Effects of essential oil on growth performance, digestibility, immunity, and intestinal health in broilers

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ABSTRACT Essential oils (**EO**) are concentrated hydrophobic liquids containing volatile aromatic compounds obtained from plants, which have properties as withdrawn antibiotic growth promoters. The objective of this study was to explore the effects of EO on growth performance, digestibility, immunity and intestinal health in broilers. A total of 500 1-day-old Arbor Acre broilers were randomly put into five groups with 10 replicate cages containing 10 birds each. Birds in the 5 groups were fed a basal diet (CON), and basal diet with 50, 100, 200 or 400 mg/kg EO (EO0.5, EO1, EO2 and EO4) for 42 d respectively. Birds were euthanized at 21d and 42 d, blood and tissue samples were collected. In the study, the digestibility of DM, GE and EE in groups with EO supplementation were significantly increased compared with CON group ($P < 0.05$). However, only EO2 and EO4 significantly increased the digestibility of CP compared with CON group ($P < 0.05$). In contrast to CON group, EO0.5 and EO1 in jejunum at 21 d, and EO1 in jejunum at 42 d markedly increased the activity of sucrase ($P < 0.05$). In addition, the level of SOD of EO2 and EO4 in serum at 21 d was significantly increased compared with CON group ($P < 0.05$). What's more, the concentration of intestinal mucosa SIgA in jejunum and ileum at 21 d of groups with EO supplementation was significantly increased compared with CON group ($P < 0.05$). Moreover, V/C in jejunum at 21 d of groups with EO supplementation, CD in jejunum at 42 d was also significantly increased to compare with CON group ($P < 0.05$). Furthermore, the expression levels of critical genes associated with nutrient transportation (i.e., GLUT2, SGLT1, SLC38A, SLC79A and SLC27A4) and barrier function (TJP1) were quadratically and linearly up-regulated in jejunum and ileum with EO supplementation ($P < 0.05$). These results suggest that EO has a positive impact on growth, immunity and intestinal health in broilers, and 200 mg/kg of EO was recommended in broiler diet.

Key words: broilers, digestibility, essential oil, immunity, intestine

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INTRODUCTION

Antibiotic growth promoters (**AGP**) have been used in poultry production to promote growth (Mehdi et al., 2018). With the increasing microbial resistance, there is an increased pressure to remove AGP in poultry production (Stefanello et al., 2019). Following the EU and US banned on the use of AGP, China also banned AGP in 2020. The prohibition of AGP encourages the industry to find the appropriate alternative for antibiotics (Heydarian et al., 2020). Among the potential candidates as alternatives, the plant-derived EO have been explored as they exhibit various biological properties, including antimicrobials, antioxidants, and immune-modulation (Adaszyńska-Skwirzyńska and Szczerbińska, 2017; Donsì and Ferrari, 2016; Han et al., 2017; Lee et al., 2020; Su et al., 2020). In recent years, there has been increased interest in developing EO feed additives as a potential alternative for AGP (Kishawy et al., 2019; Mahgoub et al., 2019).

Many studies have also been conducted on the effects of dietary EO or combinations on the performance of poultry and swine but with varying and conflicting results (Abbasi et al., 2020; Adaszyńska-Skwirzyńska and Szczerbińska, 2017; Zeng et al., 2016). There are many factors that affected the effects of EO, but properly selecting and composing of EO were the origin of positive effects. The activities of the EO depend on their composition, functional groups, and synergistic interactions between components, for example: the hydroxyl group present in the structure of phenolic

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compounds confers antimicrobial activity and its relative position is very crucial for the effectiveness of these natural components; this can explain the superior antimicrobial activity of carvacrol, compared to other plant phenolics (Amer et al., 2018; Di Pasqua et al., 2007).

Although there are several studies showing the effects of thymol, carvacrol and cinnamaldehyde on intestinal health and growth performance of broilers, the effects were varied (Du et al., 2016; Galli et al., 2020; İpçak and Alçiçek, 2018; Reis et al., 2018). The product composition in terms of type and quantity of active compounds may be the reason for the variable results found in the literature (Stefanello et al., 2019). Therefore, the objective of the present study was to evaluate the effects of a blend of EO (3.05% thymol, 2.3% carvacrol and 0.26% cinnamaldehyde, carrier is dextrin) on growth performance, nutrient digestibility, and immunity in broilers.

MATERIAL AND METHODS

Essential Oil

The EO in this study is commercial EO provided by Tianjin NAER Bio-Tech Col., Ltd, Tianjin, China. The active ingredient of EO are 3.05% thymol, 2.3% carvacrol, and 0.26% cinnamaldehyde, carrier is dextrin.

Bird Trial

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. A total of 500 one-day-old male Arbor Acre broilers were randomly put into five groups with 10 replicate cages (120 × 70 × 60 cm) containing 10 birds each. Birds in the 5 groups were fed a basal diet (CON), and basal diet with 50, 100, 200 and 400 mg/kg EO (EO0.5, EO1, EO2 and EO4) for 42 d respectively. All birds were allowed free access to feed and water in temperature-controlled room, the temperature of the experimental room was set at 33°C at the age of 1 to 4 d and then reduced by 2 or 3°C per week to a final temperature of 26°C. Moreover, the birds were raised under white light with a light schedule of 23 h light and 1 h dark was provided during the whole period of the trial (Chen et al., 2017). The basal diet was formulated based on the recommendation by National Research Council (1994), and the components and nutrients levels of the basal diet were presented in Table 1. Feed samples were collected to detect conventional nutrients.

Table 1. Ingredient composition and nutrient levels of the basal diets (%).

Ingredient	Starter diet (day 1 to 20)	Grower diet (day 1 to 20)
Corn	54.31	59.69
Soybean oil	3.39	4.1
Soybean meal	38.11	32.58
Lysine-HCl	0.15	0.11
DL-Methionine	0.22	0.19
Calcium carbonate	1.19	1
Calcium hydrophosphate	1.9	1.6
Sodium chloride	0.35	0.35
Choline chloride	0.15	0.15
Vitamin premix[a]	0.03	0.03
Trace mineral premix[b]	0.2	0.2
Calculated analysis		
ME (Mcal/kg)	2950	3050
CP	21	19
Ca	1	0.85
Available phosphorus	0.45	0.4
Lysine	1.15	1
Methionine	0.5	0.45
Cystine	0.29	0.27
Methionine + Cystine	0.64	0.58

[a]Vitamin premix provided the following per kilogram of breeder diet: 12,000 IU of vitamin A (retinol acetate), 3,000 IU of vitamin D_3, 10 IU of vitamin E (dl-α-tocopheryl acetate), 2.2 mg of vitamin K_3, 2 mg of vitamin B_1, 6 mg of vitamin B_2, 5.5 mg of vitamin B_6, 0.013 mg of vitamin B_{12}, 44 mg of nicotinic acid, 12 mg of pantothenic acid, 1.65 mg of folic acid, 0.22 mg of biotin.

[b]Mineral premix provided the following per kilogram of breeder diet: 120 mg of manganese, 100 mg of zinc, 40 mg of iron, 8 mg of copper, 1.0 mg of iodine, 0.3 mg of selenium.

Sample Collection

In this study, the body weight (**BW**) of the broilers (in 1, 21, and 42 d of age), daily feed intake (**FI**) and mortalities were recorded. The resulting data were used to calculate average daily body weight gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**F:G**).

At 21 and 42 d of age, one broiler chicken (close to the cage average body weight) from each cage was selected. Blood sample was obtained from wing vein, and serum was separated and stored at -20°C before analysis after a centrifugation at 3,000 × g for 15 min at 4°C. Then, broiler chickens were euthanized by cervical dislocation and necropsied immediately. After that, the whole gastrointestinal tract was also rapidly removed, and the segments of the midjejunum and midileum were excised (about 2 cm) and flushed gently with 4 °C phosphate-buffered saline to remove the contents, which were thereafter placed in 4% paraformaldehyde for morphology measurement. The remaining jejunum and ileum sections were subsequently opened longitudinally, and the contents were flushed with ice-cold phosphate-buffered saline. Mucosa of each sample was collected using a sterile glass microscope slide, rapidly stored in liquid nitrogen, and then frozen at -80°C for further analysis. At 39 d of age, the ileum contents were collected and stored at -20°C for measuring the digestibility of the nutrients, acid-insoluble ash was used as an endogenous indicator. Feed and excreta samples were analyzed for dry matter (**DM**), gross energy (**GE**), crude protein (**CP**) and ether extract (**EE**) according the methods of previous study (Wu et al., 2019).

Intestinal Histomorphology

The harvested segments of the jejunum and ileum were dehydrated, cleared, and embedded in paraffin

after a 24-h fixation in buffered formalin. They were then cut into serial sections at 5-mm depth for subsequent staining with hematoxylin and eosin stain. The villus height (**VH**) and crypt depth (**CD**) were determined using a light microscope equipped with a computer-assisted morphometric system (Nikon Corporation, Tokyo, Japan).

ELISA

Secreted immunoglobulin A (**SIgA**) of intestinal mucosa (the jejunum and ileum), immunoglobulin A (**IgA**), immunoglobulin G (**IgG**) and immunoglobulin M (**IgM**) of serum were determined using chicken-specific ELISA kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, P. R. China) in accordance with the descriptions by manufacturers. The results were normalized against total protein concentration in each sample for intersample comparison.

Enzyme Activity

Concentrations of malondiadehyde (**MDA**), superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**) and total antioxidant capacity (**T-AOC**) in serum were determined in accordance with the manufacturer's instructions using available commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu Province, P. R. China). Total protein (**TP**), the activity of maltase and sucrase in jejunal and ileal mucosa were measured by assay kits A045-3, A082-3 and A082-2 from Nanjing Jiancheng Bioengineering Institute according to the manufacturer's instructions. The absorbance of assay kits was determined by a UV-spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). The results were normalized against total protein concentration in each sample for intersample comparison.

Gene Expression Analysis

Total RNA was extracted from samples of duodenum, jejunum and ileum samples using TRIzol reagent (TaKaRa, Dalian, China). RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The integrity of RNA was verified by eletrophoretic analysis. Reverse transcription was run with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) with 1 mg RNA sample according to the manufacturer's instructions. The final reaction volume of 20 μL cDNA was then adjusted to 250 μL using nuclease-free water and stored at -20°C. The cDNA was used as the template for PCR. Real-time quantitative PCR reactions used SYBR Green reagent (TaKaRa, Dalian, China) and were performed in an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems). The primers were synthesized commercially by Invitrogen (Shanghai, China) and the primers of β-actin, occludin (**OCLN**), tight junction protein 1 (**TJP1**), glucose transporter 2 (**GLUT2**), sodium-glucose cotransporter 1 (**SGLT1**), solute carrier family 38 (**SLC38A**), solute carrier family 7 member 9 (**SLC7A9**), and fatty acid transport protein 4 (**SLC27A4**) are shown in Table 2. The gene β-actin was used as house-keeping gene. The melting peaks of the amplification products were determined by melting curve which indicated only one expected amplification products had been generated. Each primer pair used yielded a single peak in the melting curve and a single band with the expected size in agarose gel. The relative gene expressions compared with the house-keeping gene β-actin were calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), and CON group was used as a reference.

Statistical Analysis

All data were analyzed by one-way analysis of variance using SPSS, version 20.0, for windows (2010, SPSS

Table 2. Genes and primer sequences.

Gene	Primer sequence 5′-3′	GenBank	Product length/ bp
β-actin	F:GAGAAATTGTGCGTGACATCA R:CCTGAACCTCTCATTGCCA	L08165.1	152
Occludin	F: GGCCCACACCTCTGGGAA R: GCCTTCCCAAAAAGCCCTGA	NM_205128.1	179
TJP1 XM_015278975.2	F:CTTCAGGTGTTTCTCTTCCTCC 131 R:CTGTGGTTTCATGGCTGGATC		
SGLT1	F:CAGAACGTTTGAGGGCTTTGT R:AGCAAGTGGAGCCAATCAGA	NM_001293240	186
GLUT2	F:CCAGTTCGCCTGGATGAGTT R:CCCACAATGAAGTTGCAGGC	NM_207178.1	180
SLC38A NM_001199603.1	F:CGCTAAATGCAACATCACCT 106 R:GTGGGCAAAGCATACACAGT		
SLC7A9 NM_001199133.1	F:GGATGGACAATCCATTCAGA 116 R:CAAAGATGCCTGAGCCAATA		
SLC27A4	F:ATACCTCTGGCACTACGGGAAT R:CATACATCACATCATCGGGTCT	FJ868804.1	117

Abbreviations: GLUT2, glucose transporter 2; SGLT1, sodium-dependent glucose transporters 1; SLC38A, solute carrier family 38; SLC7A9, solute carrier family 7 member 9; SLC27A4, fatty acid transport protein 4; TJP1, tight junction protein 1.

Inc., Chicago, IL). Replicate was defined as an experimental unit for the trial. Polynomial contrasts were used to test the linear and quadratic response to the increasing levels of EO in diets, "quadratic response" means the effects would reach maximum with a level of EO in diets. Multiple comparisons were conducted using the Tukey test to evaluate the difference between CON and other groups. Statistical significance was considered if $P < 0.05$ in all analyses. Results are presented as means and standard error of means.

RESULTS

Growth Performance

ADG was quadratically increased ($P < 0.05$) at 1 to 21 d with EO supplementation (Table 3), but no significant increasing was gained with EO supplementation compared with CON group.

Nutrient Digestibility

As shown in Table 4, the digestibility of DM, GE, and EE were significantly increased with all level of EO supplementation compared with CON group ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg quadratically and linearly increased DM, GE, CP and EE ($P < 0.05$). However, only EO2 and EO4 significantly increased the digestibility of CP compared with CON group ($P < 0.05$), not all groups with EO supplementation.

Disaccharidase Activity

Effects of EO on intestinal mucosa disaccharidase activities in broilers were shown in Table 5. Compared with CON, the activities of sucrase of ileal mucosa at 1 to 21 d were markedly increased ($P < 0.05$) with all level of EO supplementation. Increasing the level of EO from 0 to 400 mg/kg quadratically increased the activities of sucrase of ileal mucosa at 1-21 d ($P < 0.05$). What's more, the activities of sucrase of ileal mucosa in EO1 group at 1 to 21 d were significantly higher than CON group ($P < 0.05$).

Serum Antioxidant

The effects of EO supplementation on serum antioxidant were presented in Table 6. Compared with CON group, the activities of SOD in EO2 and EO4 were significantly increased ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg significantly increased the activities of SOD in serum at 21 d (linear and quadratic; $P < 0.05$). However, the level of MDA, TAOC, GPX did not markedly affected by EO addition.

Immunoglobulin

The effects of EO supplementation on serum immunoglobulin and intestinal mucosa SIgA were shown in Tables 7 and 8. Compared with CON group, EO supplementation tended to increase the level of IgG in serum at 42 d ($P = 0.06$), However, increasing the level of EO from 0 to 400 mg/kg significantly increased the level of IgG in serum at 42 d (quadratic; $P < 0.05$). However, the levels of IgA, IgM and IgG at 21 d, IgA and IgM at 42 d did not markedly affected by EO addition. Compared with CON group, the level of intestinal mucosa SIgA in jejunum and ileum were significantly increased with all level of EO supplementation ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg quadratically and linearly increased the concentration of intestinal mucosa SIgA in jejunum and ileum at 21 d ($P < 0.05$).

Intestinal Morphology

As shown in Table 9, compared with CON group, V/C in jejunum at 21 d of groups with all levels of EO supplementation were significantly higher than CON group ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg quadratically and linearly increased ($P < 0.05$) V/C in jejunum at 21 d. At 42 day, the depth of CD in jejunum of

Table 3. Effects of EO on the growth performance of broiler.

	Essential oil (mg/kg)						*P*-value		
Items	0	50	100	200	400	SEM[1]	L	Q	ANOVA
IBW (g)	47.28	47.27	47.37	47.28	47.40	0.03	0.28	0.54	0.66
21 BW (g)	642.00	638.04	673.12	669.16	680.80	7.62	0.07	0.14	0.28
42 BW (g)	2491.29	2562.37	2515.01	2617.14	2592.39	30.17	0.28	0.44	0.68
ADG (g/d)									
Days 1−21	28.32	28.13	29.80	29.61	30.16	0.36	0.07	0.01	0.28
Days 22−42	88.06	91.63	87.71	92.76	91.03	1.25	0.48	0.66	0.65
Days 1−42	116.38	119.77	117.51	122.37	121.19	1.44	0.28	0.44	0.68
ADFI (g/d)									
Days 1−21	48.61	49.96	50.02	50.81	48.29	0.05	0.62	0.18	0.47
Days 22−42	145.26	158.58	141.52	152.49	148.81	3.56	0.97	0.98	0.62
Days 1−42	100.08	107.63	99.06	104.91	101.58	1.80	0.95	0.89	0.55
F:G									
Days 1−21	1.72	1.80	1.68	1.73	1.61	0.03	0.09	0.20	0.30
Days 22−42	1.73	1.82	1.73	1.72	1.74	0.05	0.84	0.97	0.97
Days 1−42	1.72	1.80	1.70	1.72	1.69	0.03	0.53	0.82	0.85

Abbreviations: ADFI, average daily feed intake; ADG, average daily body weight gain; BW, body weight; F:G, the ration of feed/body weight gain; IBW, initial body weight; L, linear; Q, quadratic.

[1]SEM, total standard error of means (n = 10).

Table 4. Effects of EO on nutrient digestibility in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
DM	88.01	90.76*	91.62*	92.92*	92.88*	0.39	<0.01	<0.01	<0.01
GE	91.19	92.82*	93.40*	94.31*	94.39*	0.26	<0.01	<0.01	<0.01
EE	96.34	97.53*	97.71*	97.96*	97.74*	0.14	0.01	<0.01	<0.01
CP	84.23	84.68	87.23	90.01*	89.00*	0.60	<0.01	<0.01	<0.01

Abbreviations: CP, crude protein; DM, dry matter; EE, ether extract; GE, gross energy; L, linear; Q, quadratic.
*Means differences between this group and the control group was different at $P < 0.05$.
[1]SEM, total standard error of means (n = 10).

Table 5. Effects of EO on intestinal mucosa disaccharidase activities in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
maltase									
Jejunum of days 1−21	26.90	28.73	30.83	29.53	27.05	1.84	0.87	0.78	0.96
Jejunum of days 22−42	24.20	27.31	29.46	26.14	23.48	1.04	0.38	0.27	0.39
Ileum of days 1−21	45.07	51.51	51.86	53.28	50.23	1.90	0.61	0.42	0.74
Ileum of days 22−42	46.19	45.57	49.00	48.08	49.12	2.90	0.72	0.93	0.99
Sucrase									
Jejunum of days 1−21	9.88	8.42	10.66	8.81	8.35	0.56	0.42	0.71	0.66
Jejunum of days 22−42	9.53	14.39	11.22	11.40	10.99	0.81	0.85	0.85	0.46
Ileum of days 1−21	6.83	12.28*	13.99*	14.56*	9.54	0.81	0.79	<0.01	<0.01
Ileum of days 22−42	5.56	8.34	12.32*	5.66	5.68	0.75	0.28	0.24	<0.01

Abbreviations: L, linear; Q, quadratic.
*Means differences between this group and the control group was different at $P < 0.05$.
[1]SEM, total standard error of means (n = 10).

Table 6. Effects of EO on serum antioxidant in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
Days 21									
MDA (nmol/mg protein)	2.42	2.11	2.23	2.28	2.17	0.07	0.57	0.82	0.73
TAOC (U/mg protein)	1.15	1.05	1.26	1.19	1.33	0.06	0.21	0.46	0.63
SOD (U/mg protein)	129.88	130.42	149.96	192.54*	185.14*	8.86	<0.01	0.01	0.04
GPX (U/mg protein)	513.64	513.40	607.79	527.08	529.09	19.53	0.99	0.75	0.54
Days 42									
MDA (nmol/mg protein)	2.37	2.15	2.17	2.33	2.18	0.06	0.68	0.92	0.74
TAOC (U/mg protein)	1.14	0.97	1.11	1.09	0.85	0.05	0.12	0.24	0.35
SOD (U/mg protein)	178.01	250.61	256.33	210.30	214.10	12.09	0.95	0.55	0.23
GPX (U/mg protein)	850.33	892.25	858.71	876.33	905.33	40.59	0.72	0.94	0.99

Abbreviations: GPX, glutathione peroxidase; L, linear; MDA, malonaldehyde; Q, quadratic; SOD, superoxide dismutase; TAOC, antioxidant capacity.
*Means differences between this group and the control group was different at $P < 0.05$.
[1]SEM, total standard error of means (n = 10).

Table 7. Effects of EO on serum immunoglobulin in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
Days 21									
IgA	1427.18	1453.63	1480.44	1448.75	1491.64	47.09	0.73	0.94	1.00
IgM	744.08	747.93	757.30	790.82	751.10	20.82	0.84	0.79	0.96
IgG	14.79	17.19	13.90	13.79	13.69	0.59	0.22	0.43	0.29
Days 42									
IgA	1389.13	1467.80	1451.74	1576.60	1393.11	28.37	0.98	0.09	0.22
IgM	1087.39	1269.38	1135.41	1473.22	1226.30	58.21	0.43	0.21	0.26
IgG	13.64	17.07	15.90	19.38	16.80	0.64	0.17	0.03	0.06

Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; L, linear; Q, quadratic.
[1]SEM, total standard error of means (n = 10).

Table 8. Effects of EO on intestinal mucosa SIgA in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
Jejunum of days 21	4.49	5.19*	5.14*	5.24*	5.41*	0.09	0.01	0.01	0.01
Jejunum of days 42	5.01	5.78	5.33	5.44	5.77	0.12	0.16	0.37	0.19
Ileum of days 21	4.19	4.90*	4.73*	4.83*	5.27*	0.10	<0.01	<0.01	<0.01
Ileum of days 42	3.29	3.56	3.73	3.71	3.63	0.08	0.34	0.19	0.44

Abbreviations: L, linear; Q, quadratic.
*Means differences between this group and the control group was different at $P < 0.05$.
[1]SEM, total standard error of means (n = 10).

EO0.5, EO1 and EO2 were significantly lower than CON group ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg quadratically increased ($P < 0.05$) CD in jejunum at 42 d. Compared with CON group, the ratio of V/C in jejunum at 42 d in EO4 was significantly higher than CON group ($P < 0.05$). Increasing the level of EO from 0 to 400 mg/kg quadratically increased ($P < 0.05$) the ratio of V/C of jejunum at 42 d in jejunum.

Intestinal Gene Expression Levels

The effects of EO supplementation on the levels of intestinal gene expression were presented in Table 10. The expression levels of SGLT1 of EO1, GLUT2 of EO0.5 and EO1, SLC38A of EO0.5 and EO1, SLC27A4 of EO1 and EO2, Occludin of EO0.5 and EO1, TJP1 of EO1 in jejunum at d 21 were significantly higher than CON group ($P < 0.05$). And with increasing the level of EO from 0 to 400 mg/kg, the expression levels of SGLT1 and SLC79A in jejunum at d 21 were quadratically and linearly increased ($P < 0.05$), the expression levels of SLC27A4 and TJP1 in jejunum at d 21 were quadratically increased ($P < 0.05$). However, the levels of SLC7A9 in EO1 and EO2 were significantly lower than CON group ($P < 0.05$). And with increasing the level of EO from 0 to 400 mg/kg, the expression levels of SLC7A9 were quadratically and linearly decreased ($P < 0.05$).

The expression levels of SGLT1 of EO1, GLUT2 of EO1, SLC7A9 of EO1, EO2 and EO4, SLC27A4 of EO1, TJP1 of EO1 in jejunum at d 42 were significantly higher than CON group ($P < 0.05$). And with increasing the level of EO from 0 to 400 mg/kg, the expression levels of SLC7A9 in jejunum at d 42 were quadratically and linearly increased ($P < 0.05$), the expression levels of GLUT2, SLC38A, and TJP1 in jejunum at d 42 were quadratically increased ($P < 0.05$).

The expression levels of GLUT2 of EO1, SLC38A of EO1, EO2 and EO4, SLC79A of EO1, EO2 and EO4, SLC27A4 of EO1, EO2, and EO4, TJP1 of EO1 in jejunum at d 21 were significantly higher than CON group ($P < 0.05$). And with increasing the level of EO from 0 to 400 mg/kg, the expression levels of SLC79A and SLC27A4 in jejunum at d 42 were quadratically and linearly increased ($P < 0.05$), the expression levels of GLUT2 and SLC38A in ileum at d 21 were quadratically increased ($P < 0.05$).

The expression levels of GLUT2 of EO0.5 and EO1, SLC38A of EO0.5, SLC79A of EO1 and EO2, *SLC27A4* in EO0.5, EO1, EO2 and EO4, TJP1 in EO0.5 and EO1 of ileum at d 42 were significantly higher than CON group ($P < 0.05$). And with increasing the level of EO from 0 to 400 mg/kg, the expression levels of SLC27A4

Table 9. Effects of EO on intestinal morphology in broilers.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
Jejunum of days 21									
VH	1050.78	1062.05	1105.15	1153.61	1191.28	29.53	0.08	0.21	0.54
CD	241.17	210.94	185.00	207.52	206.33	32.13	0.33	0.12	0.08
V/C	4.38	5.10*	5.96*	5.62*	5.87*	0.89	0.02	0.01	0.01
Ileum of days 21									
VH	850.71	940.77	868.32	865.50	853.49	18.85	0.54	0.80	0.57
CD	180.79	162.67	162.35	160.89	166.74	3.92	0.53	0.29	0.51
V/C	4.77	5.77	5.39	5.43	5.18	0.15	0.98	0.42	0.28
Jejunum of days 42									
VH	1119.52	1119.98	1123.77	1231.64	1139.31	27.52	0.61	0.51	0.69
CD	206.40	166.09*	174.64*	157.41*	178.42	5.15	0.30	0.01	0.02
V/C	5.42	6.78	6.55	7.98*	6.43	0.28	0.35	0.02	0.05
Ileum of days 42									
VH	1169.76	1297.17	1446.02	1211.06	1223.90	36.69	0.68	0.50	0.12
CD	209.51	191.01	205.18	177.18	181.49	6.95	0.19	0.34	0.53
V/C	5.75	7.13	7.05	6.92	6.78	0.26	0.54	0.38	0.45

Abbreviations: CD, crypt depth; L, linear; Q, quadratic; VH, villus height; V/C, the ratio of villus height/crypt depth.
*Means differences between this group and the control group was different at $P < 0.05$.
[1]SEM, total standard error of means (n = 10).

Table 10. Effects of EO on intestinal function genes in broiler.

	Essential oil (mg/kg)						*P*-value		
Items[1]	0	50	100	200	400	SEM	L	Q	ANOVA
Jejunum of days 21									
SGLT1	1.00	1.10	1.34*	1.07	0.88	0.03	0.03	<0.01	<0.01
GLUT2	1.00	1.56*	1.29*	1.15	1.03	0.04	0.09	0.07	<0.01
SLC38A	1.00	1.31*	1.33*	1.10	1.08	0.04	0.42	0.25	0.02
SLC7A9	1.00	1.02	1.13	0.61*	0.54*	0.05	<0.01	<0.01	<0.01
SLC27A4	1.00	1.11	1.37*	1.23*	1.03	0.04	0.69	<0.01	<0.01
Occludin	1.00	1.39*	1.61*	1.12	1.03	0.06	0.20	0.06	<0.01
TJP1	1.00	1.02	1.35*	1.12	0.96	0.03	0.38	0.01	<0.01
Jejunum of days 42									
SGLT1	1.00	1.03	1.58*	0.93	0.79	0.06	0.09	0.08	<0.01
GLUT2	1.00	1.47	1.63*	1.51	1.11	0.06	0.66	0.01	0.02
SLC38A	1.00	1.11	1.25	1.36	1.07	0.05	0.77	0.05	0.20
SLC7A9	1.00	1.11	1.59*	1.47*	1.40*	0.06	0.04	<0.01	<0.01
SLC27A4	1.00	0.90	1.55*	1.19	1.00	0.06	0.86	0.10	<0.01
Occludin	1.00	1.28	1.33	1.01	0.98	0.04	0.09	0.11	0.18
TJP1	1.00	1.05	1.51*	1.29	1.20	0.05	0.35	0.03	<0.01
Ileum of days 21									
SGLT1	1.00	1.15	1.28	1.02	0.97	0.04	0.24	0.25	0.10
GLUT2	1.00	1.17	1.40*	1.17	1.01	0.04	0.32	<0.01	<0.01
SLC38A	1.00	1.17	1.94*	1.55*	1.45*	0.08	0.14	<0.01	<0.01
SLC7A9	1.00	1.46	1.94*	2.85*	1.95*	0.12	0.01	<0.01	<0.01
SLC27A4	1.00	1.09	1.40*	1.57*	1.87*	0.06	<0.01	<0.01	<0.01
Occludin	1.00	1.08	1.28	1.19	0.97	0.04	0.56	0.08	0.18
TJP1	1.00	1.31	2.12*	1.35	1.17	0.09	0.71	0.07	<0.01
Ileum of days 42									
SGLT1	1.00	1.06	1.11	1.05	0.98	0.03	0.52	0.44	0.71
GLUT2	1.00	1.43*	1.56*	1.24	1.13	0.05	0.54	0.09	0.01
SLC38A	1.00	1.53	2.25*	1.52	1.26	0.11	0.75	0.02	<0.01
SLC7A9	1.00	1.37	2.20*	1.54*	1.17	0.07	0.68	<0.01	<0.01
SLC27A4	1.00	1.58*	1.63*	1.78*	1.86*	0.08	<0.01	<0.01	<0.01
Occludin	1.00	1.04	1.23	1.16	1.11	0.04	0.41	0.17	0.25
TJP1	1.00	1.43*	1.56*	1.24	1.10	0.04	0.35	0.02	<0.01

Abbreviations: GLUT2, glucose transporter 2; L, linear; Q, quadratic; SGLT1, sodium-dependent glucose transporters 1; SLC38A, solute carrier family 38; SLC7A9, solute carrier family 7 member 9; SLC27A4, fatty acid transport protein 4.

*Means differences between this group and the control group was different at $P < 0.05$.

[1]SEM, total standard error of means (n = 10).

in ileum at d 42 were quadratically and linearly increased ($P < 0.05$), the expression levels of SLC38A, SLC79A and TJP1 in jejunum at d 21 were quadratically increased ($P < 0.05$).

DISCUSSION

The current study was carried out to explore the effects of EO on growth performance, digestibility, immunity and intestinal health in broilers. Our findings showed that EO supplementation quadratically increased ADG at 1 to 21 d, and dietary supplementation with 200 mg/kg EO increased body weight by 5% at 42 d of age. These findings were in agreement with previous studies (Adaszyńska-Skwirzyńska and Szczerbińska, 2019; Saleh et al., 2014; Upadhaya et al., 2019), who reported the positive impact of EO on poultry production performance. The positive effects of EO on the avian digestive system maybe one factor of improved production performance, since they help to restore the microbiota balance and increase nutrient absorption (Mountzouris et al., 2011). Our results also confirm the theory that the digestibility of DM, GE, and EE were significantly increased with all levels of EO supplementation, and EO2 and EO4 significantly increased the digestibility of CP compared with CON group. On the one hand, EO affected the taste and smell of the feed, which stimulates the secretion of saliva and gastric juices (Gopi et al., 2013), on the other hand, EO also boost the production and enhance the activity of digestive enzymes (Mnafgui et al., 2016; Zhang et al., 2020). In agreement with previous study (Xu et al., 2018), in present study the activity of sucrase was improved by EO addition. However, the application of EO as growth stimulator substitutes in broiler diets does not always improve production performance, and sometimes even makes it worse (Adaszyńska-Skwirzyńska and Szczerbińska, 2017; Kırkpınar et al., 2011; Zeng et al., 2016). Some of the oils may be irritant to the mucous lining of the gut, resulting in inflammation. Therefore, it is important to appropriately select, compose, and dose EO supplementation.

Sodium-glucose cotransporter 1 (SGLT1) and glucose transporter 2 (GLUT2) are two important transporters in intestine, are responsible for transporting glucose from the intestinal lumen to the enterocyte and then to the blood stream (Gorboulev et al., 2012). Our findings showed that the expression levels of GLUT2 and SGLT1 in jejunum and ileum were upregulated with EO supplementation, which was similar with other reports (Su et al., 2018). What's more, the expression levels of SLC38A, SLC79A and SLC27A4 in jejunum and ileum

were upregulated with EO supplementation. SLC38A and SLC79A are the member of solute carriers, known to control the uptake and flow of various substances such as sugar, amino acids, nucleotides, inorganic ions, and drugs over the cell membrane (Sundberg, et al., 2008). SLC27A4 is a member of the fatty acid transport protein (FATP) family, a group of evolutionarily conserved proteins that are involved in cellular uptake and metabolism of long and very long chain fatty acids (Kim et al., 2019). The up-regulated expression of these nutrient transporters may be another of the reasons for the increased digestibility of nutrients.

Villus and crypts are two important components of the small intestine and their geometry provides an indicator of the absorptive capacity of the small intestine (Heydarian et al., 2020). Turnover of the intestinal epithelium reflects a dynamic equilibrium between the production of enterocytes in the crypts and their subsequent desquamation from the villus. The villus height: crypts depth (VH: CD) ratio is an available criterion for evaluating intestinal health and function (Su, et al., 2018). Our results showed that CD and V/C were improved with EO supplementation in jejunum, which is similar with previous researches (Kishawy et al., 2019; Yarmohammadi Barbarestani, et al., 2020). As the largest barrier between the host and the external environment, intestinal structural integrity is also an important factor that ensures nutrient absorption and intestinal health. Its barrier function is very complex and comprises multiple protective mechanisms, including the tight junction (Occludin and TJP1, and others) structure, the mucus layer, the microbial community, and abundant gut-associated lymphoid tissues (Wang et al., 2020). Our results find that the expression of Occludin and TJP1 were up-regulated with EO addition, agree with the reports (Liu, et al., 2018; Liu, et al., 2020b; Shang et al., 2020; Song et al., 2017).

The mucosal immune system in the gut faces the formidable task of eliminating potential pathogens while maintaining a mutually beneficial relationship with the commensal microbiota. Antibodies of the SIgA class act as the first line of antigen-specific immunity in the gut, and can recognize both pathogens and commensals (Liu et al., 2020a). In present study, the level of SIgA was increased by EO addition in jejunum and ileum at 21 d of age. Furthermore, EO supplementation also improved the level of serous IgG in broilers at 42 d of age. As we all know, the intestine tissue is the largest immunity organ, as well as an important mediator of immunity and oxidative status (Kelly and Coutts, 2000). Excessive oxidative stress and inflammation are common features in the occurrence and development of intestinal diseases. Excessive oxidative stress could cause intestinal inflammation and even cell apoptosis within intestine tissue, following the dysfunctions of intestinal barrier (Xue et al., 2020). In the study, EO addition not only increases the production of immunoglobulin, but also enhances the antioxidant ability of serum. The result was similar with S. Yarmohammadi Barbarestani who reported that dietary supplementation of lavender essential oil at both levels increased the activities of SOD and GSH-Px and decreased the content of MDA in the serum (Yarmohammadi Barbarestani et al., 2020). The enhanced immunity and antioxidant ability with EO supplementation may improve intestinal integrity, function, and health. Improved immunity can encourage more nutrients to be used for growth; these may partly explain the superior growth performance in groups receiving EO in the present study.

CONCLUSION

Based on the conducted study, a blend EO (3.05% thymol, 2.3% carvacrol and 0.26% cinnamaldehyde) supplementation improved broiler performance by increasing nutrients digestibility, up-regulating transport protein, modulating intestinal morphology, enhancing immunity and antioxidant ability. Herein, a level of 200 mg/kg of EO was recommended in broiler diet according growth performance and health.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

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