Effects of oregano essential oil as an antibiotic growth promoter alternative on growth performance, antioxidant status, and intestinal health of broilers

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ABSTRACT This experiment was conducted to assess the comparative effects of dietary antibiotics and oregano essential oil (**OEO**) addition on growth performance, antioxidant status and intestinal health of broilers. A total of 384 one-day-old broilers were randomly allocated to 4 treatments with 6 replicates of 16 broilers each. The 4 treatments were: an antibiotic-free control diet (control), control + 20 mg/kg colistin sulfate and 20 mg/kg virginiamycin (antibiotics), control + 200 mg/kg natural oregano essential oil (**NOEO**), and control + 200 mg/kg synthetic oregano essential oil (**SOEO**). The experiment lasted for 42 d. Results showed that birds fed with OEO had greater ($P < 0.05$) average daily gain (**ADG**) and lower ($P < 0.05$) feed conversion ratio (**FCR**) than those fed with control diet during d 1 to 21. Besides, birds fed with NOEO had the greatest ($P < 0.05$) ADG in the four groups during d 22 to 42. The serum oxidative stress parameters showed that OEO improved ($P < 0.05$) the activities of glutathione peroxidase (**GSH-Px**), superoxide dismutase (**SOD**) and glutathione reductase (**GR**) of birds on day 21 and the activity of total antioxidant capacity (**T-AOC**) of birds on d 42. Relative to control, NOEO increased ($P < 0.05$) the activity of T-AOC in jejunum and decreased ($P < 0.05$) the level of malondialdehyde (**MDA**) in serum and jejunum. Moreover, OEO supplementation increased ($P < 0.05$) the concentrations of sIgA in duodenum and jejunum, *Lactobacillus* and total anaerobes in cecum, as well as activities of trypsin, chymotrypsin, lipase and amylase in duodenum, but restrained ($P < 0.05$) the amount of *Escherichia coli*. The NOEO supplementation increased ($P < 0.05$) total anaerobes of broilers on d 42 and the villus height to crypt depth ratio (**VH/CD**) of ileum. These results suggest that OEO improved antioxidant status and intestinal health of broilers which contributed to the growth performance improvement of broilers. Dietary OEO supplementation can be a promising alternative to antibiotic growth promoters for improving poultry production.

Key words: oregano essential oil, broiler, growth performance, antioxidant status, intestinal health

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INTRODUCTION

Antibiotic growth promoters (**AGP**) have been used in animal production for many years to improve intestinal health, promote growth performance, and prevent diseases (Dibner and Richards, 2005). However, with the long-term abuse of AGP, public concerns have increased regarding antibiotic drug residues and resistant bacteria (Yang et al., 2019). In recent years, the European Union has banned the use of antibiotics as growth promoters for poultry (Barug et al., 2006). The use of AGP in animal feed has been banned in China since July 1st, 2020. Consequently, animal husbandry suffered a great challenge including reduction in growth performance, disease resistance and financial profit loss, which increased pressure to develop safe and effective strategies to maintain intestinal health of animals. At present, the use of phytochemicals is gaining more attention worldwide for animals to improve production performance and modulate antioxidant status (Dhama et al., 2015). Bozkurt et al. (2009) reported that some effective supplements such as medical plants and their extracts have been gradually used in diet to replace AGP.

Oregano essential oil (**OEO**) extracted from plants of the *Origanum* genus has been known to contain mainly thymol and carvacrol (Oniga et al., 2018). In recent years, OEO has drawn increasing attention due to its beneficial effects in maintaining gut health. However, results regarding the effects of OEO on the performance of poultry have some inconsistency. Several studies

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claimed that there was no effect of dried oregano leaves or OEO supplementation on animal performance and gut characteristics (Bampidis et al., 2005; Barreto et al., 2008; Cerisuelo et al., 2014). Nevertheless, Peng et al. (2016) found that the use of OEO had a positive influence over the intestinal health, and thereby improved the growth performance and carcass traits of broilers. In recent years, Reyer et al. (2017) found that an essential oil mixture from star anise, rosemary, thyme and oregano improved performance of broilers by an overlapping mode of action including local effects at the intestinal border and systemic alterations of macronutrient metabolism. It has been reported that oregano, thymol and carvacrol had antioxidant activity in vitro and in vivo (Milos et al., 2000; Martinez-Tome et al., 2001; Kulisic et al., 2004). Zou et al. (2016) reported that OEO alleviated the negative effects of transportation on pigs by improving antioxidant status. The live weight of chickens and resistance to oxidation of meat were influenced by dietary oregano aqueous extract addition positively (Forte et al., 2018). Previous studies with other species have shown that oregano, thymol and carvacrol had improved intestinal barrier function and modulated intestinal bacterial and morphology (Wei et al., 2015; Cheng et al., 2018; Abdel-Latif et al., 2020). Liolios et al. (2009) testified the antimicrobial effects of carvacrol and thymol isolated from oregano on many kinds of bacteria including *Escherichia coli, Staphylococcus aureus* and *Streptococcus mutans* in vitro. Furthermore, another experiment showed that OEO had inhibited the oxidant of fatty acids except for antimicrobial activity on *Escherichia coli, Salmonella typhimurium* and *Clostridium perfringens* (Henn et al., 2010).

Chemical analysis of OEO has shown its constituents to be principally carvacrol and thymol which exhibited important antimicrobial properties (Burt et al., 2005). However, there is evidence that the antimicrobial activity of OEO is greater than the additive effect of its major antimicrobial components (Lattaoui and Tantaoui-Elaraki, 1994). In a study comparing the effect of the combination of carvacrol and thymol to natural oregano essential oil (**NOEO**), NOEO was observed to be more efficient in acting as a growth promoter of channel catfish than the combination of carvacrol and thymol (Zheng et al., 2009), indicating that the other components in NOEO may also play a great role for animal performance. Therefore, it may be more useful to supplement NOEO extracted from natural oregano than synthetic oregano essential oil (**SOEO**) composed of chemically synthesized carvacrol and thymol in poultry diets.

Since oregano had been generally recognized as safe, it is of great importance to explore the impact of OEO as an alternative to AGP to be used in poultry diets. Meanwhile, considering the inconsistency of results from earlier studies and the limited studies on the comparison of effects of NOEO and SOEO on growth performance and antioxidant status of broilers, more related studies are needed to investigate the specific function of OEO on broilers. Considering phytogenic essential oil may have beneficial effects on the antibacterial activity and antioxidative properties of broilers, analyses of intestinal health and some antioxidative parameters were believed to be important tools to evaluate the health status of poultry. The purpose of this study, thus, was to evaluate the effects of NOEO and SOEO in comparison to AGP on growth performance, antioxidant activity, and intestinal health in broilers.

METHODS AND MATERIALS

All the experimental procedures were agreed by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China; No. AW09089104-1).

Birds, Diets, and Management

A total of 384 1-day-old Arbor Acre broilers (half male and half female) obtained from a local commercial hatchery (Beijing Arbor Acres poultry breeding Co. Ltd.), were randomly assigned into 4 treatments with 6 replicate cages of 16 broilers each (8 male and 8 female). Diet treatments were as follows: an antibiotic-free control diet (control), control + 20 mg/kg colistin sulfate and 20 mg/kg virginiamycin (antibiotics), control + 200 mg/kg natural oregano essential oil (NOEO), and control + 200 mg/kg synthetic oregano essential oil (SOEO). The colistin sulfate (purity, 10%) was obtained from Shandong Lukang Pharmaceutical Group Co. Ltd. (Jining, Shandong, China). The virginiamycin (purity, 50%) was purchased from Phibro Animal Health Co. (Teaneck, NJ, USA). The NOEO preparation, ORSENTIAL Dry and SOEO were obtained from Kemin Technologies Co. Ltd. (Zhuhai, Guangdong, China). Based on the obtained information, 2.64% of primary active ingredients were carvacrol and 1.3% were thymol in the NOEO. The SOEO was a premix made mainly from thymol (98%, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and carvacrol (98%, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The preparation process and effective thymol and carvacrol contents of SOEO were same with that of NOEO. The difference was that the thymol and carvacrol in NOEO were from the essential oil extracted from proprietary oregano clonal lines, but which in SOEO were chemically synthesized. Then, both NOEO and SOEO were adsorbed with silica to preserve it because of the volatile properties, and next diluted with defatted rice bran as the carrier. The feeding process was consisted of 2 phases: starter phase (d 1 to 21) and grower phase (d 22 to 42). The initial temperature of the room was set at 32°C and reduced by 2 or 3°C weekly until reaching at 22°C in wk 4, and then the temperature was maintained consistently to the end of the experiment. Continuous light was maintained. The broilers were provided with ad libitum access to mash feed and water. The compositions of basal diets and nutrients level are presented in

Table 1. The experiment diets were based on maize-soybean meal and met the NRC (1994) requirements of broiler chickens. All broilers were cared for in accordance with the guidelines for the care and use of laboratory animals issued by the National Institute of Health and China's Ministry of Agriculture.

Growth Performance

Individual body weight and feed consumption per cage were recorded on d 1, 21 and 42 after 12-h fast to calculate the average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**), and these parameters were corrected for mortality.

Sample Collection

On d 21 and 42, 2 birds per cage with the body weight close to the mean body weight of the cage (12 birds per treatment) were selected. Blood samples were collected by puncturing the vein of wings and clot in polypropylene tubes. Serum samples were separated after blood samples were centrifuged at 1000 × *g* for 10 min at 4°C and stored at -20°C until analysis. After blood sample collection, birds were slaughtered by dislocation of the neck vertebrae and bleeding of the carotid artery. Subsequently, 2 cm segments from the median sections of the intestine (duodenum, jejunum and ileum) were collected and preserved in buffered formalin (10% neutral buffered formalin; Sigma-Aldrich, St. Louis, MO) for intestine morphological measurements. Another 2 cm segments from duodenum and jejunum were isolated and rapidly frozen in liquid nitrogen, then stored at -80°C until the analysis of the secretory immunoglobulin A (**sIgA**) level. The remaining portion of the intestine was opened longitudinally and the mucosa was scraped from the middle portion of the duodenum and jejunum; and stored in sterile centrifuges tube, snap-frozen in liquid nitrogen, and stored at -80°C for the assay of antioxidant indices. The intestinal (duodenum and cecum) tract digesta was collected to evaluate microbial populations and digestive enzyme activities.

Table 1. Dietary composition and nutrient levels of the basal diets.

Ingredients, %	Starter diet (d 1 to 21)	Grower diet (d 22 to 42)
Corn	57.67	59.80
Soybean meal	28.30	25.65
Extruded soybean	8.0	8.0
Soybean oil	1.9	3.0
Limestone	1.3	1.1
Calcium hydrophosphate	1.7	1.6
Salt	0.3	0.3
Lysine 98.5%	0.1	0
DL-methionine	0.25	0.12
Threonine	0.05	0
Poultry vitamin mix[1]	0.03	0.03
Poultry mineral mix[2]	0.3	0.3
Choline chloride	0.1	0.1
Total	100.0	100.0
Calculated nutrient level		
Crude protein, %	20.51	19.28
Metabolizable energy, MJ/kg	12.55	12.97
Calcium, %	1.01	0.90
Total phosphorus, %	0.66	0.63
Nonphytate phosphorus, %	0.43	0.41
Methionine, %	0.56	0.42
Methionine + Cystine, %	0.91	0.76
Lysine, %	1.15	1.01
Tryptophan, %	0.24	0.22
Threonine, %	0.81	0.72

[1]Vitamin mix provided the following (per kg of diet): vitamin A, 10000 IU; vitamin D_3, 2,000 IU; vitamin E, 20 IU; vitamin K_3, 3 mg; vitamin B_2, 2.5 mg; vitamin B_6, 0.4 mg; vitamin B_{12}, 0.015 mg; vitamin B_5, 8 mg; nicotinic acid, 25 mg; folic acid, 1.2 mg; choline chloride, 450 mg.

[2]Trace mineral mix provided the following (per kg of diet): copper ($CuSO_4 \cdot 5H_2O$), 15 mg; iron ($FeSO_4 \cdot 7H_2O$), 20 mg; zinc (ZnO), 80 mg; manganese ($MnSO_4 \cdot H_2O$), 80 mg; iodine (from calcium iodate), 1.5 mg; selenium (from sodium selenite), 0.3 mg.

Determination of Oxidative Stress Parameters in the Serum/Duodenum/Jejunum

The contents of glutathione peroxidase (**GSH-Px**), superoxide dismutase (**SOD**), total antioxidant capacity (**T-AOC**), glutathione reductases (**GR**) and malondialdehyde (**MDA**) in serum were measured using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Intestinal mucosa was homogenized in ice-cold phosphate-buffered saline (**PBS**) and then centrifuged at 10,000 × *g* at 4°C for 10 min, and the supernatant was stored at -80°C. The activities of GSH-Px, SOD, T-AOC and GR, and the level of MDA in the supernatant of the intestine homogenate were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All procedures were performed according to the manufacturer's instructions.

Determination of sIgA Content in the Duodenum/Jejunum

sIgA concentration in jejunal mucosa was measured using a commercially available chicken ELISA kit (China Institute of Atomic Energy, Beijing, China) following the manufacturer's protocol and expressed as units/g protein. The concentration of protein in the sample was determined using a bicinchoninic acid (BCA) protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Intestinal Microbial Populations

Samples of the chyme from the cecum were immediately collected into glass containers under CO_2, sealed, and put on ice until they were transported to the laboratory for microbial populations enumeration. The cecum chyme was diluted by sterile physical saline by 10-folds serial dilutions (from 10^{-1} to 10^{-7}). These dilutions were then inoculated on labeled selective agar medium for target bacterial groups, including MacConkey agar (Beijing Aoboxing Biotech. Co., Beijing, China) for

Escherichia coli, Eosin Methylene Blue agar (Beijing Aoboxing Biotech. Co., Beijing, China) for *Salmonella*, Tryptose Sulfite Cycloserine agar (Beijing Aoboxing Biotech. Co., Beijing, China) for *Clostridium perfringens*, and De Man, Rogosa and Sharpe agar (Beijing Aoboxing Biotech. Co., Beijing, China) for *Lactobacillus*. The plates for *Escherichia coli, Salmonella*, and *Clostridium perfringens* were incubated at 37°C for 24 h, whereas the plates for *Lactobacillus* were incubated at 30°C for 48 h. Total aerobe and total anaerobe were inoculated on Nutrient agar (Beijing Aoboxing Biotech. Co., Beijing, China). Afterwards, the inoculated plates were incubated at 37°C for 24 h under aerobic and anaerobic conditions, respectively.

Duodenal Digestive Enzyme Activities

The frozen samples of chyme were thawed in a refrigerator at 4°C. Bovine serum albumin was used as the protein standard. The activities of trypsin, chymotrypsin, lipase and amylase in the thawed samples were measured using corresponding business kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the procedure was performed in accordance with the manufacturer's instructions.

Intestinal Morphological Measurements

To carry out a histological morphometric analysis of the intestine, formalin-fixed tissue samples removed from the duodenum, jejunum and ileum were dehydrated, washed with physiological saline solution, treated in tissue-processor apparatus and embedded in paraffin wax. Three cross-sections for each intestinal sample (total of 15 samples for each of the 3 intestinal segments per dietary treatment) were prepared after staining with hematoxylin and eosin. Morphological indices including villus height (**VH**) and crypt depth (**CD**) were determined using an Olympus IX81 microscope and CellSens Imaging software (Olympus America Inc., Center Valley, PA, USA). The ratio of VH to CD (**VH/CD**) can be finally calculated.

Statistical Analysis

The test data were analyzed with one-way ANOVA using the SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, USA) statistical software. Duncan's multiple comparison test was performed. A significance level of $P < 0.05$ was used. The graphs were generated with GraphPad Prism 8.00 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Growth Performance

The effects of dietary treatments on growth performance of broilers are shown in Table 2. During d 1 to 21, the ADG of broilers was affected ($P < 0.05$) by dietary treatments. Specifically, dietary NOEO and SOEO supplementation increased ($P < 0.05$) ADG compared with control. The highest ADG was obtained in NOEO group, whereas ADG was lowest for the control group. Broilers in antibiotics group had lower ($P < 0.05$) ADG than that in NOEO group. During d 1 to 42, the ADG of broilers from control or SOEO group was less ($P < 0.05$) than that from NOEO group. The ADFI was similar among treatments during each phase, but the FCR was decreased ($P < 0.05$) in groups with NOEO or SOEO relative to control or antibiotics group during d 1 to 21.

Antioxidant Variables in the Serum

The results of the effects of OEO on antioxidant variables in the serum of broilers are summarized in Table 3. On d 21, compared with the control group, broilers fed with antibiotics, NOEO and SOEO exhibited higher ($P < 0.05$) concentrations of GSH-Px and SOD. Moreover, dietary NOEO and SOEO supplementation increased ($P < 0.05$) serum GR concentration. The level of MDA was decreased ($P < 0.05$) with dietary antibiotics and NOEO

Table 2. Effect of dietary OEO supplementation on growth performance of broiler chickens.

	Treatments[1]					
Items	Control	Antibiotics	NOEO	SOEO	SEM[2]	*P*-value
ADG, g hydrophosphate						
1 to 21 d	24.59[c]	24.91[b,c]	26.18[a]	25.68[a,b]	0.193	0.003
22 to 42 d	71.04	73.46	75.66	69.96	0.872	0.078
1 to 42 d	47.74[b]	49.18[a,b]	50.92[a]	47.82[b]	0.475	0.046
ADFI, g						
1 to 21 d	42.27	42.37	42.49	41.87	0.171	0.633
22 to 42 d	138.4	141.3	144.8	135.7	1.556	0.195
1 to 42 d	88.87	90.36	92.15	87.32	0.807	0.170
FCR, g/g						
1 to 21 d	1.72[a]	1.70[a]	1.63[b]	1.63[b]	0.014	0.001
22 to 42 d	1.95	1.92	1.91	1.94	0.018	0.604
1 to 42 d	1.86	1.84	1.81	1.83	0.008	0.089

[a,b,c]Means with different superscripts in each row differ significantly ($P < 0.05$).

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[1]Control, without antibiotics, NOEO or SOEO; antibiotics, 20 mg colistin sulfate and 20 mg virginiamycin/kg of feed; NOEO, 200 mg natural oregano essential oil/kg of feed; SOEO, 200 mg synthetic oregano essential oil/kg of feed.

[2]SEM: standard error of the means.

Table 3. Effect of dietary OEO supplementation on antioxidant indices in serum of broiler chickens.

	Treatments[1]					
Items	Control	Antibiotics	NOEO	SOEO	SEM[2]	*P*-value
21 d						
GSH-Px (U/mL)	789.5[c]	811.8[b]	840.9[a]	838.8[a]	5.22	0.001
SOD (U/mL)	70.88[b]	75.06[a]	75.89[a]	76.60[a]	0.674	0.006
T-AOC (U/mL)	8.15	8.63	8.70	8.68	0.090	0.091
GR (U/L)	4.23[b]	4.34[b]	4.80[a]	4.82[a]	0.085	0.011
MDA (nmol/mL)	4.97[a]	4.24[b]	4.10[b]	4.61[a,b]	0.123	0.039
42 d						
GSH-Px (U/mL)	985.5[b]	1,054.0[a]	1,097.0[a]	1,072.0[a]	13.6	0.015
SOD (U/mL)	84.98[b]	88.43[a,b]	91.39[a]	91.74[a]	0.995	0.045
T-AOC (U/mL)	9.02[b]	10.04[a]	10.31[a]	10.69[a]	0.196	0.009
GR (U/L)	4.88[b]	5.25[b]	5.70[a]	5.72[a]	0.096	0.001
MDA (nmol/mL)	4.52	4.44	4.16	4.33	0.132	0.817

[a,b,c]Means with different superscripts in each row differ significantly ($P < 0.05$).

Abbreviations: GR, Glutathione reductase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, super-oxide dismutase; T-AOC, total antioxidant capacity.

[1]Control, without antibiotics, NOEO or SOEO; antibiotics, 20 mg colistin sulfate and 20 mg virginiamycin/kg of feed; NOEO, 200 mg natural oregano essential oil/kg of feed; SOEO, 200 mg synthetic oregano essential oil/kg of feed.

[2]SEM: standard error of the means.

supplementation but was not affected ($P > 0.05$) by dietary SOEO supplementation at d 21.

On d 42, the serum level of MDA did not differ ($P > 0.05$) among the 4 treatments. However, NOEO and SOEO supplementation increased ($P < 0.05$) serum levels of SOD and GR compared with control. Besides, birds fed with antibiotics, NOEO and SOEO had increased ($P < 0.05$) serum concentrations of GSH-Px and T-AOC than those fed with control diet.

Antioxidant Variables and sIgA Content in the Intestinal Mucosa

As indicated in Table 4, dietary SOEO addition increased ($P < 0.05$) SOD activity in duodenum of birds on d 21. Dietary antibiotics, NOEO and SOEO addition increased ($P < 0.05$) the activities of GSH-Px, T-AOC and GR of birds on d 42.

As indicated in Table 5, on d 21, dietary NOEO and SOEO supplementation increased ($P < 0.05$) GSH-Px activity in jejunum of broilers. Moreover, the activity of T-AOC in jejunum of broilers on d 21 was increased ($P < 0.05$) with dietary antibiotics or NOEO supplementation but was not affected ($P > 0.05$) by dietary SOEO supplementation. Antibiotics, NOEO and SOEO increased ($P < 0.05$) the activities of GSH-Px and SOD in jejunum of broilers on d 42. However, the level of MDA in jejunum of broilers was decreased ($P < 0.05$) with antibiotics or NOEO but was not affected ($P > 0.05$) by dietary SOEO supplementation on d 42.

Broilers fed with the OEO-supplemented diets showed higher ($P < 0.05$) sIgA content in duodenum and jejunum than that in the control group both on d 21 and 42 (Figure 1).

Cecal Microbial Population

The effects of different dietary treatments on cecal microbial population in broilers are shown in Figure 2. On d 21, dietary antibiotics, NOEO and SOEO addition

Table 4. Effect of dietary OEO supplementation on antioxidant indices in duodenum of broiler chickens.

	Treatments[1]					
Items	Control	Antibiotics	NOEO	SOEO	SEM[2]	*P*-value
21 d						
GSH-Px (U/mg)	92.9	94.82	97.55	101.2	1.264	0.101
SOD (U/mg)	51.52[b]	54.98[ab]	53.95[ab]	56.41[a]	0.650	0.044
MDA (nmol/mg)	3.30	3.07	2.79	2.69	0.146	0.465
T-AOC (U/mg)	3.93	5.24	5.40	5.68	0.259	0.066
GR (U/g)	2.71	2.99	3.37	3.46	0.134	0.170
42 d						
GSH-Px (U/mg)	75.18[b]	85.83[a]	90.63[a]	90.76[a]	1.839	0.002
SOD (U/mg)	84.70	88.58	94.95	94.92	1.843	0.125
MDA (nmol/mg)	5.82	5.26	4.93	4.98	0.145	0.098
T-AOC (U/mg)	7.11[b]	8.42[a]	8.21[a]	8.14[a]	0.183	0.039
GR (U/g)	3.02[b]	5.21[a]	4.86[a]	5.39[a]	0.308	0.013

[a,b]Means with different superscripts in each row differ significantly ($P < 0.05$).

Abbreviations: GR, Glutathione reductase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, super-oxide dismutase; T-AOC, total antioxidant capacity.

[1]Control, without antibiotics, NOEO or SOEO; antibiotics, 20 mg colistin sulfate and 20 mg virginiamycin/kg of feed; NOEO, 200 mg natural oregano essential oil/kg of feed; SOEO, 200 mg synthetic oregano essential oil/kg of feed.

[2]SEM: standard error of the means.

Table 5. Effect of dietary OEO supplementation on antioxidant indices in jejunum of broiler chickens.

Items	Treatments[1]				SEM[2]	*P*-value
	Control	Antibiotics	NOEO	SOEO		
21 d						
GSH-Px (U/mg)	95.36[c]	100.8[bc]	104.8[ab]	108.5[a]	1.367	0.001
SOD (U/mg)	49.96	54.68	55.78	54.33	1.113	0.274
MDA (nmol/mg)	2.96	2.61	2.38	2.54	0.093	0.195
T-AOC (U/mg)	4.89[b]	5.53[a]	5.49[a]	5.24[ab]	0.023	0.044
GR (U/g)	2.48	2.69	3.26	3.05	0.148	0.250
42 d						
GSH-Px (U/mg)	101.0[b]	120.3[a]	119.8[a]	112.2[a]	2.15	0.001
SOD (U/mg)	81.15[b]	91.31[a]	92.37[a]	91.23[a]	1.517	0.017
MDA (nmol/mg)	5.38[a]	4.09[b]	4.40[b]	4.87[ab]	0.173	0.035
T-AOC (U/mg)	7.91	8.69	8.80	9.17	0.180	0.083
GR (U/g)	4.22	5.70	5.69	5.16	0.229	0.061

[a,b,c]Means with different superscripts in each row differ significantly ($P < 0.05$).

Abbreviations: GR, Glutathione reductase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, super-oxide dismutase; T-AOC, total antioxidant capacity.

[1]Control, without antibiotics, NOEO or SOEO; antibiotics, 20 mg colistin sulfate and 20 mg virginiamycin/kg of feed; NOEO, 200 mg natural oregano essential oil/kg of feed; SOEO, 200 mg synthetic oregano essential oil/kg of feed.

[2]SEM: standard error of the means.

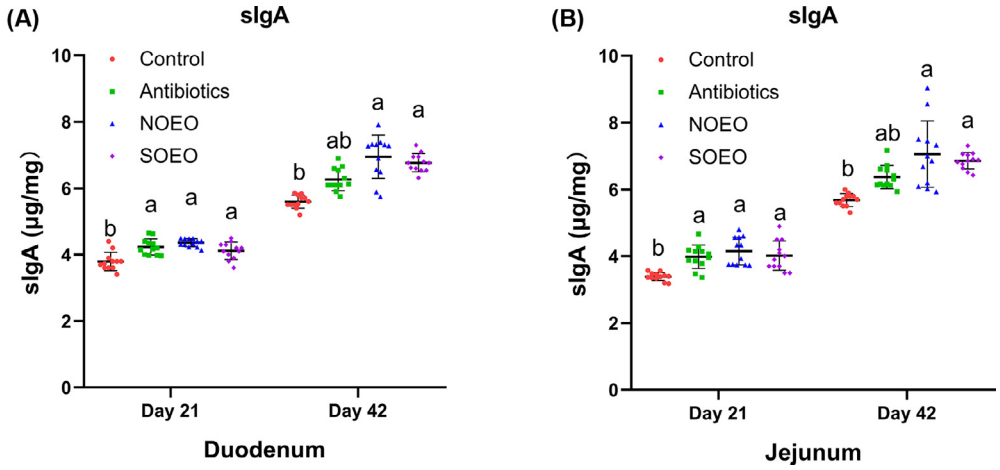

Figure 1. Effect of dietary OEO supplementation on sIgA content in the intestinal mucosa. [a,b]Different letters show significant differences among the treatments ($P < 0.05$). (A) sIgA content in duodenum; (B) sIgA content in jejunum.

increased ($P < 0.05$) the counts of *Lactobacillus* and total anaerobe but decreased ($P < 0.05$) the counts of *Escherichia coli* and *Salmonella* in cecum of broilers. On d 42, dietary supplementation of antibiotics, NOEO and SOEO increased ($P < 0.05$) the counts of *Lactobacillus* but decreased ($P < 0.05$) the counts of *Escherichia coli* in cecum of broilers. Besides, an increase ($P < 0.05$) of total anaerobe population in cecum of broilers was observed in NOEO group compared with control on d 42.

Duodenal Enzyme Activity

The effects of dietary supplementation of OEO on duodenal enzyme activity in broilers are shown in Table 6. The activity of lipase measured in the broilers on d 21 was increased ($P < 0.05$) by antibiotics supplementation. However, the dietary treatments had no significant effects ($P > 0.05$) on activities of trypsin, chymotrypsin and amylase of broilers on d 21. The activity of trypsin in broilers was increased ($P < 0.05$) with dietary NOEO and SOEO addition on d 42. In addition, the activities of chymotrypsin, lipase and amylase in the broilers on d 42 were increased ($P < 0.05$) with antibiotics, NOEO and SOEO supplementation.

Intestinal Morphology

The results of the effects of different dietary treatments on intestinal morphology in broilers are shown in Figure 3. On d 21, there were no statistically significant differences ($P > 0.05$) among treatments regarding to VH of duodenum and ileum, CD of duodenum, jejunum and ileum, and VH/CD of duodenum. On the contrary, the VH of jejunum and VH/CD of jejunum and ileum were increased ($P < 0.05$) with dietary antibiotics, NOEO and SOEO supplementation (as shown in Figure S1 to Figure S3).

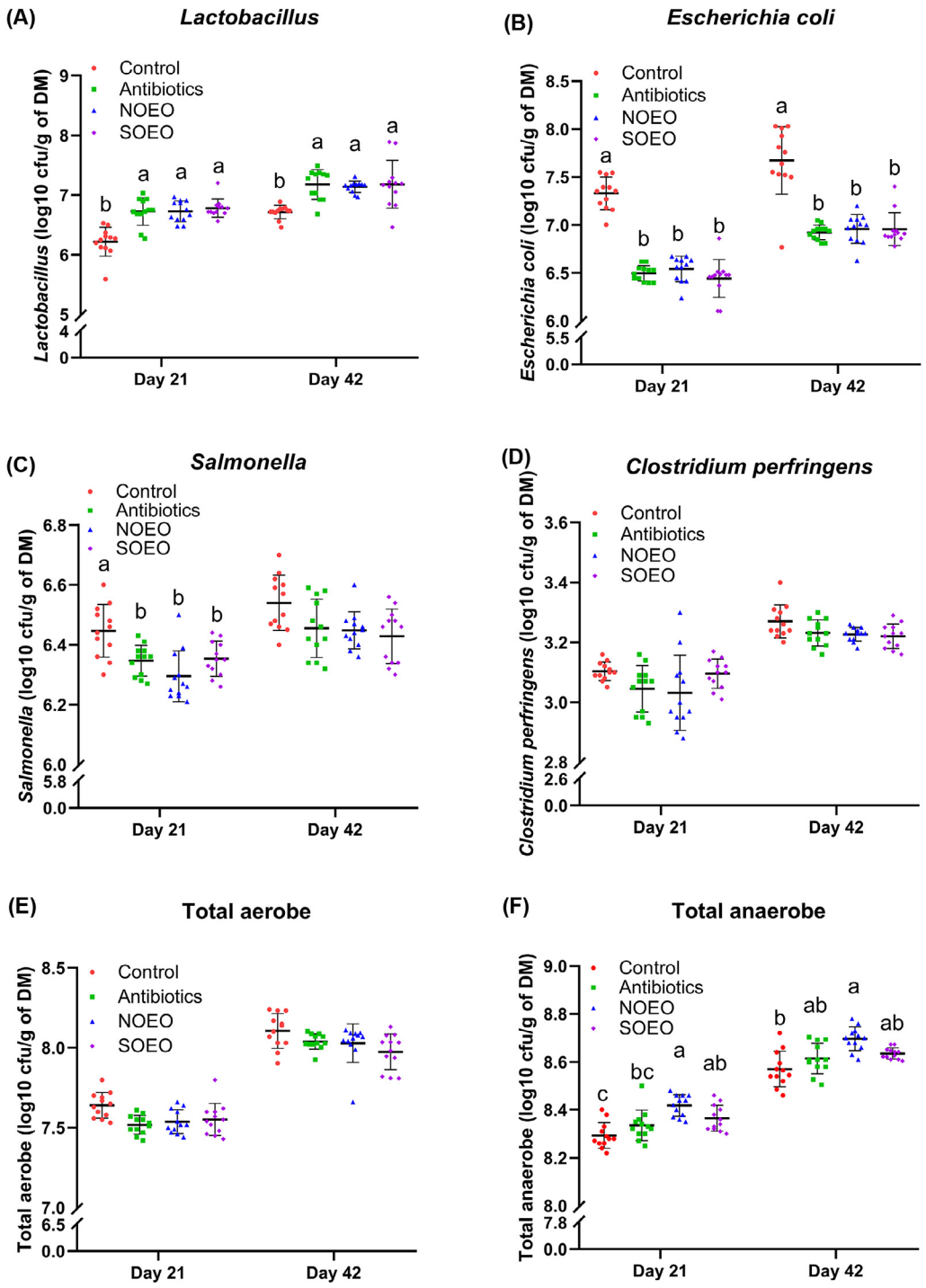

Figure 2. Effect of dietary OEO supplementation on cecal microflora of broiler chickens. [a,b,c]Different letters show significant differences among the treatments ($P < 0.05$). (A) *Lactobacillus* population; (B) *Escherichia coli* population; (C) *Salmonella* population; (D) *Clostridium perfringens* population; (E) Total aerobe population; (F) Total anaerobe population.

On d 42, the VH of duodenum was increased ($P < 0.05$) with dietary antibiotics, NOEO and SOEO addition (as shown in Figure S4). The CD of duodenum was decreased ($P < 0.05$) with NOEO and SOEO supplementation. However, VH and CD of jejunum and ileum were not significantly influenced by different treatments ($P > 0.05$) as shown in Figure S5 and Figure S6. In addition, the VH/CD of duodenum and jejunum were all increased ($P < 0.05$) with dietary antibiotics, NOEO and SOEO addition, and the VH/CD of ileum was increased ($P < 0.05$) with antibiotics and NOEO supplementation. VH/CD of ileum was not significantly affected ($P > 0.05$) by the dietary SOEO supplementation.

DISCUSSION

In the present study, dietary OEO supplementation increased the ADG of broilers during d 1 to 21 compared

Table 6. Effect of dietary OEO supplementation on duodenum digestive enzyme activities of broiler chickens.

Items	Treatments[1]				SEM[2]	*P*-value
	Control	Antibiotics	NOEO	SOEO		
21 d						
Trypsin, U/mg	2,909	3,055	2,945	3,183	44	0.107
Chymotrypsin, U/mg	3.88	4.17	4.51	4.52	0.099	0.051
Lipase, U/g	34.52[b]	40.97[a]	37.66[ab]	38.02[ab]	0.742	0.012
Amylase, U/mg hydrophosphate	3.66	4.51	4.45	4.54	0.154	0.122
42 d						
Trypsin, U/mg	4,650.0[b]	5,094.0[ab]	5,566.0[a]	5,440.0[a]	108.4	0.005
Chymotrypsin, U/mg	7.71[b]	8.73[a]	8.84[a]	8.56[a]	0.127	0.001
Lipase, U/g	97.73[b]	114.5[a]	119.4[a]	1,17.1[a]	2.58	0.004
Amylase, U/mg hydrophosphate	5.44[b]	5.88[a]	5.98[a]	6.02[a]	0.073	0.009

[a,b]Means with different superscripts in each row differ significantly ($P < 0.05$).
[1]Control, without antibiotics, NOEO or SOEO; antibiotics, 20 mg colistin sulfate and 20 mg virginiamycin/kg of feed; NOEO, 200 mg natural oregano essential oil/kg of feed; SOEO, 200 mg synthetic oregano essential oil/kg of feed.
[2]SEM: standard error of the means.

with control. Because OEO supplementation reduced FCR without influencing ADFI of broilers in the starter phase, the improved ADG of broilers was likely due to increased nutrient utilization during d 1 to 21. Furthermore, broilers fed with NOEO in the starter phase had better growth performance than those fed antibiotics, indicating that NOEO may be an alternative to antibiotics in poultry diets and may be more effective on growth performance than antibiotics. Similar ADG results were also reported by Peng et al. (2016) at 300 and 600 mg/kg of feed of broilers and Ariza-Nieto et al. (2018) at 250 mg/kg of feed of broilers. Nevertheless, there were some conflicting findings suggesting that dietary OEO addition had no significant effects on the growth performance of broilers (Hernandez-Coronado et al., 2019) and ducks (Ding et al., 2020). Differential effects of ADG may depend upon differences in the levels and components of OEO. Hernandez-Coronado et al. (2019) found that there was no effect for OEO at 400 mg/L drinking water. This result presented low ADG yields for low level of OEO, which in contrast with the ADG in the current study at 200 mg/kg OEO. In our study, dietary NOEO addition improved the growth performance of broilers during d 1 to 42. However, there was no significant improvement of the growth performance of broilers fed with SOEO. This result indicated that some other components except for carvacrol and thymol in NOEO may play a great role for the growth performance of the broilers. Cross et al. (2003) pointed out that the addition of thyme oil did not affect the ADG of broilers. Additionally, Lee et al. (2003) indicated that 200 mg/kg of thymol in diet had no effect on the growth performance of broilers. The contradictory result suggested that the effect of OEO on the growth performance of broilers may be different from that of thymol which was the important component of OEO. Therefore, since the addition level and composition of OEO may be the main factors affecting the performance of broilers, these two important factors should be taken into account in production practice.

The activities of antioxidant enzymes and concentrations of oxidative products are important for assessing the oxidant status of animals. In our present study, the inclusion of OEO in the broilers' diet caused an increase in serum, duodenal and jejunal GSH-Px and SOD, as well as serum and duodenal T-AOC and GR contents. The activities of these enzymes were regarded as sensitive indicators related to oxidative stress, so that the increase of antioxidant enzyme activity reflects effective defense systems (Jos et al., 1999). These results are in line with Ri et al. (2017) who reported that using oregano powder leads to an increase in T-AOC concentration in broilers. Ding et al. (2020) also observed that supplementation of OEO increased SOD activity in ducks. Carvacrol and thymol, two main phenols of OEO, were believed to contribute antioxidant activity by scavenge radicals in blood, and then affected the antioxidant defense system of animals (Yanishlieva et al., 1999). Additionally, NOEO addition decreased the MDA content in serum at 21 d and in jejunum at 42 d, which suggested that OEO could improve lipid antioxidation status. Furthermore, Basmacioglu et al. (2004) showed that the dietary OEO addition was effective in preventing lipid oxidation. Based on these results, we can speculate that maybe some antioxidant components presented in OEO entered the circulatory system of broilers, remained in the tissues and then exhibited protective ability on oxidative stress by induction of antioxidant enzymes.

Meanwhile, as a vital index of immune function to some extent, antioxidant function is closely related to immune function (Min et al., 2016). sIgA was secreted by animal plasma cells and was the most prominent antibody presented in mucosal surfaces and protected intestinal mucosa against invading pathogens in the gastrointestinal tract (Shang et al., 2020). However, limited studies have been conducted to evaluate the effects of OEO on broiler intestinal mucosa sIgA content. In the present study, greater sIgA concentrations in birds fed with OEO indicated that intestinal mucosal immunity was enhanced to protect the intestine against pathogen adherence. As reported by Tilg and Moschen (2015), the gut microbiota interacts directly or indirectly with the host immune system. There was speculation that dietary OEO supplementation may alter the intestinal microbial composition and stimulate the humoral immune system

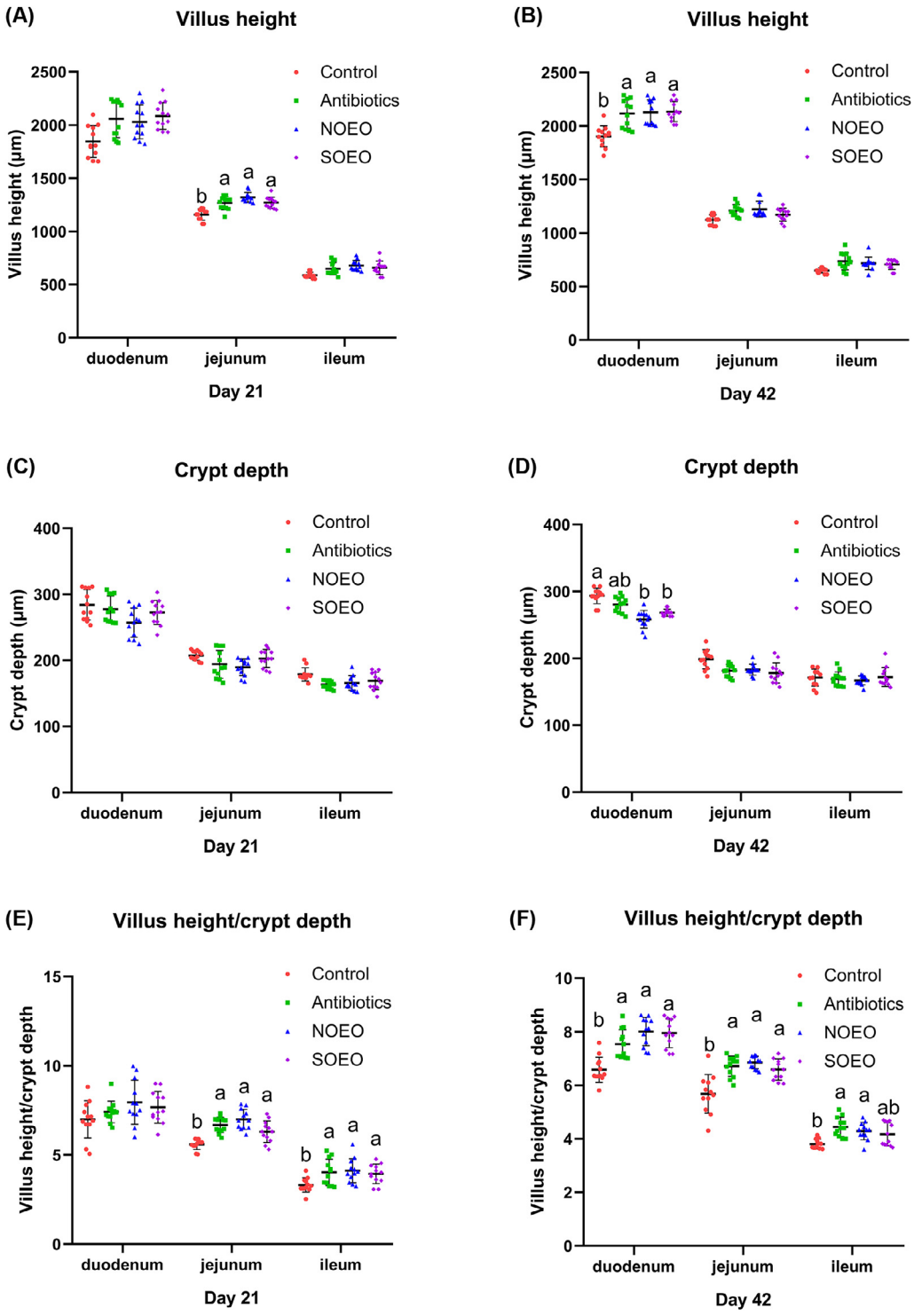

Figure 3. Effect of dietary OEO supplementation on intestinal morphology of broiler chickens. [a,b]Different letters show significant differences among the treatments ($P < 0.05$). (A) Villus height on d 21; (B) Villus height on d 42; (C) Crypt depth on d 21; (D) Crypt depth on d 42; (E) Villus height/crypt depth on d 21; (F) Villus height/crypt depth on d 42.

to produce and secrete more antibodies and therefore increased antibodies cover the surface of intestinal mucosa, protected villi from damage and further promoted the healthy functioning of the intestinal system of broilers. Ding et al. (2020) observed that addition of 100 mg/kg OEO of diet decreased the mRNA expression of sIgA genes in jejunum of ducks, the result of which is different from our study. The divergent results may be connected with the different animals and the addition levels of dietary OEO between these studies. Further studies deserve to be carried out to extend these findings.

In our work, the influence of OEO on *Lactobacillus* should be emphasized. As the role of *Lactobacillus* in protecting the intestinal environment against invasions by pathogens had been known for a long time (Mead, 2000), the stimulation of *Lactobacillus* with dietary OEO supplementation could contribute to balanced

gut microflora and provide a favorable condition for digestion processes (Franciosini et al., 2016). On the other hand, supplementation of experimental broilers with OEO lowered populations of cecal *Escherichia coli* and *Salmonella*, respectively, which were regarded as pathogens. Similarly, an in vitro study showed that two major components of OEO, carvacrol and thymol, were able to restrain the proliferation of *Escherichia coli* (Helander et al., 1998). The presence of carvacrol and thymol may have antibacterial effect. These two primary components of OEO enable to disturbance in the structures of the bacterial cell membrane, rendering it more permeable and leading to the leakage of ions and other cell contents, and finally causing bacteria to die (Ultee et al., 2002; Burt, 2004). Considering the large number of different groups of chemical compounds present in OEO, it is most likely that the antibacterial activity could be also attributed to some other different mechanisms. More studies are needed to evaluate the specific antibacterial mechanism of OEO in vivo. Thus, dietary OEO supplementation may modulate the intestinal health by promoting the growth of beneficial bacteria and inhibiting the growth of harmful bacteria. In terms of the gut, what is hard to interpret is that dietary inclusion of antibiotics containing virginiamycin, which was active against gram-positive bacteria, resulted in significantly higher count of *Lactobacillus* in cecum. This result was different from most of the other studies in which the count of *Lactobacillus* in the cecum of chickens was hardly affected by virginiamycin (Salim et al., 2013; Rostami et al, 2015). It may be partially explained by the addition of both virginiamycin and colistin sulfate in our study. The growth of *Escherichia coli* was inhibited by the supplementation of colistin sulfate, thus the status of cecal microbe was changed, and then it may result in the proliferation of *Lactobacillus*.

In the current study, the activities of trypsin, chymotrypsin, lipase and amylase in the duodenum contents of broilers on d 42 were all improved with dietary OEO supplementation. These results suggested that maybe OEO supplementation promoted the expression of digestive enzymes, and thereby increased the nutrient digestibility, eventually resulting in the improved growth performance of broilers. Little is known about the underlying mechanisms by which dietary supplementation of OEO modulates digestive enzymes, especially in the duodenum. Malayoglu et al. (2010) noted that dietary OEO supplementation at 250 or 500 mg/kg increased intestinal chymotrypsin activity as well as the crude protein digestibility coefficient. Unlike the present results, these researchers noted that intestinal amylase and lipase activities were not significantly affected by OEO. In addition, Lee et al. (2003) reported that thymol did not affect the intestinal enzyme activities, but a commercial preparation of essential oil components containing thymol increased the amylase activity of broilers on d 21. The same commercial preparation was used in the study of Jang et al. (2006), in which total and specific activities of trypsin and total α-amylase activity in pancreas were increased. Therefore, further research is needed to elucidate the mechanism by which OEO modulate the intestinal digestive enzymes of broilers.

In our current study, we found that broilers fed with OEO supplementation had higher VH and lower CD in duodenum on d 42, and had higher VH/CD in duodenum, jejunum and ileum on d 21 and d 42. These results were consistent with previous findings by Ding et al. (2020), who found a significant reduction of CD and increased VH/CD in jejunum of ducks. Moreover, supplementing 600 mg/kg OEO to basal diet showed higher VH in laying hens (Gul et al., 2019). Behnamifar et al. (2018) found that oregano extract improved the VH in ileum of quails as a beneficial and low-risk additive for poultries. An increased VH and VH/CD in jejunum may increase absorptive surface area and efficiency of digestion and absorption (Mohammadi et al., 2014). In addition, Windisch et al. (2008) suggested that herbal essential oil-induced improvement in intestinal morphology result from their antioxidant activities. The change of intestinal morphology affected by dietary OEO supplementation in our present study was in accordance with the improved antioxidant activities of serum and intestine.

It has been suggested that the lipophilic properties and the chemical structure of OEO played a role in the antimicrobial activity (Helander et al., 1998). The main components of OEO are carvacrol and thymol, which together represent up to 85% of its composition (Burt, 2004). Helander et al. (1998) investigated how OEO, carvacrol and thymol exert their antibacterial effects on *Escherichia coli* O_{157} and *Salmonella typhimurium*. It was indicated that these phenols can penetrate the membrane of the bacteria and reach the inner parts of the cell because of their lipophilic properties, and then lead to the release of membrane-associated material from the cells to the external medium by dissolving the bacterial membrane (Helander et al., 1998). Although the functions of NOEO and a combination of carvacrol and thymol were believed to be similar, minor components appeared to play a significant role. Some researchers showed that the functional groups (Farag et al., 1989) and aromaticity (Bowles and Miller, 1993) are responsible for their antibacterial activity. The effect of NOEO on the performance of broilers were compared with that of SOEO which containing chemically synthesized thymol and carvacrol in this study. Results of our study showed that although both NOEO and SOEO supplementation in broiler diets could improve the growth performance of broilers, still it was NOEO that showed the better performance as an antibiotics alternative. The serum MDA concentration and jejunum T-AOC concentration on d 21, as well as jejunum MDA concentration, ileum VH/CD and cecum total anaerobe population on d 42 of broilers from NOEO group were also higher than that from SOEO group. Similarly, there is evidence that the dietary NOEO supplementation improved the growth performance and antioxidant activity of channel catfish more effectively than the supplementation of carvacrol extract or the combination of

carvacrol and thymol extracts (Zheng et al., 2009). In the present study, the growth performance, antioxidant activity and intestinal health of broilers fed with NOEO was superior to SOEO. It is most likely that the other minor functional groups and aromatic compounds in NOEO may play a role in modulating intestinal health, improving oxidative status and growth performance, so that the NOEO may be more effective in promoting the growth of broilers than SOEO in which the active components only including thymol and carvacrol. Nevertheless, the exact mechanism for other minor active constituents in NOEO which played an important role in improving these factors needs further and more advanced study.

In conclusion, supplementation of OEO could enhance growth by improving intestinal immune and exerting protective effects on oxidant stress of broilers. Moreover, dietary 200 mg/kg NOEO addition showed better performance as a growth promoter and an antioxidant. Therefore, it can be concluded that the NOEO may play a pivotal role in replacing antibiotics in broilers; however, more studies are needed to assess the mechanism of action of OEO on the growth performance of broilers.

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DISCLOSURES

The authors declared that they have no conflicts of interest to this work.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101163.

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