Peer-reviewed version available at *Animals* **2020**, *10*, 481; doi:10.3390/ani10030481

Article

Dietary encapsulated essential oils improve production performance of coccidiosis vaccine-challenged broiler chickens

Jeong-Woo Lee [1], Da-Hye Kim [1], Yoo Bhin Kim [1], Su-Been Jeong [1], Sungtaek Oh [1,3], Seung-Yeol Cho [2], and Kyung-Woo Lee [1,*]

[1] Department of Animal Science and Technology, Sanghuh College of Life Sciences, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, South Korea; dlwjddn0123@naver.com (J.W.L.); kdh142536@naver.com (D.H.K.); ybin51@naver.com (Y.B.K.); jsbjsy317@naver.com (S.B.J.)

[2] EUGENE BIO, Suwon-si, Gyeonggi-do 16675, South Korea; eugenebio@naver.com (S.Y.C.)

[3] Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, US Department of Agriculture, Beltsville, MD, 20705, USA; ostpoultry@gmail.com (S.T.O)

* Correspondence: kyungwoolee@konkuk.ac.kr; Tel.: +82-2-450-0495

Simple Summary: The in-feed antibiotics have been banned worldwide and anticoccidial drug is also expected to be removed from the formulated, complete feeds. Thus, looking for alternatives to anticoccidials has been on the increase. Essential oils are naturally derived substances containing the aromatic components of herbs and spices and exhibit antibacterial/anticoccidial, antioxidant and immune modulating effects, of which properties render them being considered potential anticoccidial agents. Encapsulated essential oils are known to slowly release their active components upon passing the gastrointestinal tract for efficacy. This study was conducted to examine the effects of encapsulated thymol- and carvacrol- based essential oils on productivity and gut health of chickens challenged with high dose of coccidiosis vaccine.

Abstract: The present study was conducted to evaluate encapsulated essential oils as an alternative anticoccidial in coccidiosis vaccine challenged broiler chickens. A total of 600 day-old male broiler chicks were provided with no-added corn-soybean meal-based control diet or diets that contained either salinomycin or EO at 60 and 120 mg per kg of diet. On day 21, half of the control groups were orally challenged with a coccidiosis vaccine at 25 times higher than the recommended vaccine dose. During 22 to 28 days (i.e., one-week post coccidiosis vaccine challenge), the challenged chickens had decrease ($P < 0.05$) in body weight gain and feed intake but increase in feed conversion ratio compared with the non-challenged, naïve control chickens. However, dietary EO significantly counteracted ($P < 0.05$) coccidiosis vaccine-induced depression in body weight gain and feed intake. Increasing dietary EO linearly decreased ($P < 0.05$) the concentrations of the volatile fatty acids. Dietary SAL and EO affected gut morphology in chickens at 20 days posthatch. Increasing dietary EO linearly ($P = 0.073$) increased serum catalase activity. Collectively, our study shows that dietary EO increased coccidiosis vaccine-induced growth depression and altered gut physiology in broiler chickens.

Keywords: encapsulated essential oils; coccidiosis vaccine; growth performance; broiler chickens; gut health

1. Introduction

Avian coccidiosis is caused by several species of Eimeria, which are infectious protozoa that pe penetrate and damage the epithelial cells of intestinal tissue, resulting in intestinal inflammation and hemorrhage [1]. The intestinal damage decreases feed intake (FI), retards growth and suppress cell-

Peer-reviewed version available at *Animals* **2020**, *10*, 481; doi:10.3390/ani10030481

mediated immune response, all of which have significant adverse implications for the commercial poultry industry [2]. The financial loss by coccidiosis in the poultry industry worldwide has been estimated annually at US $3 billion, which is mainly due to prophylactic or therapeutic in-feed medications and compromised health status of afflicted chickens [3].

Nutrition-based strategies have been implemented to control avian coccidiosis caused by the *Eimeria* spp. [4]. Of note, the plant-derived essential oils (EO) have been explored as they exhibit antimicrobials, antioxidants, and immune regulation [5]. Among the EO studied, thymol and carvacrol are the major components of thyme or oregano EOs and likely to have similar mechanisms of antimicrobial activity [6]. In addition, [7] reported that the EO addition to feed increased the productivity of the *Eimeria*-infected broiler chickens. Due to the chemical nature of EO and their components being low molecular weights, they are rapidly absorbed in the upper segments of intestine upon ingestion, and known to directly or indirectly affect intestinal microflora and secretion of endogenous digestive enzymes [8]. In order to maximize the effect of EO as an anticoccidial agent, it is necessary to reach the infected area proximally to distally. Therefore, it is expected that the anticoccidial effect of EO will be enhanced if they are specially encapsulated to release active components slowing during passage of the gut thus enable to act on the *Eimeria* present in duodenum to ceca. Similar strategies with encapsulated EO (EEO) on necrotic enteritis or Salmonella has been reported [9,10].

The purpose of this study was to investigate the effects of thymol and carvacrol-based EEO on the productivity and gut health of broiler chicks inoculated with high doses of coccidiosis vaccine. In this study, we used live coccidiosis vaccine to induce experimental coccidiosis as documented elsewhere [11-13]. Earlier conflicting reports that Eimeria infection decreased rectal temperature [14] or dietary EEO relieved heat-stress chickens [15,16] attempted us to measure the body surface temperature of the challenged chickens.

2. Materials and Methods

2.1. Animal care

The experimental procedure was approved by the Institutional Animal Care and Use Committee of Konkuk University (KU18095).

2.2. Experimental design, animals and diets

A total of 600 day-old feather-sexed male broiler chicks (Ross 308) were obtained from a local hatchery. Upon arrival, they were individually weighed and randomly placed into 50 floor pens. The chicken facility was initially set at 32^{0}C, was gradually decreased to 25^{0}C at 3 weeks and then kept constant thereafter. The light was set with one-hour darkness per day. The windowless chicken facility was thoroughly disinfected before experiment, and fresh rice husks as a bedding material was used.

A corn and soybean meal-based diet was used as a control diet (Table 1) and the experimental diets were formulated by mixing the control diet with salinomycin (60 mg/kg)(SAL) or EEO preparations at the levels of 60 and 120 mg/kg of diet. The EEO preparations used contained equal concentration of thymol and carvacrol at the level of 140 g per kg of preparation as active components and microencapsulated (Vetagro SpA, Italy) to prevent loss during the pelleting process and/or to allow slow release upon ingestion to reach to distal intestine (e.g., cecum). It is reported that EEO added into mash or pellet diets were stable and able to release its active component throughout the intestine [17].

Day-old chicks were provided with either control or experimental diets from the beginning. Each treatment had ten replicates of 12 chicks each (n=120 chicks/treatment) except for the control group which had 20 replicates. At 21 days, half of control groups (n=10 replicates/treatment) and all experimental groups were orally gavaged with 25x recommended dose to induce coccidiosis [11-13]. Chickens not inoculated with coccidiosis vaccine were considered non-challenged control groups

(n=10 replicates/treatment). Feed intake and body weight per pen were measured weekly. Mortality was recorded when occurred and used to calculate mortality-adjusted feed conversion ratio.

Table 1. Ingredients and composition of the basal diets (as-fed basis)

Ingredient (g/kg)	Starter (0-21 d)	Finisher (22-35 d)
Maize, 8.8% CP[1]	570.0	636.5
Corn soybean meal, 44.8% CP	320	255
Corn gluten meal, 60% CP	40	40
Soybean oil	25	30
Salt	3.0	2.4
Dicalcium phosphate	17	13
DL-methionine, 99%	3.1	2.0
L-lysine, 78%	2.0	2.2
L-threonine	0.5	0.5
Limestone	13	13
Sodium bicarbonate	2.4	1.4
Choline chloride, 50%	2.0	2.0
Vitamin premix[2]	1.0	1.0
Mineral premix[3]	1.0	1.0
Total	1000.0	1000.0
Nutrient composition[4]		
AMEn[4] (kcal/kg)	3,049	3,152
Dry matter, %	89.1	89.3
Crude protein, %	22.1	19.8
Lysine, %	1.26	1.10
Met + Cys, %	1.00	0.84
Calcium, %	1.00	0.9
Available phosphorus, %	0.45	0.37

[1] CP = crude protein, AMEn = nitrogen-corrected apparent metabolizable energy.

[2] Vitamin premix provided following nutrients per kg of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; Vitamin E, 40 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_6, 3 mg; vitamin B_{12}, 0.02 mg; niacin, 40 mg; biotin, 0.15 mg; pantothenic acid, 10 mg.

[3] Mineral premix provided following nutrients per kg of diet: Fe, 88 mg; Mn, 66 mg; Zn, 60 mg; I, 0.99 mg; Se, 0.22 mg; Cu, 72.6 mg; Co, 0.33 mg.

[4] Calculated value (as-fed basis).

2.3. Sampling

At 21, and 28 days, one bird per pen was randomly selected and euthanized by overdose of CO_2 gas. At 21 days, immediately after euthanasia, small intestine was excised and sampled for the measurement of gut morphology. At 28 days post-hatch (i.e., one week post coccidiosis vaccine challenge), blood was collected into vacutainer tubes by heart puncture immediately after euthanasia. Serum samples were obtained by gentle centrifugation 200×*g* for 15 min and stored at -20ºC until use. Immediately after blood sampling, small intestine was sampled for counting *Eimeria*-specific lesion scores and a pair of ceca were excised for measurement of volatile fatty acids. In addition, at 28 days two birds per pen were randomly selected to record the chicken's surface temperature (FLIR-300 Infrared Camera).

2.4. Gut morphology

Mid-sections (approximately1-cm-long segment) of duodenum, jejunum, and ileum sampled at 21 days were fixed in 10% neutral-buffered-formalin for a minimum of 48 h and 4.0 µm sections were

prepared. The sections were dyed with standard hematoxylin-eosin solution. The villus height (VH) was measured from the villus tip to the villus bottom. The crypt depth (CD) was defined from villus bottom to the crypt. The ratio of villus height and crypt depth (VH:CD) was then calculated.

2.5. Body surface temperature index

On one-week (i.e., 28 days post-hatch) post coccidiosis vaccine challenge, two birds were selected for measuring the surface temperature. The surface temperature of the body was measured by taking a head of the broiler, a breast (abdomen) and a leg portion using a thermally sensed image cam (FLIR-300).

2.6. Lesion score

Approximately 20-cm-long mid-segments of duodenum and jejunum sampled at 28 days (i.e., one-week post vaccine infection) were taken and cut longitudinally. Intestinal contents were gently removed and lesion scores from 0 to 4 in ascending order of severity as described elsewhere [18] were independently made by 3 observers in a blinded fashion with no knowledge of treatment groups.

2.7. Measurement of volatile fatty acids

Approximately 1 g of cecal content sampled at 28 days was homogenized with 0.05 ml of saturated solution $HgCl_2$ and 0.2 ml of 2% pivalic acid and centrifuged. Then, the supernatant was collected and stored at -20ºC before analysis. Volatile fatty acids (VFA) were measured using gas chromatography (6890 Series GC System, HP, Palo Alto, CA, USA) as described elsewhere [19].

2.8. Measurement of biochemical and antioxidant parameters in serum samples

Serum samples collected at 28 days were analyzed for glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, triglyceride, total cholesterol, and uric acid using an automatic dry biochemical analyzer (Film DRI CHEM 7000i, Fuji film, Tokyo, Japan). The concentrations of NO in serum samples were determined as described elsewhere . NO concentration was calculated from standard curve with sodium titrate as described [20]. For antioxidant markers in serum samples, malondialdehyde contents using TBARS assay kit (OxiSelect™ TBARS Assay Kit-MDA Quantitation, Cell Biolabs Inc., San Diego, CA, USA), catalase (OxiSelect™ Catalase Activity Assay kit, Cell Biolabs Inc., San Diego, CA, USA), superoxide dismutase (SOD) (SOD determination assay kit-WST, Sigma, St. Louis, MO, USA), and total antioxidant capacity (TAC) (QuantiChrom™ antioxidant assay kit-DTAC 100, BioAssay Systems, Hayward, CA, USA) were measured per the manufacturers' recommendation.

2.9. Statistical analysis

The pen was considered an experimental unit. All data were evaluated by one-way analysis of variance using general linear model (GLM) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). If the F-test for treatment effect was significant, differences between treatment means were determined using the Duncan's multiple range test [21]. In addition, orthogonal polynomial contrasts were used to assess the significance of graded EEO addition against either non-challenged or challenged controls depending on the variables measured before or after coccidiosis vaccine challenge. The significance was pre-set at $P < 0.05$.

3. Results

3.1. Growth performance

None of dietary treatments affected body weight gain, feed intake and feed conversion ratio during the starter phase (Table 2). Coccidiosis vaccine challenge significantly impaired body weight gain compared with the naïve control group during the finisher phase. However, chickens fed diets containing SAL or EEO increased body weight gain ($P < 0.05$) compared with the challenged control group and did not differ from the naïve control groups (Table 2). The challenged control chickens ate least ($P = 0.087$) during the finisher period, being lower by on average 11.5% compared with the non-challenged control group and by 6.5 to 7.9% compared with the challenged/treated groups. Increasing dietary EEO quadratically increased ($P = 0.007$) feed intake. None of treatments affected feed conversion ratio during the finisher phase.

Table 2. Effect of dietary encapsulated essential oils on growth performance in coccidiosis vaccine-challenged broiler chickens[1]

Item	N CON[4]	P CON	SAL	EO60	EO120	SEM[6]	*P*-value		
							ANOVA	L[5]	Q[5]
Before challenge (0 to 21 days post-hatch)									
Body weight gain, g/d/bird	32.5[2]	-	32.5	31.6	31.4	0.49	0.179	0.141	0.371
Feed intake, g/d/bird	43.4[2]	-	44.2	42.2	42.4	0.80	0.238	0.251	0.134
Feed conversion ratio, g/g	1.34[2]	-	1.36	1.34	1.35	0.02	0.751	0.907	0.315
After challenge (22 to 35 days post-hatch)									
Body weight gain, g/d/bird	85.3[a]	76.2[b]	83.8[a]	81.8[a]	84.7[a]	1.59	0.018	0.561	0.002
Feed intake, g/d/bird	131	116	126	123	124	2.97	0.087	0.357	0.007
Feed conversion ratio, g/g	1.53	1.52	1.51	1.50	1.47	0.02	0.220	0.407	0.869

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] Values are least squares means of 20 replicates.
[3] BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio.
[4] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[5] L, linear; Q. quadratic.
[6] SEM = pooled standard errors of the means.
[abc] Means without a common superscript letter differ ($P < 0.05$).

3.2. Intestinal morphology

Duodenal villus heights were significantly increased in SAL-fed chickens compared with the control group and dietary EEO quadratically increased duodenal villus height (Table 3). On the other hand, jejunal and ileal villus heights were not affected by dietary treatments. Duodenal crypt depth was not affected by dietary treatments. Jejunal crypt depth was lowest in SAL-fed chickens and was quadratically increased with increasing dietary EEO. However, crypt depth at duodenum and ileum was not altered by dietary treatments. Duodenal villus height : crypt depth ratio was quadratically

increased as dietary EEO increased. Its ratio at jejunum was highest in a SAL-added diet-fed chicken and quadratically decreased with increasing EEO. Dietary EEO at 120 mg/kg had highest ileal villus height : crypt depth ratio ($P < 0.05$).

Table 3. Effect of supplementation of encapsulated essential oil on morphology of small intestine in naïve broiler chickens[1]

Item	N CON[4]	P CON	SAL	EO60	EO120	SEM[6]	*P*-value		
							ANOVA	L[5]	Q[5]
Villus height, μm									
Duodenum	1,678[b2]	-	1,884[a]	1,794[ab]	1,758[ab]	53.1	0.044	0.128	0.042
Jejunum	1,541[2]	-	1,476	1,577	1,252	95.0	0.063	0.746	0.454
Ileum	871[2]	-	836	949	922	57.6	0.440	0.255	0.238
Crypt depth, μm									
Duodenum	345[2]	-	321	350	320	12.9	0.231	0.783	0.120
Jejunum	225[a2]	-	185[b]	243[a]	222[a]	12.6	0.014	0.213	0.002
Ileum	193[2]	-	182	210	174	12.2	0.178	0.236	0.174
VH: CD ratio[3] μm: μm									
Duodenum	5.00[2]	-	6.01	5.14	5.56	0.29	0.067	0.742	0.018
Jejunum	6.98[ab2]	-	8.20[a]	6.52[b]	5.73[b]	0.48	0.007	0.426	0.013
Ileum	4.55[b2]	-	4.39[b]	4.31[b]	5.49[a]	0.19	0.001	0.315	0.854

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] Values are least squares means of 20 replicates.
[3] VH: CD ratio = villus height to crypt depth ratio.
[4] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[5] L, linear; Q. quadratic.
[6] SEM = pooled standard errors of the means.
[abc] Means without a common superscript letter differ ($P < 0.05$).

3.3. Gut lesion score

Gut lesions at duodenum and jejunum were scored at 7 days post coccidiosis vaccine challenge. No *Eimeria*-specific lesion was noted in the naïve control chickens. Duodenal lesion was linearly lowered ($P = 0.054$) with increasing EEO (Table 4). Chickens fed a diet containing EEO at 60 mg/kg of diet exhibited ($P < 0.05$) highest jejunal lesion (Table 4).

Table 4. Effect of dietary encapsulated essential oils on gut lesion scores in coccidiosis vaccine-challenged broiler chickens[1]

Item	N CON[2]	P CON	SAL	EO60	EO120	SEM[4]	*P*-value		
							ANOVA	L[3]	Q[3]
28 days on lesion score									
Duodenum	0.00	0.48	0.50	0.60	0.21	0.18	0.133	0.054	0.312
Jejunum	0.00[b]	0.21[b]	0.00[b]	0.63[a]	0.00[b]	0.12	0.002	1.000	0.203

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[3] L, linear; Q. quadratic.
[4] SEM = pooled standard errors of the means.

3.4. Cecal volatile fatty acids

No difference in concentrations of cecal volatile fatty acids was detected between the non-challenged control and the challenged control groups (Table 5). The concentration of volatile fatty acids (i.e., acetate, valerate, branched-chain fatty acids, and total short-chain fatty acids) in cecal contents linearly decreased as the EEO increased. It was noted that chickens fed diet containing EEO oils at 120 mg per gram of diet had lowest concentration ($P < 0.05$) of acetate and total short-chain fatty acids.

Table 5. Effect of dietary encapsulated essential oils on concentrations (mM/g) of cecal volatile fatty acids (VFA) in coccidiosis vaccine-challenged broiler chickens[1]

Item	N CON[3]	P CON	SAL	EO60	EO120	SEM[5]	*P*-value		
							ANOVA	L[4]	Q[4]
Acetate	23.5[a]	22.4[a]	19.5[ab]	21.6[a]	16.2[b]	1.47	0.010	0.009	0.238
Propionate	2.12	2.08	1.68	2.00	1.36	0.28	0.283	0.077	0.425
Isobutyrate	0.10	0.09	0.08	0.09	0.09	0.01	0.501	0.766	0.699
Butyrate	6.42	7.04	8.74	7.97	5.90	0.82	0.206	0.405	0.210
Isovalerate	0.22	0.30	0.26	0.20	0.21	0.02	0.086	0.028	0.146
Valerate	0.40	0.44	0.40	0.38	0.33	0.04	0.317	0.052	0.925
Lactate	3.01	2.92	2.67	2.80	2.77	0.27	0.951	0.688	0.889
BCFA[2]	0.73	0.82	0.74	0.67	0.63	0.05	0.102	0.020	0.481
Total SCFA[2]	35.8[a]	35.2[a]	33.4[ab]	35.0[a]	26.9[b]	2.11	0.027	0.021	0.189

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] BCFA = branched-chain fatty acid (isobutyrate + valerate + isovalerate; total SCFA = total short-chain fatty acid (acetate + propionate + butyrate + isobutyrate + valerate + isovalerate).
[3] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[4] L, linear; Q. quadratic.
[5] SEM = pooled standard errors of the means.
[abc] Means without a common superscript letter differ ($P < 0.05$).

3.5. Body surface temperature

In this study, broiler's body surface temperature was measured using infrared camera at 7 days post coccidiosis vaccine challenge (Table 6). No difference in body surface temperature at three locations between the naïve and challenged control groups was noted. On the other hand, the body surface temperatures of the head, chest and leg were significantly low ($P < 0.05$) in chickens fed EEO-added diets compared with the challenged-control group, and linearly decreased ($P < 0.05$) with increasing EEO (Table 6). Dietary SAL did not affect the body surface temperature.

Table 6. Effect of dietary encapsulated essential oil on body surface temperature (ºC) in coccidiosis vaccine-challenged broiler chickens[1]

Item	N CON[2]	P CON	SAL	EO60	EO120	SEM[4]	*P*-value		
							ANOVA	L[3]	Q[3]
Day 28									
Head	35.6[a]	36.1[a]	35.8[a]	33.9[b]	32.4[c]	0.26	<.0001	<.0001	0.374
Chest	35.2[ab]	35.3[a]	35.6[a]	34.0[bc]	33.5[c]	0.40	0.002	0.002	0.448
Leg	36.4[a]	37.0[a]	36.7[a]	35.2[b]	34.6[b]	0.38	<.0001	0.000	0.206

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[3] L, linear; Q, quadratic.
[4] SEM = pooled standard errors of the means.
[abc] Means without a common superscript letter differ ($P < 0.05$).

3.6. Serum parameters

None of serum parameters including total cholesterol, triglycerides, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, uric acid and nitric oxide was affected by coccidiosis vaccine challenge or dietary treatments (Table 7). Serum concentrations of uric acid tended linearly ($P = 0.065$) to decrease with increasing dietary EEO.

Table 7. Effect of supplementation of encapsulated essential oil on serum biochemical parameters in coccidiosis vaccine-challenged broiler chickens[1]

Item[2]	N CON[3]	P CON	SAL	EO60	EO120	SEM[5]	*P*-value		
							ANOVA	L[4]	Q[4]
TCHO, mg/dl	111	104	97.7	109	101	4.80	0.287	0.692	0.299
TG, mg/dl	146	151	142	194	144	25.2	0.598	0.828	0.139
GOT, IU/L	170	169	156	160	168	7.92	0.669	0.918	0.467
GPT, IU/L	3.10	3.70	3.40	3.33	3.40	0.29	0.691	0.377	0.476
UA, mg/dl	7.19	8.07	6.96	7.03	6.56	0.53	0.372	0.065	0.692
Nitric oxide, μM	16.8	21.7	14.5	19.3	19.2	2.82	0.500	0.619	0.768

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] TCHO = total cholesterol; TG = triglyceride; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; UA = urea acid.
[3] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[4] L, linear; Q. quadratic.
[5] SEM = pooled standard errors of the means.

3.7. Antioxidant marker assays

Concentration of malondialdehyde was significantly increased in the challenged control *vs.* naïve control groups (Table 8). However, dietary SAL or EEO did not affect malondialdehyde content although chickens fed a diet containing EEO at 60 mg/kg tended to decrease it by on average 16.3% compared with the challenged control group. Catalase, SOD and TAC were not affected by

coccidiosis vaccine challenge or dietary treatments (Table 8). However, serum catalase activity exhibited tended linearly to increase ($P = 0.073$) as the EEO increased.

Table 8. Effect of supplementation of encapsulated essential oil on serum antioxidant parameters in coccidiosis vaccine-challenged broiler chickens[1]

Item[2]	N CON[3]	P CON	SAL	EO60	EO120	SEM[5]	*P*-value		
							ANOVA	L[4]	Q[4]
MDA, μM/L	12.6[b]	25.2[a]	27.8[a]	21.1[ab]	30.6[a]	3.67	0.012	0.369	0.201
Catalase, U/mL	21.0	11.4	14.9	15.1	16.6	3.04	0.310	0.073	0.665
SOD, U/mL	80.0	77.5	69.7	76.3	74.9	3.88	0.431	0.662	0.990
TAC, μM Trolox equivalents	601	633	564	589	553	28.6	0.378	0.361	0.191

[1] Values are least squares means of 10 replicates unless otherwise stated.
[2] MDA = malondialdehyde; SOD = superoxide dismutase; TAC = total antioxidant capacity.
[3] N CON = non-challenged (naïve) control; P CON = challenged control; SAL = salinomycin at 60 mg/kg; EO 60 = encapsulated essential oil at 60 mg/kg, EO120 = encapsulated essential oil at 120 mg/kg.
[4] L, linear; Q. quadratic.
[5] SEM = pooled standard errors of the means.
[abc] Means without a common superscript letter differ ($P < 0.05$).

4. Discussion

It is clear from this study that coccidiosis vaccine overdose can be used alternative challenge strain to *Eimeria* field isolates [11-13] and dietary SAL exhibited anticoccidial effect [22]. Addition of EEO into the diets of broilers challenged with coccidiosis vaccine mitigated *Eimeria* vaccine-induced depression in body weight gain and feed intake without affecting feed conversion ratio. Thus, it is likely that dietary EEO mainly overcome the negative effect of coccidiosis vaccine challenge by increasing feed intake. In line with our study, dietary EO increased growth performance, enhanced nutrient digestion and altered body composition in broiler chickens [23]. In addition, it was reported that dietary thymol or carvacrol is known to increase amino acid digestibility [24], and activities of endogenous digestive enzymes [23,25] in broilers. Finally, EO preparations including carvacrol or thymol are known to increase *Eimeria*-induced growth depression in chickens [26].

Protective effect of EO on *Eimeria*-specific lesions was reported elsewhere [27]. However, no clear effect of SAL or EEO on gut lesion scores was noted. In general, duodenal and jejunal lesions were kept low in the challenged chickens. This might be related to the live/attenuated vaccine strain used to induce avian coccidiosis in this study and/or delayed sampling which conducted at 7 days post challenge. Thus, the reported coccidiosis lesions [13] by vaccine strain might have been weakened or partially recovered. In general, it is reported that field isolates of *Eimeria* could induce more severe gut lesions compared with coccidiosis vaccine overdose [13]. It would need to use more vaccine doses if it is considered feasible to induce severe gut lesion scores.

Villus height, crypt depth or their ratios are considered the best indicators for the health and function of the intestine in chickens [28]. Dietary SAL increased duodenal villus height and jejunal villus height : crypt depth ratio, but decreased jejunal crypt dept compared with the control group. Our study corroborates with earlier studies [29,30], which reported that dietary antimicrobials improved gut morphology in broiler chickens. Increasing dietary EEO quadratically increased duodenal villus heights, jejunal crypt depth, and duodenal villus height : crypt depth ratio, but quadratically lowered jejunal and ileal villus height : crypt depth. In line with our findings, dietary EO are known to alter gut morphology in naïve chickens [31] or those challenged with coccidiosis [32]. Based on these findings, it can be speculated that dietary SAL and EEO might help to mitigate

coccidiosis-induced deterioration in gut morphology enable to better feed digestion and absorption that linked to improved production performance in these groups.

Volatile fatty acids are used as a nutrient source for colon epithelium cells and have inhibitory effect of pathogenic bacteria in intestine [33]. In this study, cecal volatile fatty acids were not affected by coccidiosis vaccine challenge, but linearly decreased with increasing EEO in diets. In contrast to our finding, recent studies [34,35] showed that *Eimeria* challenge altered cecal volatile fatty acids. The difference may be due to the strains used; vaccine vs. field isolates. Nonetheless, dietary EEO linearly lowered acetate, valerate, BCFA and total SCFA in cecal contents. This effect might be related to either direct inhibitory effect on gut bacteria or indirect effect on enhanced nutrient digestibility or both. Earlier studies [36,37] showed that dietary thyme or oregano EO or their combinations modified gut volatile fatty acids in broiler chickens. If the direct inhibitory effect of EEO on volatile fatty acids was considered dominant as acting mechanism, then it is likely as encapsulation used in this study would release or supply its active components to the distal part of the intestine [38].

As to body surface temperature, an interesting result was emerged from this study. Coccidiosis challenge did not affect the body surface temperature, but dietary EEO significantly lowered surface temperature of head, chest and leg, being their effect dose-dependent ($P < 0.05$). Our study indicates that dietary EO may regulate or alter themo-regulation of the chicken so as to relieve negative effect on heat stress. It has been reported that dietary peppermint or oregano EO at the level of 250 mg/kg increased growth performance in broiler chickens under heat stress [15,16]. In addition, [39] reported that dietary EO alleviate the stress indicators induced by high stocking density in broiler chickens. Thus, our study and earlier studies provide potential EO applications as a stress reliever in environmental stress conditions (e.g., heat and cold stress or immune compromise) afflicted in poultry production that needs to be assessed.

As to serum parameters, coccidiosis challenge or dietary EEO treatments did not affect any of parameters including total cholesterol, triglycerides, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, uric acid, and nitric oxide in serum samples. It is however noted that dietary EEO tended to linearly lower uric acid levels in broiler chickens. Whether this reduction in uric acid is related to low levels of amino acid oxidation [40] needs to be verified.

It is well reported that *Eimeria* infection disrupted oxidative balance leading to pathogenic oxidative stress in broiler chickens [41]. In this study, coccidiosis vaccine challenge increased serum concentration of malondialdehyde in broiler chickens, which supports that coccidiosis induced host oxidative stress. Dietary EEO consisting components of thymol and carvacrol at the level of 60 mg/kg tended to lower serum malondialdehyde levels compared with the challenged control group ($P < 0.05$). In addition, dietary EEO linearly increased serum catalase activity although statistical significance was not reached. Dietary EO or their combinations have been known to increase antioxidant markers in naïve chickens or those challenged with lipopolysaccharide [42], *Clostridium perfringens* [43], *Eimeria* [26] or *Salmonella* spp. [44]. Thus, antioxidative properties of EO are considered an important prerequisite as an alternative anticoccidial that may be responsible for mitigating the coccidiosis-induced growth depression and/or altered physiological responses.

5. Conclusions

In conclusion, dietary SAL and EEO significantly increased coccidiosis vaccine-induced growth depression in broiler chickens, which was mediated by increase in feed intake. Increasing dietary EEO clearly lowered body surface temperature, marginally improved antioxidant systems in chickens and lowered the concentrations of volatile fatty acids. Taken together, our study suggests that dietary EEO can be used as an alternative anticoccidial agent to mitigate coccidiosis-induced growth depression in broiler chickens. Further studies are warranted whether dietary EEO could counteract environmental stressors such as heat stress or stocking density and improve gut microbiome, antioxidative defense system, and gut barrier functions in broiler chickens.

Conflicts of Interest: The authors declare that they have no conflict interests.

References

1. Lillehoj, H.S.; Trout, J.M. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. *Clin Microbiol Rev* **1996**, *9*, 349-360.
2. Lillehoj, H.S.; Lillehoj, E.P. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. *Avian Dis* **2000**, *44*, 408-425.
3. Williams, R.B. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *Int J Parasitol* **1999**, *29*, 1209-1229.
4. Williams, R.; Bushell, A.; Reperant, J.; Doy, T.; Morgan, J.; Shirley, M.; Yvore, P.; Carr, M.M.; Fremont, Y. A survey of Eimeria species in commercially-reared chickens in France during 1994. *Avian Pathol* **1996**, *25*, 113-130.
5. Cowieson, A.J.; Roos, F.F. Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants. *Anim Feed Sci Technol* **2016**, *221*, 331-340.
6. Gavaric, N.; Mozina, S.S.; Kladar, N.; Bozin, B. Chemical profile, antioxidant and antibacterial activity of thyme and oregano essential oils, thymol and carvacrol and their possible synergism. *J Essential Oil Bear plants* **2015**, *18*, 1013-1021.
7. Peek, H.W.; van der Klis, J.D.; Vermeulen, B.; Landman, W.J.M. Dietary protease can alleviate negative effects of a coccidiosis infection on production performance in broiler chickens. *Anim Feed Sci Technol* **2009**, *150*, 151-159.
8. Jang, I.; Ko, Y.; Yang, H.; Ha, J.; Kim, J.; Kang, S.; Yoo, D.; Nam, D.; Kim, D.; Lee, C. Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. *Asian-Australas J Anim Sci* **2004**, *17*, 394-400.
9. Liu, X.; Diarra, M.S.; Zhang, Y.; Wang, Q.; Yu, H.; Nie, S.-P.; Xie, M.-Y.; Gong, J. Effect of encapsulated carvacrol on the incidence of necrotic enteritis in broiler chickens. *Avian Pathol* **2016**, *45*, 357-364.
10. Cui, H.; Yuan, L.; Ma, C.; Li, C.; Lin, L. Effect of nianoliposome-encapsulated thyme oil on growth of Salmonella enteritidis in chicken. *J Food Proc Pres* **2017**, *41*, e13299.
11. Shojadoost, B.; Vince, A.R.; Prescott, J.F. The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. *Vet Res* **2012**, *43*, 74.
12. Zhang, Q.; Chen, X.; Eicher, S.D.; Ajuwon, K.M.; Applegate, T.J. Effect of threonine deficiency on intestinal integrity and immune response to feed withdrawal combined with coccidial vaccine challenge in broiler chicks. *Br J Nutr* **2016**, *116*, 2030-2043.
13. Mathis, G.F.; Newman, L.J.; Fitz-Coy, S.; Lumpkins, B.; Charette, R.; Fuller, L. Comparison of breeder/layer coccidiosis vaccines: Part 1-precocity and pathogenicity. *J Appl Poult Res* **2017**, *27*, 33-37.
14. Hamzic, E.; Bed'Hom, B.; Juin, H.; Hawken, R.; Abrahamsen, M.S.; Elsen, J.-M.; Servin, B.; Pinard-van der Laan, M.-H.; Demeure, O. Large-scale investigation of the parameters in response to Eimeria maxima challenge in broilers. *J Anim Sci* **2015**, *93*, 1830-1840.
15. Akbari, M.; Torki, M. Effects of dietary chromium picolinate and peppermint essential oil on growth performance and blood biochemical parameters of broiler chicks reared under heat stress conditions. *Int J Biometeorol* **2014**, *58*, 1383-1391.
16. Ghazi, S.; Amjadian, T.; Norouzi, S. Single and combined effects of vitamin C and oregano essential oil in diet, on growth performance, and blood parameters of broiler chicks reared under heat stress condition. *Int J Biometeorol* **2015**, *59*, 1019-1024.
17. Piva, A.; Pizzamiglio, V.; Morlacchini, M.; Tedeschi, M.; Piva, G. Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. *J Anim Sci* **2007**, *85*, 486-493.

18. Lee, K.W.; Lillehoj, H.S.; Jang, S.I.; Li, G.; Lee, S.H.; Lillehoj, E.P.; Siragusa, G.R. Effect of Bacillus-based direct-fed microbials on Eimeria maxima infection in broiler chickens. *Comp Immunol Microbiol Infect Dis* **2010**, *33*, e105-110.
19. Meimandipour, A.; Shuhaimi, M.; Soleimani, A.F.; Azhar, K.; Hair-Bejo, M.; Kabeir, B.M.; Javanmard, A.; Muhammad Anas, O.; Yazid, A.M. Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of Lactobacillus. *Poult Sci* **2010**, *89*, 470-476.
20. Kim, D.H.; Han, S.M.; Keum, M.C.; Lee, S.; An, B.K.; Lee, S.R.; Lee, K.W. Evaluation of bee venom as a novel feed additive in fast-growing broilers. *Br Poult Sci* **2018**, *59*, 435-442.
21. Duncan, D.B. Multiple range and F tests. *Biometrics* **1955**, *11*, 1-42.
22. Abdelrahman, W.; Mohnl, M.; Teichmann, K.; Doupovec, B.; Schatzmayr, G.; Lumpkins, B.; Mathis, G. Comparative evaluation of probiotic and salinomycin effects on performance and coccidiosis control in broiler chickens. *Poult Sci* **2014**, *93*, 3002-3008.
23. Basmacioğlu Malayoğlu, H.; Baysal, Ş.; Misirlioğlu, Z.; Polat, M.; Yilmaz, H.; Turan, N. Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat–soybean meal diets. *Br Poult Sci* **2010**, *51*, 67-80.
24. Ragaa, N.M.; Korany, R.M.; Mohamed, F. Effect of thyme and/or formic acid dietary supplementation on broiler performance and immunity. *Agri and Agri Sci Proc* **2016**, *10*, 270-279.
25. Hashemipour, H.; Kermanshahi, H.; Golian, A.; Veldkamp, T. Effect of thymol and carvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid composition, digestive enzyme activities, and immune response in broiler chickens. *Poult Sci* **2013**, *92*, 2059-2069.
26. Giannenas, I.; Florou-Paneri, P.; Papazahariadou, M.; Botsoglou, N.; Christaki, E.; Spais, A. Effect of diet supplementation with ground oregano on performance of broiler chickens challenged with Eimeria tenella. *Arch Geflugelkunde* **2004**, *68*, 247-252.
27. Barbour, E.; Bragg, R.; Karrouf, G.; Iyer, A.; Azhar, E.; Harakeh, S.; Kumosani, T. Control of eight predominant Eimeria spp. involved in economic coccidiosis of broiler chicken by a chemically characterized essential oil. *J Appl microbiol* **2015**, *118*, 583-591.
28. Nabizadeh, A. The effect of inulin on broiler chicken intestinal microflora, gut morphology, and performance. *J. Anim Feed Sci* **2012**, *21*, 725-734.
29. Miles, R.; Butcher, G.; Henry, P.; Littell, R. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poult Sci* **2006**, *85*, 476-485.
30. Nabian, S.; Arabkhazaeli, F.; Seifouri, P.; Farahani, A. Morphometric Analysis of the Intestine in Experimental Coccidiosis in Broilers Treated with Anticoccidial Drugs. *Iran J Parasitol* **2018**, *13*, 493-499.
31. Jamroz, D.; Wertelecki, T.; Houszka, M.; Kamel, C. Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. *J Anim Physiol Anim Nutr* **2006**, *90*, 255-268.
32. Reisinger, N.; Steiner, T.; Nitsch, S.; Schatzmayr, G.; Applegate, T. Effects of a blend of essential oils on broiler performance and intestinal morphology during coccidial vaccine exposure. *J Appl Poult Res* **2011**, *20*, 272-283.
33. Van Immerseel, F.; De Buck, J.; Pasmans, F.; Velge, P.; Bottreau, E.; Fievez, V.; Haesebrouck, F.; Ducatelle, R. Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. *Int J Food microbiol* **2003**, *85*, 237-248.
34. Leung, H.; Yitbarek, A.; Snyder, R.; Patterson, R.; Barta, J.; Karrow, N.; Kiarie, E. Responses of broiler chickens to Eimeria challenge when fed a nucleotide-rich yeast extract. *Poult Sci* **2018**, *98*, 1622-1633.

35. Wu, S.B.; Rodgers, N.J.; Cui, G.; Sun, Y.; Choct, M. Dynamics of intestinal metabolites and morphology in response to necrotic enteritis challenge in broiler chickens. *Avian Pathol* **2016**, *45*, 346-356.
36. Cross, D.E.; McDevitt, R.; Acamovic, T. Herbs, thyme essential oil and condensed tannin extracts as dietary supplements for broilers, and their effects on performance, digestibility, volatile fatty acids and organoleptic properties. *Br Poult Sci* **2011**, *52*, 227-237.
37. Hashemipour, H.; Khaksar, V.; Rubio, L.; Veldkamp, T.; Van Krimpen, M. Effect of feed supplementation with a thymol plus carvacrol mixture, in combination or not with an NSP-degrading enzyme, on productive and physiological parameters of broilers fed on wheat-based diets. *Anim Feed Sci Technol* **2016**, *211*, 117-131.
38. Grilli, E.; Bodin, J.; Gatta, P.; Tedeschi, M.; Piva, A. Microencapsulation allows slow release of organic acids in the GI tract of broilers. *Proc, 16th Eur Symp on Poult Nutr, Strasbourg, France* **2007**, pp. 463-466.
39. Hosseini, S.; Farhangfar, H.; Nourmohammadi, R. Effects of a blend of essential oils and overcrowding stress on the growth performance, meat quality and heat shock protein gene expression of broilers. *Br Poult Sci* **2018**, *59*, 92-99.
40. Darsi, E.; Shivazad, M.; Zaghari, M.; Namroud, N.; Mohammadi, R. Effect of reduced dietary crude protein levels on growth performance, plasma uric acid and electrolyte concentration of male broiler chicks. *J Agr Sci Tech* **2012**, 14, 789-797.
41. Georgieva, N.V.; Koinarski, V.; Gadjeva, V. Antioxidant status during the course of Eimeria tenella infection in broiler chickens. *Vet J* **2006**, *172*, 488-492.
42. Liu, S.; Song, M.; Yun, W.; Lee, J.; Kim, H.; Cho, J. Effect of carvacrol essential oils on immune response and inflammation-related genes expression in broilers challenged by lipopolysaccharide. *Poult Sci* **2018**, *98*, 2026-2033.
43. Sun, Q.; Liu, D.; Guo, S.; Chen, Y.; Guo, Y. Effects of dietary essential oil and enzyme supplementation on growth performance and gut health of broilers challenged by Clostridium perfringens. *Anim Feed Sci Technol* **2015**, *207*, 234-244.
44. Hoffman-Pennesi, D.; Wu, C. The effect of thymol and thyme oil feed supplementation on growth performance, serum antioxidant levels, and cecal Salmonella population in broilers. *J Appl Poult Res* **2010**, *19*, 432-443.