, and these reactive oxygen radicals (ROS) can attack the surface of the intestinal mucosa, impairing the absorption of nutrients (Mishra and Jha, 2019). The same authors described cell components are susceptible to the action of ROS; however, the cell membrane is one of the most severely affected, because of lipid peroxidation that culminates in changes in membrane structure and permeability. The enzyme SOD catalyzes O_2 dismutation, converting it to H_2O_2 (Cao et al., 2019), which has a lower reactive potential and can be degraded by other enzymes. In the present study, we found that 50 mg/kg of carvacrol and cinnamaldehyde contributed to increased activity of GPx ($P = 0.007$) and GST ($P<0.001$), and reduced activity of SOD was ($P = 0.012$) at 20 days in birds fed 100, 150, and 200 mg/kg of the blend than the control.

The amounts of ROS and TBARS were significantly higher at doses of 50 and 200 mg/kg of carvacrol and cinnamaldehyde ($P<0.001$ and $P = 0.008$, respectively). This suggests that the effects of carvacrol and cinnamaldehyde in the first collection stage were not sufficient to control the enzymes responsible for cellular oxidative stress. Values of ROS in the control treatment were statistically similar to those of other treatments, except for 50 mg/kg. At 42 days, carvacrol and cinnamaldehyde were more efficient when compared with the control treatment in terms of ROS ($P<0.001$) at the dose of 50 mg/kg, as well as the activities of GPx, GST, and SOD, which presented lower values at the same dose but did not differ statistically; the assumption is there was less enzymatic activity with this carvacrol and cinnamaldehyde level. The enzyme GST reduces oxidative stress via the regulation of homeostasis, and herbal extracts were positively correlated with the activity of this enzyme ($P = 0.023$), while for TBARS, the effect was greatest at 200 mg/kg.

When evaluated in non-challenging conditions, phytogenic additives may not have a substantial impact. The low stocking density and longer periods of sanitary emptying may contribute to birds' health maintenance and productive performance. Some positive effects of phytogenics additives have been reported in commercial crops; however, there remains a considerable lack of knowledge regarding the mechanisms of action of these products, which may influence the results and efficiency of several studies carried out in this area.

5. Conclusions

The dose of 200 mg/kg of microencapsulated carvacrol and cinnamaldehyde has the best results for the metabolizability of nutrients, increasing metabolizable energy. However, supplementation at doses

of 100 and 150 mg/kg have the best effects on intestinal and serum antioxidant enzyme response, protecting against free radicals and having no deleterious effects on serum makers. Overall, the doses can be safely used in broiler diets.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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