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Thymol-carvacrol supplementation in broilers: impact on performance, blood biomarkers, and gut health

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Abstract The stress conditions are induced by a high-dose live coccidiosis vaccine, and thymol-carvacrol supplementation in broilers were significantly improved the feed conversion ratio (FCR) during the starter and grower phases as compared to the negative control. In contrast, antibiotic supplementation resulted in better growth performance throughout the rearing period. In addition, antibiotic supplementation lowered serum levels of the oxidative stress marker malondialdehyde (MDA) and the pro-inflammatory cytokine interleukin-17 (IL-17), with thymol-carvacrol supplementation showing comparable but less pronounced effects, followed by the negative control. In addition, antibiotic supplementation improved intestinal morphology as evidenced by the decreased crypt depth and increased villus height to crypt depth ratio, followed by thymol-carvacrol supplementation compared to the non-supplemented groups. These results suggest that thymol-carvacrol supplementation can improve the gut health and performance of broilers under stress conditions.

Keywords: Phytobiotics, Oxidative stress substances, Pro-inflammatory cytokine, Intestinal morphology

Introduction

Chicken meat is an important source of protein as it is easy to produce, has a short production time and provides high-quality nutrition at low cost. Chicken production relies on fast-growing breeds and intensive farming practices to

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achieve high yields (Brevik *et al*., 2020). However, intensive farming can lead to stress in broilers. To mitigate stress, producers often supplement chicken feed with antibiotics to reduce negative effects and promote growth (Paintsil *et al*., 2021). However, the use of antibiotics can lead to drug resistance and antibiotic residues in broiler products, posing a potential risk to consumers (Christy *et al*., 2018).

To solve this problem, natural growth stimulants with similar properties to antibiotics have been used as alternatives in broiler production. These natural growth promoters are derived from plants and include phytobiotic compounds, which are biologically active substances. For example, essential oils extracted from plants containing thymol and carvacrol are added to broiler feed (Suganya *et al*., 2016). These substances are known to promote production efficiency (Youssef *et al*., 2021), have antimicrobial effects (Xu *et al*., 2008; Chavan and Tupe, 2014), stimulate the immune system (Acamovic and Brooker, 2005; Mosleh *et al*., 2013), increase antioxidant enzyme activity (Hashemipour *et al*., 2013) and possibly improve intestinal morphology (Jamroz *et al*., 2006; Michiels *et al*., 2008).

Research is currently limited to determining the optimal levels of thymol and carvacrol as an alternative to antibiotics for rearing broilers under stress conditions. Determining the optimal dosage of these compounds is critical to their effective use as a replacement for antibiotics in broiler rearing. However, the benefit of additional feed additives to boost the animals' immunity to stress is not readily apparent under normal conditions. It is, therefore, necessary to stress the animals under controlled conditions. In this study, we exposed broilers to stress by administering a high dose of a live coccidiosis vaccine. The aim was to investigate the effects of antibiotics and thymol-carvacrol in broiler feed under high-dose vaccination stress on production performance, levels of the oxidative stress marker malondialdehyde (MDA), the proinflammatory cytokine interleukin 17 (IL-17) and intestinal morphology in broilers that had received a high-dose live coccidiosis vaccine.

Materials and methods

Animal ethics

All animal experiments were approved by the Ethics Committee at Kasetsart University, Thailand (ACKU66-AGK-028).

Animals, diets, treatments, and raising procedures

A total of 1,000 day-old male Ross chicks were reared for 35 days at the Animal Science Research and Development Center, Department of Animal Science, Faculty of Agriculture Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom Province. The chicks were randomly divided into four groups, each receiving the same basal diet supplemented with antibiotics and varying amounts of Next Enhance 150 (50% active component of 1:1thymol-carvacrol; Novus International Inc., St. Louis, MO). Treatments were T1 (positive control) was the basal diet supplemented with zinc-bacitracin (667 mg/kg feed) and salinomycin (500 mg/kg diet), T2 (negative control) was the basal diet only, T3 was the basal diet supplemented with thymol-carvacrol at 15 mg/kg diet and T4 was the basal diet supplemented with thymol-carvacrol at 30 mg/kg diet.

There were three feeding phases as starter (1-10 days old) with 22% protein, grower (11-21 days old) with 21% protein and finisher (22-35 days old) with 19% protein. The composition of the basal diet is shown in Table 1.

Table 1. Ingredient compositions and calculated analysis of experimental basal diet

Ingredient (%)	Starter	Grower	Finisher
Corn	52.43	55.86	61.77
Soybean meal (46% CP)	32.85	30.18	26.72
Full fat soybean	8	8	6
Rice bran oil crude	1.79	2.32	2.35
L-lysine	0.7	0.21	0.16
DL-methionine	0.39	0.33	0.29
Choline chloride 60%	0.01	0.01	0.01
Mono-dicalcium phosphate 21%	1.83	1.44	1.17
Calcium carbonate	1.39	1.05	0.93
Salt	0.41	0.41	0.41
Premix	0.2	0.2	0.2
Metabolizable energy (kcal/kg)	2,975.00	3,050.00	3,100.00
Crude protein (%)	22.54	21.11	19.26

Each treatment consisted of 10 replicates with 25 chicks in each replicate. The chicks were housed in a 1.95 m x 1.45 m room in a house with evaporative cooling and received water and feed ad libitum. In addition, a live coccidiosis vaccine (FORTEGRA®, *E. tenella*, *E. acervulina*, *E. mivati*, *E. maxima* strains) was administered orally to all chicks at 14 days of age at 20 times the usual dose to create stress conditions.

Data collection

Production performance

The body weight (BW) of all chickens was recorded at 1, 10, 21, and 35 days of age to calculate body weight gain (BWG) and average daily gain (ADG) periodically. Feed intake (FI) per bird per day was determined by recording each replicate's given feed and leftover feed, using the formula FI = (given feed – leftover feed) / number of days. From these data, the feed conversion ratio (FCR) was calculated using the formula FCR = feed intake/body weight gain.

Blood sampling and methods for measuring MDA and IL-17

At 12 and 22 days of age, one chicken was randomly selected from each replicate for blood collection. A volume of 3 milliliters of blood per chicken was drawn from the wing vein. Each blood sample was centrifuged at 3,000 rpm at 25 °C for 5 minutes. The serum was separated and stored at -20 °C for analysis.

Malondialdehyde (MDA) levels were measured using a thiobarbituric acid reactive substances (TBARs) assay. A UV-visible spectrophotometer was used to monitor the eluent's absorbance at 532 nanometers, following the procedure of Grotto *et al*. (2007), to determine MDA as an indicator of oxidative stress.

Interleukin 17 (IL-17) was analyzed as a pro-inflammatory cytokine marker according to the MyBioSource Version 13.0 manual, Chicken Interleukin 17 (IL17) ELISA Kit (MBS2020130) using a microplate reader ELISA with a wavelength of 450 nanometers.

Jejunum sampling and methods for measuring intestinal morphology

One chicken was randomly selected from each replicate at 12, 22, and 29 days of age to study the jejunum morphology. The method was modified by Iji *et al*. (2001). A 2 cm section from the middle of the jejunum was collected and fixed in 10% neutral buffered formalin. Two tissue sections, approximately 5 micrometers thick, were cut and placed on slides, then stained using Hematoxylin and Eosin staining techniques.

The stained tissue slides were examined under a microscope at 400x magnification. Images were analyzed using the Cellsens Digital Imaging software, Olympus, Tokyo, Japan, to study the histology. Villus height (VH) was measured from the brush-border membrane to the villi-crypt junction, and villus width (VW) was measured at the villi-crypt junction from left to right. The villus surface area was calculated using the formula (2π) $(VW/2)$ (VH) (Sakamoto *et al*., 2000). Crypt depth (CD) was measured from the basement membrane to the villi-crypt junction, and the ratio of villus height to crypt depth (VH/CD) was calculated (Rahman *et al*., 2017).

Statistical analysis

The experimental design used a completely randomized approach, with each treatment comprising 10 replicates, each containing 25 chicks. For the purposes of measuring other parameters, one chick was randomly selected from each pen and treated as an experimental unit. The statistical model's equation is as follows:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where Y_{ij} is Experimental group observation, μ is the overall mean, τ_i is the effect of treatment, and ε_{ij} is the random error.

Descriptive statistics was used to describe the data. The F-test analysis of variance (ANOVA) was used to analyze the mean differences between treatments. The Duncan new multiple range test was used to compare the means between the two groups. The values $P < 0.05$ were considered statistically significant. SPSS statistics software version 26 (SPSS Inc, Chicago, IL, USA) was used to analyze the data.

Results

Production performance

The effects of the antibiotic and thymol-carvacrol supplements on broiler performance from day 1 to 35 are shown in Table 2. During this period, there were not statistically significant differed ($P > 0.05$) in BW, BWG, ADG, and FI between chickens fed thymol-carvacrol supplements and those fed a basal diet. However, after 10 and 21 days, the chickens fed a diet supplemented with thymol-carvacrol at doses of 15 and 30 mg/kg showed significantly better FCR than those fed the basal diet ($P = 0.000$). Despite these improvements, their FCR remained higher than that of the chickens receiving antibiotics. Notably, chickens fed an antibiotic-enriched diet had significantly higher BW, BWG, ADG and FCR throughout the rearing period than the other groups.

Oxidative stress marker and Pro-inflammatory cytokine

The results of antibiotic and thymol-carvacrol supplementation on the levels of oxidative stress substances (MDA) and pro-inflammatory cytokines (IL-17) in broilers are summarized in Table 3. At 9 days of age, significant differences ($P < 0.05$) were found in MDA levels among the groups. The positive control group receiving antibiotics (T1) had the lowest MDA levels, which were not statistically different from those of group T4 supplemented with thymol-carvacrol at a dose of 30 mg/kg diet. Group T1 also had lower MDA levels than group T2 without any supplementation, which had the highest MDA levels. In addition, group T2 had significantly higher MDA levels than group T3 supplemented with thymol-carvacrol at a dose of 15 mg/kg diet (P < 0.05).

Table 2. Effect of antibiotics and thymol-carvacrol on production performance in broiler

Trait	T1	T2	T3	T4	Pooled SE	P-value
Initial weight	44.56	44.75	44.62	44.53	0.111	0.908
1-10 days of age						
Body Weight (g)	298.89	293.37	294.89	295.57	1.019	0.277
Body Weight Gain (g/b)	254.34	248.62	250.26	251.04	1.028	0.257
Average dairy gain (g/d)	25.44	24.86	25.03	25.11	0.103	0.256
Feed Intake (g/b/d)	291.93	294.18	292.80	293.15	1.137	0.925
FCR	1.15^{c}	1.18^{a}	1.17^{b}	1.17^{b}	0.002	0.000
11-21 days of age						
Body Weight (g)	1012.29^{a}	979.76^{b}	985.02^{b}	986.20^{b}	3.956	0.011
Body Weight Gain (g/b)	713.40^{a}	686.39^{b}	690.13^{b}	690.63^{b}	3.323	0.011
Average dairy gain (g/d)	64.86^{a}	62.40^{b}	62.74^{b}	62.78^{b}	0.302	0.011
Feed Intake (g/b/d)	959.81	949.34	943.85	944.17	4.077	0.490
FCR	1.35^{c}	1.38^{a}	1.37^{b}	1.37^{b}	0.003	0.000
22-35 days of age						
Body Weight (g)	2304.37^{a}	2180.97^{b}	2198.17^{b}	2203.30^{b}	11.518	0.000
Body Weight Gain (g/b)	1292.08^{a}	1201.21^{b}	1213.16^{b}	1217.10^{b}	9.385	0.001
Average dairy gain (g/d)	92.29^{a}	85.80^{b}	86.65^{b}	86.94^{b}	0.670	0.001
Feed Intake (g/b/d)	1979.32	2033.07	2025.75	2029.04	9.554	0.156
FCR	1.53^{b}	1.69^{a}	1.67^{a}	1.67^{a}	0.013	0.000
1-35 days of age						
Body Weight (g)	2304.37^{a}	2180.97^{b}	2198.17^{b}	2203.30^{b}	11.518	0.000
Body Weight Gain (g/b)	2259.81^{a}	2136.22^{b}	2153.55^{b}	2158.77^{b}	11.546	0.000
Average dairy gain (g/d)	64.57^{a}	61.04^{b}	61.53^{b}	61.68^{b}	0.329	0.000
Feed Intake (g/b/d)	3231.05	3276.59	3262.39	3266.36	9.625	0.385
FCR	1.43^{b}	1.53^{a}	1.51^{a}	1.51^{a}	0.008	0.000

[a,b,c] Means in column with different superscripts are significant different (P < 0.05).

IL-17 levels in broilers in group T2 without additives were significantly higher than in the positive control group receiving antibiotics (T1) and in groups T3 and T4 supplemented with thymol-carvacrol at a dose of 15 and 30 mg/kg diet ($P < 0.05$).

Under normal rearing conditions for nine days, thymol-carvacrol supplementation effectively reduced stress markers in chicken feed, comparable to antibiotics. In particular, thymol-carvacrol supplementation at a dose of 30 mg/kg showed similar efficacy to antibiotics in reducing MDA levels.

When broilers were exposed to stressful conditions at 14 days of age with a high dose of live coccidiosis vaccine, significant differences ($P < 0.05$) in MDA levels were observed between all experimental groups at 21 days of age. Broilers in the positive control group receiving antibiotics (T1) again had the lowest MDA values, which were not significantly different from those in the T4 group supplemented with thymol-carvacrol at a dose of 30 mg/kg diet and lower than those in the T2 group without supplementation. The MDA values in the T3 and T4 groups supplemented with thymol-carvacrol at a dose of 15 and 30 mg/kg diet did not differ significantly from each other. However, they were significantly lower than in the T2 group without supplementation ($P < 0.05$).

IL-17 levels in broilers of groups T3 and T4 supplemented with thymol-carvacrol at a dosage of 15 and 30 mg/kg diet were significantly lower than those of group T2 without supplementation ($P < 0.05$). Nevertheless, IL-17 levels in the positive control group receiving antibiotics (T1) differed significantly from those in the T2 group without supplementation and in the T3 and T4 groups supplemented with thymol-carvacrol at a dose of 15 and 30 mg/kg diet ($P < 0.05$).

Table 3. Effect of antibiotics and thymol-carvacrol levels on oxidative stress substance (MDA) and pro-inflammatory cytokine (IL-17) in broiler

Trait	T1	T2	T3	T4	Pooled SE	P-value
9 days of age						
Malondialdehyde (nmol/ml)	13.77^{c}	17.25^{a}	15.90^{b}	14.99^{bc}	0.313	0.000
Interleukin 17 (pg/ml)	9.25^{b}	12.01^{a}	10.18^{b}	9.90^{b}	0.158	0.000
21 days of age						
Malondialdehyde (nmol/ml)	11.03^{c}	12.59^{a}	12.17^{ab}	11.62^{bc}	1.424	0.000
Interleukin 17 (pg/ml)	17.21^{c}	24.46^{a}	21.30^{b}	21.10^{b}	2.953	0.000

[a,b,c] Means in column with different superscripts are significant different ($P < 0.05$).

Jejunum morphology

The effects of the antibiotic and thymol-carvacrol supplements on the morphology of the jejunum of broiler chickens are summarized in Table 4. At 9 days of age, no significant differences in intestinal development were observed among the experimental groups in terms of VH, VW, villus surface area, CD, and VH/CD ratio (P > 0.05). However, at 28 days of age, two weeks after inducing stress by administering a high-dose vaccine, dietary supplementation with antibiotics was found to be effective in promoting intestinal development. In addition, 30 mg/kg of thymol-carvacrol supplementation resulted in increased VW compared to broilers fed a basal diet (P = 0.000). The day of 35th, supplementation with thymol-carvacrol at both concentration levels (15 and 30 mg/kg) led to a reduced CD. Moreover, the 30 mg/kg significantly improved the VH/CD ratio (P = 0.000) compared to the basal diet group.

Table 4. Effect of antibiotics and thymol-carvacrol levels on intestinal morphology in broiler

Trait	T1	T2	T3	T4	Pooled SE	P-value
9 days of age						
Villus height (µm)	903.60	883.36	892.27	894.79	8.643	0.876
Villus width (µm)	133.53	131.06	131.21	131.55	1.131	0.866
Surface area (mm^2)	378.87	363.53	367.60	369.60	5.329	0.762
Crypt depth (µm)	142.95	146.83	145.48	145.01	1.554	0.852
VH/CD ratio	6.32	6.02	6.13	6.17	0.099	0.762
28 days of age						
Villus height (µm)	1393.10^{a}	1261.08^{b}	1302.34^{b}	1317.53^{ab}	15.955	0.028
Villus width (µm)	115.84^{a}	108.74^{c}	110.17^{bc}	110.69^{b}	0.565	0.000
Surface area (mm^2)	506.73^{a}	430.59^{b}	450.52^{b}	457.92^{b}	6.672	0.000
Crypt depth (µm)	143.43^{b}	151.15^{a}	148.55^{a}	148.35^{a}	0.790	0.003
VH/CD ratio	9.71^{a}	8.34^{b}	8.77^{b}	8.88^{b}	0.132	0.001
35 days of age						
Villus height (µm)	971.28	951.49	962.05	965.64	3.823	0.308
Villus width (µm)	114.40^{a}	109.95^{b}	111.20^{b}	111.62^{b}	0.464	0.001
Surface area (mm^2)	348.90^{a}	328.49^{b}	335.91^{b}	338.44^{b}	2.148	0.002
Crypt depth (µm)	143.19^{c}	150.35^{a}	148.01^{b}	147.92^{b}	0.637	0.000
VH/CD ratio	6.78^{a}	6.33^{c}	6.50^{bc}	6.53^{b}	0.043	0.000

^{a,b,c} Means in column with different superscripts are significant different (P < 0.05).

Discussion

The high-stress coccidiosis vaccine experiments revealed that the supplementation with 15 and 30 mg/kg thymol-carvacrol improved the FCR of the broilers during the first 21 days. However, the use of thymol-carvacrol was not as good as antibiotics, which improved the overall production performance of the broilers. In order to enhance growth performance with thymol-carvacrol, we might need to use a higher concentration. This was suggested by Hashemipour *et al*. (2013), who utilized thymol-carvacrol at levels ranging between 60 and 200 mg/kg. This concentration led to increased weight gain and improved feed efficiency in broiler chickens, although feed intake was reduced.

In addition, El-Ghousein and Al-Beitawi (2009) demonstrated that higher doses are required to significantly affect feed conversion ratio (FCR) and weight gain. In contrast, Hernández-Coronado *et al*. (2019) found no significant effect on the growth performance of broilers when supplemented with 400 mg thymol-carvacrol per liter of drinking water. This suggests that the efficacy of thymol-carvacrol may vary depending on the amount and route of administration, highlighting the importance of consistent diet or water supplementation protocols in influencing broiler performance.

In addition, it was observed that the broilers in the group without antibiotics and thymol-carvacrol supplementation (T2) had the highest MDA and IL-17 levels, which are indications of stress levels. In contrast, the broilers supplemented with antibiotics and thymol-carvacrol had lower MDA and IL-17 levels. This underlines the positive effect of antibiotics and thymol-carvacrol as dietary supplements in mitigating stress responses. Li *et al*. (2013) state that thymol carvacrol supplementation effectively scavenges free radicals and prevents peroxidation. Hashemipour *et al.* (2013) also reported that thymol-carvacrol supplementation reduces free radical levels, as evidenced by a reduction in MDA levels. Malondialdehyde is the principal and most significant final product of lipid peroxidation among free radicals, commonly utilized to assess oxidative damage. In addition, Amirghofran *et al*. (2016) suggested that thymol-carvacrol supplementation may have anti-inflammatory effects by promoting immune response and eliminating diseased cells. In addition, Gholijani and Amirghofran (2017) found that carvacrol and thymol have the potential to alleviate inflammatory symptoms by possibly downregulating IL-17 expression in serum and tissue. IL-17 is a significant pro-inflammatory cytokine known for its potent role as a mediator of inflammation, actively promoting inflammatory responses.

Thymol-carvacrol supplementation improved intestinal morphology, promoting nutrient absorption by increasing VW on day 28. Additionally, the 30 mg/kg Next Enhance 150 supplement (containing 15 mg/kg thymol and 15 mg/kg carvacrol) reduced CD and increased the VH/CD ratio on day 35. According to Peng *et al*. (2016), supplementation with thymol-carvacrol from oregano essential oil (13 mg/kg thymol and 6.50 mg/kg carvacrol) significantly reduced CD and increased the VH/CD ratio in broilers at 42 days of age, with no significant effect on villus height. Guo *et al*. (2021) emphasized the relationship between intestinal morphology, gastrointestinal health, and the growth of broilers. Factors such as CD, VW, and the VH/CD ratio play a crucial role in nutrient absorption and digestive processes.

In conclusion, under stress induced by a high-dose coccidiosis vaccine, thymol-carvacrol supplementation significantly improved feed conversion ratio (FCR) during the starter and grower phases compared to the negative control. Antibiotics led to better growth performance throughout the rearing period, reduced serum malondialdehyde (MDA) and interleukin-17 (IL-17) levels, and enhanced intestinal morphology by decreasing crypt depth and increasing villus height-to-crypt depth ratio. Thymol-carvacrol showed similar, though less pronounced, effects. These findings suggest that thymol-carvacrol supplementation can improve gut health and performance in stressed broilers.

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