

Broiler responses to dietary 3,4,5-trihydroxybenzoic acid and oregano extracts under *Eimeria* challenge conditions

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Abstract

This study was conducted to evaluate the efficacy of a combination 3,4,5-trihydroxybenzoic acid (THB) and oregano extracts (i.e., Carvacrol and Thymol) at intake/dietary different levels on growth performance, intestinal health indicators, immune responses and fecal oocyst shedding in broiler chickens under *Eimeria* challenged condition. A total of 336 one-day-old broilers were randomly assigned to one of six dietary treatments with seven replications per treatment. Dietary treatments were: i) Non-challenged bird without any dietary treatment (NCNT), ii) Challenged bird without any dietary treatment (CNT), iii) Challenged birds fed a THB diet (0.1 g/kg, THB), iv) Challenged birds fed a combination of THB and oregano extracts diet (0.1 g/kg, COM 100), and a gradual increase of combination of THB and oregano extracts likely v) 0.15 g/kg (COM 150), and 0.2 g/kg (COM 200). On day 14, all groups except for NCNT have orally challenged with a 10-fold dose of Livacox[®] T anticoccidial vaccine to trigger coccidiosis. The results indicated that *Eimeria*-challenged broilers fed COM 100 and COM 200 diets increased ($p < 0.05$) body weight than CNT diet on day 35. Furthermore, birds fed COM 100 and COM 200 diets increased ($p < 0.05$) average daily gain compared to those fed CNT diets for the entire experimental period. There is no significant ($p > 0.05$) in average daily feed intake, feed efficiency between NCNT and birds fed with combined THB and oregano extracts for the entire experimental period. A combination of THB and oregano extract regardless of concentration levels or THB alone reduced ($p < 0.05$) lesion score in ileum compared to the CNT diet for 7 days post-infection (dpi). Birds fed COM 100 diet had lower ($p < 0.05$) intestinal lesion scores in jejunum and caeca on 7 dpi compared to those were in the CNT diet. No ($p > 0.05$) difference was observed in the oocysts per gram of feces count, intestinal morphology, carcass traits and blood cytokine concentration among the infected treatments. Collectively, we conclude that birds fed with a combination of THB and oregano extracts regardless of the ratios that were used demonstrated better recovery of health after the coccidial challenge than using only THB alone.

Keywords: Broiler, Coccidiosis, Growth performance, Oregano extract, 3,4,5-Trihydroxybenzoic acid

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Competing interests

The authors Hans Lee, Vannie Wan, Noele Kai Jing Ng and Chuan Hao Tan are employees of Kemin Industries Asia Pte Ltd., declare no conflict of interest.

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

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Ethics approval and consent to participate

The experimental procedures were reviewed and approved by the Animal Ethics Committee of the Chungnam National University (202006A-CNU-092).

INTRODUCTION

Coccidiosis is a major gastrointestinal parasitic disease in the poultry industry and is caused by several *Eimeria* parasite species [1]. Annually, coccidiosis causes over USD 14.5 billion losses worldwide due to high mortality and its negative effects on growth performance [2].

To control avian coccidiosis, anticoccidial drugs or attenuated vaccines are being used as the most common management practices in the poultry industry [3]. Although anticoccidial products are cost-effective in managing coccidiosis, the occurrence of drug resistance and public demands for antibiotic-free meat has encouraged the development of alternative strategies to manage coccidiosis [4]. Live vaccines are another approach to control coccidiosis, however, they cause severe reactions that may affect the performance of broilers in cases of poor management [5]. Resultingly, attenuated vaccines with reduced pathogenicity have been developed, however, these are costly to produce [6]. Consequently, the use of phytochemicals and herbals is an alternative strategy for the safe and effective control of coccidiosis [7,8]. These are natural products that have medicinal properties against *Eimeria* parasites and have not been associated with the development of antimicrobial resistance [9].

The phenolic compound 3,4,5-trihydroxybenzoic acid (THB), also known as gallic acid, is found in several vegetables and fruits [10]. According to previous studies, THB has disease-suppressing biological activities, such as anti-inflammatory, antioxidant, and antibacterial [11–13]. Although THB possesses these benefits, studies that reported the utilization of THB have been limited because of its low bioavailability, poor absorption, and rapid elimination from the body [14,15].

One of the well-established methods for controlling avian coccidiosis is the supplementation of essential oils [16,17]. Oregano is a traditional herbal remedy that enhances growth performance and intestinal integrity in broiler chickens with coccidiosis [7,18,19]. Carvacrol and thymol of plant-derived essential oils have been analyzed as they exhibit abundant biological properties, including antioxidant, antifungal, antibacterial, and antiprotozoal effects against *Eimeria* [18,20,21].

To our knowledge, studies on the efficacy of THB treatment and combination THB treatment with oregano extracts on coccidiosis in broilers remain limited. Therefore, our study aimed to investigate and compare the influence of various combinations of THB, and oregano extracts and THB, on growth performance and cocci elimination in broilers for a 35-day post-hatch period. We hypothesized that the properties of THB and oregano extracts, or the synergistic mechanism between THB and oregano extracts, could effectively achieve the anticoccidial effect and improve growth performance in infected broilers.

MATERIALS AND METHODS

The experimental procedures were reviewed and approved by the Animal Ethics Committee of the Chungnam National University (202006A-CNU-092).

Birds and housing

A total of 336 one-day-old broiler chickens (Ross 308) were received from the local hatchery (Pyeongtaek, Korea) and housed in raised battery cages (76 × 61 × 46 cm³), with similar body weights (BW) and weight distributions (47.43 ± 0.05 g). Each pen was equipped with two nipple drinkers and a metal trough. The room temperature was managed according to the Ross 308 broiler management guideline [22]. Birds were offered the experimental diets on an *ad libitum* basis and had free access to fresh clean drinking water via nipple drinkers throughout the experimental period. The lighting was continuous for 24 hours.

Experimental design, treatments and diets

Birds were allocated into one of six dietary treatments in a completely randomized design. Each treatment contained seven replicate cages with eight birds per each. Dietary treatments were as follows: i) Non-challenged bird without any dietary treatment (NCNT), ii) Challenged bird without any dietary treatment (CNT), iii) Challenged birds fed a THB diet (0.1 g/kg, THB), iv) Challenged birds fed a combination of THB and oregano extracts diet (0.1 g/kg, COM 100), and a gradual increase of combination of THB and oregano extracts likely v) 0.15 g/kg (COM 150), and 0.2 g/kg (COM 200). Diets were formulated based on corn and soybean meal to meet the Ross 308 nutritional specifications [23] (Table 1) to make dietary treatments, the basal diets were with or without supplementing either THB (VANTIPEARL™ 201, Kemin Industries Asia Pte, Senoko Drive, Singapore) alone or THB with oregano extracts (2.3:1 ratio; ORSENTIAL™ EXTEND, Kemin Industries Asia Pte). In addition, Cr₂O₃ (Chromium oxide powder, > 99.9% purity, Sigma-Aldrich, St. Louis, MO, USA) was added as an internal indigestible marker for digestibility analysis in a proportion of 0.3% to all experimental diets.

Table 1. Composition (g/kg, as-fed basis) of the experimental diets

Item	Starter feed (Week 1–3)	Grower feed (Week 4–5)
Corn	48.51	60.96
Wheat	8.40	4.12
Wheat bran	4.10	-
Soybean meal 48%	31.15	27.62
Vegetable oil	3.30	3.30
Limestone	1.20	0.95
Mono-calcium phosphate	1.65	1.39
Salt	0.30	0.35
Vitamin-mineral premix ¹⁾	0.30	0.30
Lysine-HCl	0.34	0.30
DL-Methionine	0.20	0.19
L-Threonine	0.13	0.11
L-Cystine	0.12	0.11
Cr ₂ O ₃	0.30	0.30
Calculated values ²⁾		
Crude protein (%)	22.0	20.0
Metabolizable energy (kcal/kg)	3050	3200
Lysine (%)	1.40	1.20
Methionine (%)	0.64	0.60
Methionine + cystine (%)	1.00	0.93
Calcium (%)	0.94	0.80
Phosphorus (%)	0.43	0.37
SID lysine	1.25	1.12
SID methionine	0.60	0.56
SID methionine + cystine	0.92	0.85

¹⁾Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D₃, 2,500 IU; vitamin E, 30 IU; vitamin K₃, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B₁₂, 12 µg; Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn, 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

²⁾The values were calculated based on the values of feedstuffs in [24] to meet or exceed the [23].

SID, standardized ileal digestible.

Growth performance evaluation

BW of the birds were recorded individually on day 1 of the experiment and designated as the initial BW. Subsequently, BW and feed intake were measured on day 7, 14, 21, 28 and 35. Based on the measured BW and feed intake data, average daily gain (ADG), average daily feed intake (ADFI) including mortality correction, and feed conversion ratio (FCR) were calculated on a pen basis.

Coccidiosis challenge

All the experimental groups except the NCNT group were challenged by tenfold overdosing of recommended dosage of (LIVACOX® T, Biopharm, Prague, Czech Republic) live vaccine on day 14. One milliliter of inoculum (i.e., 0.1 mL of vaccine with 0.9 mL of sterile distilled water) was orally gavaged directly into the crop to each bird in the challenged groups while the NCNT were similarly gavaged the same amount of sterile distilled water. According to manufacturer specification, the LIVACOX® T vaccine containing live oocysts of *Eimeria acevulina*, *E. maxima* and *E. tenella* strains.

Post-mortem procedure and sample collection

Seven birds per treatment that closer to the mean BW was selected, fasted and euthanized by cervical dislocation for sample collection on day 18, 21 and 35. The dressing percentage of meat with giblets (i.e., heart, gizzard and liver) was calculated by dividing it by the live weight of the birds. Drumsticks (skinless) and breast meat were removed from carcasses and weighed. The percentages of breast meat and drumstick were calculated as a percentage of the relative carcass weight of broilers [25].

Blood sample collections were carried out on days 18 and 21 of the experiment. Blood samples were collected from the brachial vein into a vacutainer coated with lithium heparin (BD Vacutainer, BD, Franklin Lakes, NJ, USA) before euthanizing the birds. Collected blood samples were quickly transferred to a laboratory for plasma separation.

Abdominal incisions were made on each sacrificed bird and the ileum was separated from the gastrointestinal tract. The ileum was defined as the segment of the small intestine that extended from Meckel's diverticulum to the ileocecal junction [26]. The removed ileal samples (3 cm piece) were flushed with ice-cold phosphate-buffered saline (PBS, pH 7.4) and placed into plastic containers that contained 10% formaldehyde for fixation and stored until microscopic slide preparation [27,28]. The remaining digesta of the ileal segment was gently stripped into labeled plastic containers and quickly stored at -20 °C freezer until further analysis.

Sample preparation and laboratory analyses

Collected blood samples were centrifuged (LABOGENE 1248R, Gyrozen, Daejeon, Korea) at 3,000×g for 10 min at 4 °C and the plasma was separated and stored at -80 °C (UniFreez U 400, DAIHAN Scientific, Wonju, Korea) until analysis. The concentrations of interleukin 1β (IL-1β), IL-10, interferon-gamma (IFN-γ), tumor necrosis factor α (TNF-α) in plasma were quantified using commercially available ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturers' instructions described by [29,30].

To analyze the ileal morphometry we followed the method described by [31]. Briefly, ileal samples fixed in 10% formaldehyde were used for sample preparation. Ring-shaped ileal tissue samples, six diagonal histological sections (4–6 μm), were excised and dehydrated, followed by impregnation in paraffin wax. The height of 10 well-align villi and their associated crypts were observed with an inverted microscope (Eclipse TE2000, Nikon Instruments, Tokyo, Japan) and the height and width of the villi and the deep of the crypts were measured through the analysis

of images of histological sections made from the computerized image-capture software (NIS-Elements Viewer software, Version: 4.20, NIS Elements, Nikon). The height of the villi is defined as the distance from their tip to the base and the width of the villi was measured at the half-height point. The depth of the crypt was defined as the distance from the top of the crypt to muscularis mucosa [32].

Intestinal lesion scoring

Before ileal samples were collected, one bird from each pen was examined for the presence and the degree of coccidiosis lesion. Lesion scores in the experimental broilers were determined from observing lesions in the digestive tract including jejunum (from the insertion of the duodenal mesentery to the Meckel's diverticulum), ileum and caeca. In general, lesions in these regions correspond grossly to natural predilection sites for *E. acervulina*, *E. maxima*, and *E. tenella*, respectively. Based on the seriousness of the lesions, a scale of 0 (no lesions), 1 (mild lesions), 2 (moderate lesions), 3 (severe lesions) or 4 (extremely severe lesions) was recorded for each chicken according to the estimation by [33].

Oocysts shedding

On days 7, 8, 9, 10 and 11 post-infection, excreta (free from feathers and feed) was collected per cage for oocyst counting and kept in separate airtight plastic bags. Sample bags were stored at 4°C until analysis. The Oocysts per gram feces (OPG) count was measured using the McMaster method of [34] and the procedures by [34] with adjustment. In summary, 4 g of fecal sample was put in a 56 mL saturated salt solution (floatation solution) and the mixture was filtered carefully. Then, the filtrate was loaded into both chambers of the McMaster counting slide using a micropipette and kept for five minutes before counting. The number of oocysts in the chambers was counted separately by observing under a 10 × 10 magnification compound microscope (Eclipse, TE 2000, Nikon Instruments). The OPG count in each replicate was calculated by multiplying the number of oocysts counted in the McMaster chambers by the factor 50 and the final results were expressed as log₁₀ oocysts count per gram of feces for each treatment [35,36].

Statistical analyses

Data were analyzed according to a completely randomized design, using a general linear model (GLM) procedure of one-way ANOVA of SPSS software (Version 26; IBM SPSS 2019, IBM, Armonk, NY, USA). A single pen was used as the experimental unit for all growth performance measurements and OPG counting. Selected individual birds were considered as the experimental unit for the proportion of carcass trait weights, blood cytokines, ileal morphology, and lesion score. When treatment effects were significant ($p < 0.05$), means were separated using Tukey's multiple range test procedures of SPSS software.

RESULTS

Growth performance

The effects of dietary THB and oregano supplementation on the growth performance of *Eimeria*-challenged broiler chickens from hatching to 35 days post-hatch are presented in Table 2. There was no difference ($p > 0.05$) in the BW of broilers from days 1 to 14. However, all *Eimeria*-challenged broilers (after day 14) had reduced BW ($p < 0.05$) compared to NCNT. On day 35, birds fed COM 100 and COM 200 diets had improved BW ($p < 0.05$).

Birds in CNT showed a lower ADG compared to NCNT birds from day 15 to 35 following

Table 2. Effect of dietary 3,4,5-trihydroxybenzoic acid and oregano supplementation on growth performance of coccidiosis challenged broiler chickens¹⁾

Period	Dietary treatment ²⁾						SEM	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
Body weight (g)								
Day 1	47.38	47.46	47.55	47.36	47.48	47.52	0.052	0.883
Day 7	176.21	167.55	168.38	168.52	168.02	173.98	1.073	0.068
Day 14	441.50	425.00	428.89	445.98	440.03	445.98	4.013	0.558
Day 21	905.50 ^b	707.07 ^a	736.04 ^a	771.44 ^a	771.28 ^a	759.90 ^a	12.190	0.001
Day 28	1,481.52 ^b	1,162.39 ^a	1,195.45 ^a	1,256.91 ^a	1,251.84 ^a	1,252.86 ^a	19.121	0.001
Day 35	2,090.51 ^c	1,692.43 ^a	1,802.57 ^{ab}	1,857.08 ^b	1,840.66 ^{ab}	1,861.68 ^b	23.482	0.001
Average daily gain (g/d)								
Day 7	18.40	17.16	17.26	17.31	17.22	18.07	0.154	0.066
Day 14	37.90	36.78	37.22	39.64	38.86	38.86	0.524	0.624
Day 21	66.29 ^b	40.30 ^a	43.88 ^a	46.49 ^a	47.32 ^a	44.85 ^a	1.502	0.001
Day 28	82.29 ^b	65.05 ^a	65.63 ^a	69.35 ^a	68.65 ^a	70.42 ^a	1.243	0.001
Day 35	87.00 ^b	75.72 ^a	86.73 ^b	85.74 ^b	84.12 ^{ab}	86.97 ^b	1.063	0.007
Day 1–14	28.15	26.97	27.24	28.47	28.04	28.46	0.286	0.552
Day 15–35	78.52 ^c	60.35 ^a	65.41 ^{ab}	67.20 ^{ab}	66.70 ^{ab}	67.42 ^b	1.058	0.001
Day 1–35	58.38 ^c	47.00 ^a	50.14 ^{ab}	51.71 ^b	51.23 ^{ab}	51.83 ^b	0.671	0.001
Average daily feed intake (g/d)								
Day 7	24.17 ^a	24.26 ^{ab}	27.16 ^{bc}	27.53 ^c	28.06 ^c	27.19 ^c	0.358	0.001
Day 14	54.87	51.82	51.26	53.73	53.20	54.62	0.668	0.569
Day 21	93.75 ^b	73.39 ^a	73.00 ^a	65.63 ^a	72.70 ^a	70.76 ^a	1.624	0.001
Day 28	117.94	113.58	110.94	113.62	115.97	113.81	0.986	0.445
Day 35	133.42 ^{ab}	131.96 ^a	140.36 ^c	139.42 ^{ab}	140.36 ^c	141.26 ^c	1.022	0.015
Day 1–14	39.52	38.04	39.21	40.63	40.63	40.91	0.439	0.393
Day 15–35	115.04 ^b	106.31 ^a	108.10 ^a	106.22 ^a	109.68 ^{ab}	108.61 ^{ab}	0.756	0.003
Day 1–35	84.83 ^b	79.00 ^a	80.54 ^{ab}	79.99 ^{ab}	82.06 ^{ab}	81.53 ^{ab}	0.540	0.026
Feed conversion ratio (g/g)								
Day 7	1.32 ^a	1.42 ^{ab}	1.58 ^{ab}	1.59 ^{bc}	1.64 ^c	1.51 ^{abc}	0.026	0.001
Day 14	1.47	1.41	1.38	1.36	1.38	1.42	0.025	0.891
Day 21	1.42 ^a	1.83 ^b	1.69 ^{ab}	1.43 ^a	1.54 ^{ab}	1.60 ^{ab}	0.036	0.002
Day 28	1.44 ^a	1.75 ^b	1.70 ^b	1.65 ^{ab}	1.70 ^b	1.62 ^{ab}	0.026	0.005
Day 35	1.54	1.75	1.62	1.64	1.68	1.63	0.022	0.135
Day 1–14	1.41	1.41	1.44	1.43	1.45	1.45	0.021	0.989
Day 15–35	1.47 ^a	1.76 ^c	1.66 ^{bc}	1.59 ^{ab}	1.65 ^{bc}	1.62 ^{abc}	0.020	0.001
Day 1–35	1.46 ^a	1.68 ^b	1.61 ^b	1.55 ^{ab}	1.60 ^{ab}	1.58 ^{ab}	0.017	0.002

¹⁾Values are the mean of six replicates per treatment.

²⁾NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

^{a-c)}Values in a row with different superscripts differ significantly ($p < 0.05$).

the *Eimeria* challenge. Nevertheless, birds fed a COM 200 diet showed higher ADG ($p < 0.05$) than birds fed a CNT diet from day 15 to 35.

Feeding of COM 150 and COM 200 diets was not significant ($p > 0.05$) in the ADFI of the broilers in the NCNT from day 15 to 35. Additionally, all birds fed a diet supplemented with THB and/or oregano extracts were not significant ($p > 0.05$) in ADFI with NCNT during the overall experimental period.

Moreover, there was no difference ($p > 0.05$) in feed efficiency between treatments during the starting period before the coccidian challenge. After the challenge, birds fed a diet supplemented with COM 100 had a better FCR ($p < 0.05$) than did the CNTs from day 15 to day 35. Furthermore, broilers fed with combined THB and oregano extracts (i.e., COM 100, COM 150, and COM 200) showed no significant ($p > 0.05$) FCR compared to NCNT during the overall experimental period.

Lesion score

Data in Table 3 represent the effects of dietary THB and oregano supplementation on separated lesion scores for the jejunum, ileum, and caeca in the broiler digestive tract. Broilers fed COM 100, COM 150, and COM 200 diets showed a lower ($p < 0.05$) lesion score for the jejunum at 4 dpi when compared with coccidiosis-infected broilers. Moreover, broilers fed the COM 100 diet had lower ($p < 0.05$) lesion scores of 0.93 for the jejunum, whereas COM 150 birds had lower ($p < 0.05$) lesion scores of 0.57 for the ileum compared with those of challenged birds at 7 dpi. Furthermore, all birds fed a diet supplemented with THB and/or oregano extracts had lower ($p < 0.05$) lesion scores for the ileum compared with those of CNT at 7 dpi. Additionally, lower ($p < 0.05$) lesion scores of 0.79 for the caeca were observed in the COM 100 group compared to the coccidiosis-infected broilers at 4 dpi.

Oocysts per gram feces count

The results presented in Table 4 indicate that oocysts are undetected in the excreta obtained from the NCNT group. The oocyst shedding pattern showed an increase ($p < 0.05$) at 7 and 8 dpi for

Table 3. Effect of dietary 3,4,5-trihydroxybenzoic acid and oregano supplementation on lesion score in caeca, jejunum and ileum of coccidiosis challenged broiler chickens¹⁾

Period	Dietary treatment ²⁾						SEM	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
Jejunum								
4 dpi	0.00 ^a	2.79 ^c	2.14 ^{bc}	1.71 ^b	1.79 ^b	1.57 ^b	0.153	0.001
7 dpi	0.00 ^a	2.00 ^c	1.14 ^{bc}	0.93 ^{ab}	1.21 ^{bc}	1.64 ^{bc}	0.134	0.001
Ileum								
4 dpi	0.00 ^a	1.43 ^b	0.57 ^{ab}	0.71 ^{ab}	0.79 ^{ab}	0.64 ^{ab}	0.121	0.032
7 dpi	0.00 ^a	1.71 ^c	1.00 ^b	0.86 ^b	0.57 ^{ab}	0.79 ^b	0.098	0.001
Caeca								
4 dpi	0.00 ^a	1.64 ^c	1.29 ^{bc}	0.79 ^b	0.93 ^{bc}	1.00 ^{bc}	0.102	0.001
7 dpi	0.00 ^a	1.79 ^c	1.00 ^{bc}	1.43 ^{bc}	1.14 ^{bc}	1.07 ^{bc}	0.107	0.001

¹⁾Values are the mean of six replicates per treatment.

²⁾NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

^{a-c}Values in a row with different superscripts differ significantly ($p < 0.05$).

dpi, days post-infection.

Table 4. Effect of dietary 3,4,5-trihydroxybenzoic acid and oregano supplementation on oocysts per gram in feces of coccidiosis challenged broiler chickens¹⁾

Period	Dietary treatment ²⁾						SEM	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
Oocysts per gram count (Log ₁₀ Oocysts)								
7 dpi	0.00 ^a	4.57 ^b	4.15 ^b	3.85 ^b	3.92 ^b	3.53 ^b	0.384	0.001
8 dpi	0.00 ^a	3.83 ^b	4.27 ^b	3.99 ^b	4.25 ^b	4.02 ^b	0.389	0.001
9 dpi	0.00 ^a	3.30 ^b	3.15 ^b	3.80 ^b	3.73 ^b	3.71 ^b	0.350	0.001
10 dpi	0.00 ^a	3.16 ^b	3.13 ^b	3.73 ^b	3.51 ^b	2.90 ^b	0.341	0.001
11 dpi	0.00 ^a	3.02 ^b	2.38 ^{ab}	2.97 ^b	2.68 ^b	2.06 ^{ab}	0.308	0.014

¹⁾Values are the mean of six replicates per treatment.

²⁾NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

^{a,b}Values in a row with different superscripts differ significantly ($p < 0.05$).

dpi, days post-infection.

all the other treatments, excluding NCNTs and CNTs. Thereafter, a reduction in oocyte shedding was observed ($p < 0.05$) at 9, 10, and 11 dpi. There was no difference ($p > 0.05$) in the OPG count among the challenged treatments from 7–11 dpi.

Ileal morphology

The effects of coccidia-challenged broilers fed diets containing different dietary treatments on intestinal morphology are summarized in Table 5. There were no differences ($p > 0.05$) in villus height, crypt depth, and villus height : crypt depth ratio (V:C ratio) of broilers between all treatments at 4 dpi. NCNT birds had a higher villus height ($p < 0.05$) but a shorter crypt depth ($p < 0.05$) at 7 dpi. Consequently, the NCNT group had a higher V:C ratio ($p < 0.05$) among all treatments.

Table 5. Effect of dietary 3,4,5-trihydroxybenzoic acid and oregano supplementation on intestinal morphology of coccidiosis challenged broiler chickens¹⁾

Period	Dietary treatment ²⁾						SEM	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
Villus height (μm)								
4 dpi	873.25	743.82	810.59	785.06	754.67	812.24	17.330	0.308
7 dpi	877.45 ^b	427.76 ^a	577.46 ^a	581.74 ^a	508.52 ^a	540.85 ^a	33.207	0.001
Crypt depth (μm)								
4 dpi	123.28	127.04	143.64	124.05	116.49	123.34	3.322	0.281
7 dpi	89.21 ^a	263.91 ^b	208.07 ^b	249.08 ^b	254.70 ^b	237.39 ^b	13.300	0.001
Villus height : Crypt depth ratio (V : C)								
4 dpi	7.38	6.07	5.83	6.53	6.65	6.92	0.210	0.322
7 dpi	10.18 ^b	1.66 ^a	2.99 ^a	2.63 ^a	2.04 ^a	2.39 ^a	0.633	0.001

¹⁾Values are the mean of six replicates per treatment.

²⁾NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

^{a,b}Values in a row with different superscripts differ significantly ($p < 0.05$).

dpi, days post-infection.

Blood parameters

The effects of the experimental treatments on blood cytokine concentrations in broiler chickens at 4 and 7 dpi are presented in Table 6. There were no differences ($p > 0.05$) in blood cytokine concentrations between dietary treatments at 4 and 7 dpi.

Carcass traits

There were no differences ($p > 0.05$) in dressing percentage and relative breast meat weight of broilers between dietary treatments at 4, 7, and 21 dpi (Table 7). The relative drumstick weight was not significantly different ($p > 0.05$) among dietary treatments at 4, 7, and 21 dpi.

DISCUSSION

The present study was designed to investigate the effects of THB and oregano extracts on growth performance, lesion score, OPG count, blood cytokine concentrations, intestinal morphology, and carcass traits in coccidia-infected broilers. To date, there are few publications on the use of THB as a mitigant treatment for coccidiosis in the diet of broiler chicks. Considering the severity of coccidiosis and the shortcomings associated with anticoccidial drugs or vaccines, any effort made toward improving poultry husbandry conditions without using anticoccidial drugs or vaccines would be of fundamental importance to the production of poultry chains.

As hypothesized, our results showed that broilers challenged with coccidian protozoa adversely affected growth performance. After exposure to *Eimeria* spp., although there were no significant differences in growth performance indices, such as ADG and ADFI, among those fed with a diet supplemented with THB and/or oregano extracts, all diets containing THB and a combination of THB and oregano extracts (COM 100, COM 150, and COM 200) improved ADG from day 15 to 35. In particular, broilers fed COM 100 and COM 200 had higher ADG during the overall experimental period. The performance of these birds was closer to the results of the NCNT group, and a similar finding was reported by [37], who stated that broilers fed commercial diets with 10% polyphenolic extract improved the ADG of coccidiosis-infected chicks over the entire experimental

Table 6. Effect of dietary 3,4,5-trihydroxybenzoic acid and oregano supplementation on blood cytokine concentration of coccidiosis challenged broiler chickens¹

Period	Dietary treatment ²						SEM	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
4 dpi								
IL-1 β (ng/mL)	1.67	8.58	8.47	7.49	7.55	8.27	1.225	0.588
IL-10 (ng/mL)	6.20	8.14	8.14	7.16	7.86	7.94	1.526	0.999
IFN- γ (ng/mL)	2.21	4.44	4.02	3.61	3.86	3.93	0.552	0.914
TNF- α (pg/mL)	13.62	22.50	20.20	18.00	18.45	18.91	2.582	0.964
7 dpi								
IL-1 β (ng/mL)	6.21	11.65	9.46	10.73	9.88	10.65	1.306	0.899
IL-10 (ng/mL)	16.96	16.82	19.07	15.65	18.36	18.15	2.013	0.998
IFN- γ (ng/mL)	1.96	4.42	3.82	3.76	4.21	4.44	0.599	0.867
TNF- α (pg/mL)	6.48	9.70	7.63	6.54	7.07	7.38	1.014	0.959

¹Values are the mean of six replicates per treatment.

²NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

dpi, days post-infection; IL-1 β , interleukin-1 beta; IL-10, interleukin-10; IFN- γ , interferon-gamma; TNF- α , tumor necrosis factor-alpha.

Table 7. Effect of 3,4,5-trihydroxybenzoic acid and oregano supplementation on dressing percentage, relative breast meat weight and relative drumstick weight of coccidiosis challenged broiler chickens¹⁾

Period	Dietary treatment ²⁾						SEM ³⁾	p-value
	NCNT	CNT	THB	COM 100	COM 150	COM 200		
Dressing ratio ⁴⁾ (%)								
4 dpi	88.69	88.24	88.12	88.32	88.45	88.72	0.113	0.594
7 dpi	88.00	88.45	87.99	88.25	87.66	88.02	0.149	0.763
21 dpi	92.26	92.65	91.87	91.02	92.49	92.17	0.251	0.484
Relative breast meat weight ⁵⁾ (%)								
4 dpi	23.05	21.93	22.00	22.02	22.32	23.24	0.203	0.251
7 dpi	23.55	22.57	24.31	23.96	24.39	23.45	0.199	0.069
21 dpi	26.40	26.24	25.53	25.80	25.42	25.76	0.288	0.928
Relative drumstick weight ⁶⁾ (%)								
4 dpi	9.97	9.68	9.71	10.01	9.74	9.92	0.067	0.592
7 dpi	9.83	9.46	9.64	9.47	9.48	9.35	0.084	0.694
21 dpi	9.55	9.26	9.97	10.07	9.94	10.12	0.100	0.077

¹⁾Values are the mean of six replicates per treatment.

²⁾NCNT, non-challenged without any dietary treatment; CNT, challenged without any dietary treatment; THB, CNT with 3,4,5-trihydroxybenzoic acid at 0.1 g per kg; COM 100, CNT with combination of THB and oregano extract at 0.1 g per kg; COM 150, CNT with combination of THB and oregano extract at 0.15 g per kg; COM 200, CNT with combination of THB and oregano extract at 0.2 g per kg.

³⁾Pooled standard error of the mean.

⁴⁾(Carcass weight / Live body weight) × 100.

⁵⁾(Breast meat weight / Carcass weight) × 100.

⁶⁾(Drumstick weight / Carcass weight) × 100.

^{a,b)}Values in a row with different superscripts differ significantly ($p < 0.05$).

dpi, days post-infection.

period. [38] reported that 1.0% of the dietary *Galla Rhois* containing various tannin-derived components, including methyl gallate and gallic acid, improved ADG of coccidiosis-infected chicks at 5 and 10 dpi. Moreover, previous studies by [7,18] reported improved growth performance of infected broiler chickens receiving diets containing carvacrol and thymol compared with the infected control group. Phenolic compounds (i.e., gallic acid), oregano, and its key components (i.e., carvacrol and thymol) preserved gut integrity while stimulating endogenous digestive enzyme secretion of bile, mucus, and saliva, which boosts digestion, leading to enhanced growth performance, as observed in infected broilers [39,40]. Therefore, it is possible that the improved growth performance observed in the birds fed a diet with a combination of THB and oregano extracts in the present study may be due to the effects of individual components and synergistic mechanisms between the mixed components.

The use of polyphenolic extracts and oregano extracts to reduce the severity of gut lesions caused by *Eimeria* spp. has been reported in several studies [18,19,37]. In the current study, we observed that supplemental THB and combinations of THB and oregano extracts had beneficial effects on gut lesion scores in challenged broilers with coccidiosis *Eimeria*. This observation might be related to the antioxidative properties of the compounds used (i.e., individual THB and combinations of THB and oregano extracts). Antioxidants are known to reduce the cytotoxic effect of scavenging reactive oxygen species and supporting host immunomodulatory effects [16]. Furthermore, the antiparasitic properties of carvacrol and thymol might have reduced coccidial oocysts by reducing the coccidiosis-induced lesions [19].

Although there was no effect on the number of oocysts shedding from 7 to 11 dpi among challenged groups, several studies [3,38,41] have demonstrated similar oocyst shedding patterns to

the current study. In particular, THB and COM 200 showed numerically better OPG counts at 11 dpi compared with those of infected broilers. This significant effect of phenolic compounds suggests antibacterial mechanisms. [18] reported that the high lipid solubility and hydrophobicity of carvacrol and thymol led to interaction with the phospholipid bilayer in the *Eimeria* cell membrane, resulting in a change in membrane permeability for cations such as H⁺ and K⁺. Consequently, the collapse of the oocyst's cell occurs through energy losses (i.e., ATPase inhibition) and ion leakage (particularly calcium), resulting in water imbalance [19,42]. THB exhibited the same mechanisms through similar decreasing results due to its hydrophobic properties [43].

Ileal morphology functions as an indicator of gut health and nutrient absorption capacity in broiler chickens [44]. Villus height, crypt depth, and their ratios (V:C) are the histomorphometric indices that can be used to evaluate the development, condition, and digestive capacity of the small intestine [10,32]. Coccidiosis in broilers disrupts intestinal integrity, causing mucosal and submucosal tissue damage, which shortens the villi and reduces digestive enzyme activities. Consequently, digestion and absorption of nutrients are reduced [45,46]. In this study, dietary THB and incorporation of THB with oregano extracts in the diets resulted in no significant difference in ileal villus height and crypt depth, as well as V:C ratio, among infected broilers. Previous studies by [47,48] reported that feeding oregano essential oils improved the V:C ratio compared with coccidial groups, whereas there is scarce information in the literature to verify whether gallic acid could have a significant effect on ileal morphology due to *Eimeria* spp. infection in broilers.

Coccidiosis challenge or supplementation did not affect any of the cytokine profiles, including proinflammatory cytokines, such as IL-1 β , IFN- γ , TNF- α , and the anti-inflammatory IL-10. Ultimately, further research related to blood cytokine parameters between induced coccidiosis in broilers is required.

In the present study, at 4, 7, and 21 dpi, all treatments showed no difference in dressing percentage or breast meat yield. Moreover, all birds used in the experiments showed no major differences in relative leg meat weight. Nevertheless, the addition of THB and combined THB and oregano extract appeared to increase the relative leg meat weight in infected broilers at 21 dpi. Consistent with our results, an earlier study [7] reported that the addition of herbal components, including carvacrol and thymol, to coccidial-challenged broiler chickens did not affect carcass weight. Alternatively, [49] observed that the addition of oregano essential oil, such as carvacrol and thymol, to broiler diets increased carcass yield. Considering this, further studies related to carcass yield between infected broilers and phenolic compounds are required.

In conclusion, dietary THB and a combination of THB and oregano extract demonstrated significantly better performance and coccidia elimination effect in broiler chickens 35 days post-hatch. However, numerically, the combination of THB and oregano extracts, regardless of the ratios used, was better than using THB alone. These findings suggest that single or synergistic anticoccidial effects could be achieved through THB or a combination of THB and oregano extract (i.e., carvacrol and thymol) in challenged broilers.

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