

The dietary combination of essential oils and organic acids reduces *Salmonella enteritidis* in challenged chicks

S. Zhang,^{*,†} Y. R. Shen,^{*,†} S. Wu,^{*,†} Y. Q. Xiao,^{*,†} Q. He,[‡] and S. R. Shi^{*,†,§,1}

^{*}Poultry Institute, Chinese Academy of Agriculture Science, Yangzhou, Jiangsu 225125, China; [†]Institute of Effective Evaluation of Feed and Feed Additive (Poultry institute), Ministry of Agriculture, Yangzhou, Jiangsu 225125, China; [‡]Shanghai Menon Animal Nutrition Technology Co., LTD, Shanghai 201800, China; and [§]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu 225000, China

ABSTRACT This study was conducted to determine the effects of essential oils and organic acids (EOA) on *Salmonella* Enteritidis (**S. Enteritidis**) challenged chickens. One-day-old specific pathogen-free (SPF) chicks (250) were randomly assigned to 5 groups, with 50 birds in each group. The treatment groups were as follows: 1) basal diet, negative control group (NC); 2) basal diet + *S. Enteritidis*, positive control group (PC); 3) PC + 4,000 g/t of enrofloxacin (5%), antibiotic group (ENR); 4) PC + 800 g/t of EOA1, thymol-benzoic acid group (TBA); and 5) PC + 800 g/t of EOA2, cinnamylaldehyde-caproic acid group (CCA). At 7 D of age, each bird, except those in NC, was orally gavaged with 0.4 mL of a suspension of 4.4×10^9 cfu *S. Enteritidis*/mL. Results revealed that ENR reduced bacterial counts in the liver and spleen on days 3, 5, and 7 post-challenge more ($P < 0.05$) than any other treatments. However, bacterial counts in cecal contents

among ENR, TBA, and CCA were similar at 5 and 7 D post-challenge but lower than those of PC. Additionally, the bacterial counts in liver, spleen, and cecum contents in TBA were lower ($P < 0.05$) than in PC at 3, 5, and 7 D post-challenge; the bacterial counts in spleen contents in TBA were lower ($P < 0.05$) than in CCA at 7 D post-challenge. Tumor necrosis factor- α contents in TBA and CCA were lower ($P < 0.05$) than those in PC. Also, the ratio of villus height to crypt depth in the ileum of CCA was higher ($P < 0.05$) than that of PC and ENR; however, there was no difference in the secretory IgA content of the jejunum among the groups. In conclusion, EOA had a bacteriostatic effect on *S. Enteritidis*, and the effect of the thymol-benzoic acid complex surpassed that of the cinnamaldehyde-caproic acid complex. Therefore, EOA may act as an effective antibiotic substitute for animals in the prevention and treatment of *Salmonella*.

Key words: essential oils and organic acids, *Salmonella* Enteritidis, bacterial count, serum index, chicken

2019 Poultry Science 98:6349–6355
<http://dx.doi.org/10.3382/ps/pez457>

INTRODUCTION

Salmonella is an important zoonotic pathogen and is one of the causes of foodborne diseases that can lead to human morbidity, death, and serious economic losses (Lin et al., 2014; Sallam et al., 2014). Poultry is considered the main host of *Salmonella*, and infected poultry and contaminated poultry products are the main sources of human *Salmonella* infection, constituting a potential risk to public health (Liljebjelke et al., 2005). *Salmonella* Enteritidis (**S. Enteritidis**) serotype, which is among more than 2,500 different *Salmonella* serovars, is one of the most important serotypes that is spread from animals to humans (WHO, 2018). Therefore, it is important for the poultry industry and for human health to prevent and control *S. Enteritidis*. However,

S. Enteritidis tends to be highly resistant to many antibiotics, such as tetracycline, chloramphenicol, and fluoroquinolones because of their excessive use and abuse (Piedrola, 2001; Piddock, 2002). As a result, many countries have banned the use of antibiotics in animal feed. For example, the European Union banned the addition of antibiotics to feed in 2006 (Giannenas et al., 2014). Thus, the development of new antibacterial and environmentally friendly antibiotic substitutes is an important task for protecting animal and human health.

Because essential oils and organic acids (EOA) also have antimicrobial and antioxidant effects, which improve animal intestinal health and promote the absorption of nutrients, they are currently commonly used as antibiotic substitutes (Mueller et al., 2012; Hafeez et al., 2016). Many essential oils have inhibitory or sterilization effects on most plant and animal pathogens, and they inhibit many gram-negative and positive bacteria (Basile et al., 2006). Essential oils easily contact bacterial lipid membranes and then penetrate

© 2019 Poultry Science Association Inc.

Received November 2, 2018.

Accepted July 29, 2019.

¹Corresponding author: ssr236@163.com

bacterial cell membranes thereby achieving a bacteriostatic effect (Shao et al., 2013). Organic acids are also bacteriostatic because they easily enter bacteria and cause disorder of the bacterial metabolic system by altering pH, which eventually leads to decreased bacterial activity (Nava et al., 2009). The successful use of EOA in vivo depends on their antibacterial activity. Thus, in this study, different EOA were selected to compare their effects on *Salmonella* reduction in Leghorn chicks, in order to provide evidence for the prevention and treatment of *Salmonella*.

MATERIAL AND METHODS

Experimental Design and Animal Management

The EOA were provided by Shanghai Menon Animal Nutrition Technology Co., LTD (Shanghai, China) and coated by microcapsules. The main components of EOA1 were thymol, carvacrol, benzoic acid, and butyric acid, accounting for 8, 8, 2, and 1%, respectively, and the remaining excipients were 40% of silicon dioxide, 25% of palm oil, and 16% of others. The main components of EOA2 were cinnamylaldehyde, caproic acid, benzoic acid, and butyric acid, accounting for 7, 7, 1.5, and 1%, respectively, and the remaining excipients were 40% of silicon dioxide, 25% of palm oil, and 18.5% of others. A total of 300 specific pathogen-free (SPF) White Leghorn eggs were purchased from the Beijing Merial Vital Laboratory at the Animal Technology Co. LTD (Beijing, China) and were maintained in an automated hatchery. After hatching, 250 healthy SPF chicks were obtained and randomly assigned to 5 groups, with 50 chicks in each group. The treatment groups were as follows: 1) basal diet, negative control group (NC); 2) basal diet + *S. Enteritidis*, positive control group (PC); 3) PC + 4,000 g/t of enrofloxacin (5%), antibiotic group (ENR); 4) PC + 800 g/t of EOA1, thymol-benzoic acid group (TBA); and 5) PC + 800 g/t of EOA2, cinnamylaldehyde-caproic acid group (CCA). The NC group was raised in a separate room with the same structure as the challenged groups to avoid cross contamination. The birds in each group were fed with different diets starting from the age of 0 D. In addition, the birds had free access to feed and water and were reared in wire cages (1.2 × 0.9 m, length × width) with 50 birds per cage, with 23 h of illumination throughout the study. The temperature in the chick house was 32 to 35°C during the first week, and then decreased by 1°C per day until the final temperature of 27°C on 14th day. The basal diets were formulated to meet or exceed the nutrient requirements recommended by the National Research Council (NRC, 1994), and the nutrient composition is shown in Table 1. The experimental protocols used in this study were approved by the Animal Care and Use Committee of the Poultry Institute at the Chinese Academy of Agriculture Science (Yangzhou, Jiangsu, China).

Table 1. Diet composition and nutrient levels (as-fed basis) from 0 to 14 D of age.

Ingredient	%
Corn	63.44
Soybean meal (46%)	31.80
Soybean oil	0.70
NaCl	0.30
Calcium hydrogen phosphate	1.50
Limestone	1.57
Methionine	0.20
Lysine (hydrochloride)	0.11
Mineral premix ¹	0.20
Vitamin premix ²	0.03
Phytase	0.15
Total	100.00
Nutrient levels (%) ³	
ME(kcal/kg)	2953.8
CP	21.00
Lys	1.11
Met	0.49
Met+Cys	0.81
Ca	1.00
Total phosphorus	0.58
Nonphytate phosphorus	0.45

¹The mineral premix provides the following per kg of diet: Fe, 80 mg; Cu, 16 mg; Mn, 120 mg; Zn, 110 mg; Se, 0.3 mg; I, 1.5 mg; and Co, 0.5 mg.

²The vitamin premix provides the following per kg of diet: vitamin A (retinyl acetate), 15,000 IU; vitamin D₃ (cholecalciferol), 3,600 IU; vitamin E (*DL*- α -tocopheryl acetate), 30 IU; vitamin K, 3 mg; vitamin B₁, 2.7 mg; vitamin B₂, 9.6 mg; vitamin B₆, 3.75 mg; VB₁₂, 0.03 mg; D-pantothenic acid, 14.1 mg; niacin, 45 mg; folic acid, 1.5 mg; and D-biotin, 0.15 mg.

³The nutrient levels were calculated values.

Challenge with *Salmonella*

At 7 D of age, each chick was orally gavaged with 0.4 mL of a suspension of 4.4×10^9 cfu *S. Enteritidis*/mL, except for the NC group, which was administered the same volume of PBS. The bacterial strain SC070 was a gift from the associate researcher Gong of the Poultry Institute of the Chinese Academy of Agriculture Science. The bacterial strain was cultured on xylose lysine desoxycholate (XLD) agar (Qingdao-Hope Bio-Technology Co., LTD, Qingdao, China) at 37°C overnight. Then, a single colony was selected from the XLD plate, was inoculated in 5 mL of advanced Martin broth, and was incubated at 37°C for 6 h on a rotary mixer at 220 rpm. Next, the bacteria solution was transferred into 250 mL of modified Martin medium and was incubated at 37°C overnight on a rotary mixer at 160 rpm to obtain the final inocula. The CFU counts in the inocula were confirmed on XLD agar before the challenge. The method described above was based on previous study (Gong et al., 2014) and improved in our laboratory.

Sample Collection

At 10, 12, and 14 D of age, namely 3, 5, and 7 D post-challenge with *S. Enteritidis*, 15 birds from each group were randomly selected and euthanized by severing the jugular vein. The liver, spleen, and cecum contents were aseptically removed from the birds to obtain *S. Enteritidis* counts. At 14 D of age, 7 D post-challenge

with *S. Enteritidis*, chick blood was collected via the jugular vein in coagulation-promoting tubes to test the serum level of inflammatory factors and the content of endotoxin. In addition, the duodenum, jejunum, and ileum contents were collected to assess tissue morphology changes, and the secretory IgA (sIgA) content in the jejunal mucosa was also determined.

Measurement of the Bacterial Counts in the Tissue

For the bacterial counts in liver, spleen, and cecum content, the samples were aseptically removed, weighed, and diluted in 3 mL of sterile PBS. Then, the samples were homogenized for 60 s at 60 Hz using a SCIENTZ-48 homogenizer (Ningbo Xingzhi Biotechnology Co., LTD, Ningbo, China). The cecum content was further diluted in sterile PBS by plating 10-fold serial dilutions. A total of 100 μ L of the homogenate liquid from the samples was plated on XLD agar and was incubated for 24 h at 37°C. Next, the bacterial counts were calculated as log CFU per gram of liver or spleen tissue or per gram of cecum content.

Measurement of Serum Biochemical Indices

To investigate the serum biochemical indices in the selected chickens, the blood was centrifuged at 3,500 g for 10 min in order to collect serum. The contents of endotoxin, interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and IgG in serum were measured by an ELISA method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Determination of Intestinal Morphology

To investigate the intestinal morphology of the selected chickens, 3 cm segments from the duodenum, jejunum, and ileum were removed carefully without chyme and were fixed in a 4% paraformaldehyde solution. Then, the segments were successively placed into an ascending gradient of ethanol for dehydration. After xylene clearing, these samples were embedded in paraffin wax and processed into slices followed by hematoxylin-eosin staining. The villus height and crypt depth were observed and measured under a positive fluorescence microscope (DM4000B, Leica Microsystems, Wetzlar, Germany), and the ratio of the villus height to crypt depth (VCR) was calculated.

Measurement of Intestinal sIgA

The jejunal mucosa was collected, and the sIgA content in the jejunum was determined by an ELISA method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Table 2. Effect of essential oils and organic acids on the bacterial counts (log cfu/g) in the livers of chicks challenged with *Salmonella* Enteritidis.¹

Groups	3 D post-challenge	5 D post-challenge	7 D post-challenge
NC	0 \pm 0 ^z	0 \pm 0 ^z	0 \pm 0 ^z
PC	3.18 \pm 0.17 ^x	3.26 \pm 0.18 ^x	3.65 \pm 0.14 ^x
ENR	0 \pm 0 ^z	0 \pm 0 ^z	0 \pm 0 ^z
TBA	1.79 \pm 0.35 ^y	2.85 \pm 0.19 ^y	2.71 \pm 0.14 ^y
CCA	2.34 \pm 0.11 ^y	2.92 \pm 0.16 ^{x,y}	2.47 \pm 0.14 ^y
<i>P</i> -value	<0.001	<0.001	<0.001

¹Results are means with n = 15 per treatment, the same as the tables below.

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group.

^{x,z}In the same column, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean \pm SEM.

Table 3. Effect of essential oils and organic acids on the bacterial counts (log cfu/g) in the spleens of chicks challenged with *Salmonella* Enteritidis.

Groups	3D post-challenge	5 D post-challenge	7 D post-challenge
NC	0 \pm 0 ^z	0 \pm 0 ^z	0 \pm 0 ^z
PC	4.42 \pm 0.11 ^x	4.11 \pm 0.03 ^x	4.18 \pm 0.07 ^x
ENR	0 \pm 0 ^z	0 \pm 0 ^z	0 \pm 0 ^z
TBA	3.23 \pm 0.48 ^y	3.58 \pm 0.11 ^y	3.26 \pm 0.21 ^y
CCA	3.84 \pm 0.18 ^{x,y}	3.85 \pm 0.12 ^{x,y}	3.98 \pm 0.09 ^x
<i>P</i> -value	<0.001	<0.001	<0.001

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group.

^{x,z}In the same column, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean \pm SEM.

Statistical Analysis

The results were expressed as the mean \pm SEM. Statistical analyses were carried out with SPSS (SPSS 20.0 for Windows, SPSS Inc., Chicago, IL). One-way ANOVA followed by a Duncan's multiple comparison test was used to analyze the significance of the differences among the treatment groups. A *P*-value of less than 0.05 was used to indicate statistical significance.

RESULTS

Bacterial Counts in the Tissue

Liver Bacterial counts in livers from the challenged chicks are shown in Table 2. No *S. Enteritidis* was found in the NC group and ENR group at any of the 3 time periods post-challenge. The bacterial counts in the TBA group and CCA group were significantly higher ($P < 0.05$) than in the ENR group at all times but were significantly lower ($P < 0.05$) than in the PC group at 3 and 7 D post-challenge. There was no significant difference ($P > 0.05$) between the TBA and CCA groups.

Spleen Bacterial counts in spleens from the challenged chicks are shown in Table 3. Again, *S. Enteritidis* was not detected in the NC group or ENR group at any of the 3 times post-challenge. Bacterial counts in the TBA and CCA groups were significantly higher ($P < 0.05$) than in the ENR group. Bacterial

Table 4. Effect of essential oils and organic acids on the bacterial counts (log cfu/g) in the cecum contents of chicks challenged with *Salmonella* Enteritidis.

Groups	3 D post-challenge	5 D post-challenge	7 D post-challenge
NC	0 ± 0 ^z	0 ± 0 ^z	0 ± 0 ^z
PC	9.17 ± 0.09 ^x	9.24 ± 0.14 ^x	9.20 ± 0.24 ^x
ENR	0 ± 0 ^z	2.37 ± 1.05 ^{y,z}	4.27 ± 1.23 ^y
TBA	4.46 ± 1.12 ^y	4.10 ± 1.15 ^y	3.82 ± 1.11 ^y
CCA	4.60 ± 1.16 ^y	4.48 ± 1.13 ^y	3.96 ± 1.14 ^y
<i>P</i> -value	<0.001	<0.001	<0.001

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group.

^{x-z}In the same column, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean ± SEM.

Table 5. Effect of essential oils and organic acids on the serum biochemical indices of chicks challenged with *Salmonella* Enteritidis (7 D post-challenge).

Groups	Endotoxin EU/mL	IL-6 ng/L	TNF- α ng/L	IgG mg/mL
NC	40 ± 5.2	90 ± 5.1 ^y	20 ± 1.9 ^z	9 ± 1.2 ^x
PC	33 ± 3.5	163 ± 12.5 ^x	31 ± 2.3 ^x	8 ± 0.7 ^x
ENR	37 ± 2.7	81 ± 10.4 ^y	28 ± 3.2 ^{x,y}	8 ± 1.9 ^{x,y}
TBA	35 ± 4.2	145 ± 24.5 ^{x,y}	22 ± 1.8 ^{y,z}	11 ± 2.1 ^x
CCA	39 ± 2.5	109 ± 19.0 ^{x,y}	23 ± 2.0 ^{y,z}	3 ± 0.8 ^y
<i>P</i> -value	0.630	0.002	0.011	0.012

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group; IL-6, interleukin-6 and TNF- α , tumor necrosis factor- α .

^{x-z}In the same column, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean ± SEM.

counts in the TBA group were significantly lower ($P < 0.05$) than in the PC group at all times. The TBA and CCA groups were only different from one another at 7 D post-challenge with *S. Enteritidis* counts in the TBA group lower ($P < 0.05$) than in the CCA group.

Cecum Content Bacterial counts in the cecum contents from the challenged chicks are shown in Table 4. Although *S. Enteritidis* was not detected in the NC group at any of the 3 times, it was present by 5 and 7 D post-challenge in the ENR group. Bacterial counts in the TBA and CCA groups were significantly higher ($P < 0.05$) than the ENR group only early after inoculation (3 D post-challenge with *S. Enteritidis*). However, there was no significant difference ($P > 0.05$) among the ENR, TBA, and CCA groups by 5 and 7 D post-challenge. The bacterial counts in the ENR, TBA, and CCA groups were significantly lower ($P < 0.05$) than that of the PC group at all times. There was no significant difference ($P > 0.05$) between the TBA and CCA groups at any time.

Serum Biochemical Indices The results from the serum biochemical indices of the inoculated chicks are shown in Table 5. There was no significant difference among the groups for the endotoxin index ($P > 0.05$); however, there were significant differences among the groups for IL-6, TNF- α , and IgG. The IL-6 and IgG contents between the NC and ENR groups were not significantly different ($P > 0.05$). On the other hand, IL-6

was greater for PC than in NC or ENR, and IgG was less in CCA than in NC, PC, and TBA. The TNF- α content in TBA and CCA was significantly lower ($P < 0.05$) than in PC but similar to NC and ENR.

Intestinal Morphology Histological results of the inoculated chicks are shown in Table 6. The VCR of the ileum in the CCA group was significantly higher ($P < 0.05$) than in the PC and ENR groups; however, there was no significant difference ($P > 0.05$) among the NC, PC, ENR, and TBA groups. Moreover, there were no significant differences ($P > 0.05$) among any of the results from the duodenal and jejunal sections or from the villus height and crypt depth of the ileum.

Intestinal sIgA As shown in Table 7, there was no significant difference ($P > 0.05$) in the sIgA content in the jejunal mucosa among the groups.

DISCUSSION

Salmonella can be transmitted horizontally through the digestive tract or respiratory tract by fecal matter, contaminants, or vectors in chickens; it can also be transmitted vertically from the diseased breeders to offspring through the egg. In this study, we used SPF chicks to establish an *S. Enteritidis* infection model to exclude the possibility of vertical infection. Previous reports have indicated that contaminated poultry and their products are the main source of human infection with *Salmonella*, which mainly colonizes the intestine, liver, and spleen (Shah et al., 2012; Wang et al., 2016). From Tables 2–4, we found that in the NC group, *S. Enteritidis* was not detected, and the bacterial counts of the PC group were always significantly higher than the NC group. These results indicate that the *S. Enteritidis* infection model was constructed successfully and that cross contamination among treatment groups during our test was unlikely.

Amerah et al. (2012) reported that colonization of *Salmonella* in the intestine of broilers was affected by adding cinnamaldehyde and thymol to the diet, thus reducing the mortality of broilers due to *Salmonella* infection. Various essential oils appear to be synergistic in their antibacterial effects, and the effects of the essential oil complex are higher than those of a single essential oil molecule (Burt, 2004; Lee et al., 2004). In addition, essential oils combined with the organic acid butyrate effectively control the proliferation of *Salmonella* (Cerisuelo et al., 2014). The bacteriostatic effect of organic acids is related to their species and concentration (Beier et al., 2017), and the bacteriostatic effect of organic acids in vivo is also related to the feeding method (Bourassa et al., 2018). In addition, the compounded use of essential oils and organic acids has also synergistic effects on antibacterial activity in vitro (Zhou et al., 2007; Zheng et al., 2013). Basmacioglu-Malayoglu et al. (2016) reported that the use of EOA was more effective than their individual use, in some respects, in broilers. In our study, the EOA groups, TBA and CCA, both exhibited a bacteriostatic effect

Table 6. Effect of essential oils and organic acids on the intestinal morphology in chicks challenged with *Salmonella* Enteritidis (7 D post-challenge).

Items	NC	PC	ENR	TBA	CCA	<i>P</i> -value
Duodenum						
Villus height (μm)	629 \pm 48	577 \pm 35	664 \pm 21	574 \pm 81	579 \pm 64	0.568
Crypt depth (μm)	116 \pm 5.9	111 \pm 3.7	128 \pm 23.1	121 \pm 10.3	107 \pm 4.3	0.787
VCR	5.8 \pm 0.42	5.2 \pm 0.34	5.9 \pm 0.61	4.8 \pm 0.66	5.4 \pm 0.54	0.697
Jejunum						
Villus height (μm)	496 \pm 40	533 \pm 26	550 \pm 35	614 \pm 29	540 \pm 20	0.109
Crypt depth (μm)	82 \pm 5.2	89 \pm 3.7	85 \pm 8.6	89 \pm 5.0	92 \pm 8.0	0.855
VCR	6.0 \pm 0.33	6.1 \pm 0.46	6.7 \pm 0.47	6.9 \pm 0.27	6.1 \pm 0.33	0.320
Ileum						
Villus height (μm)	398 \pm 29	461 \pm 68	377 \pm 49	442 \pm 21	474 \pm 26	0.449
Crypt depth (μm)	69 \pm 3.6	86 \pm 4.4	75 \pm 10.3	79 \pm 3.6	76 \pm 2.9	0.346
VCR	5.8 \pm 0.39 ^{a,b}	5.4 \pm 0.48 ^b	5.1 \pm 0.24 ^b	5.6 \pm 0.21 ^{a,b}	6.3 \pm 0.27 ^a	0.034

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group; VCR, the ratio of the villus height to crypt depth.

^{a,b}In the same row, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean \pm SEM.

Table 7. Effect of essential oils and organic acids on the intestinal secretory IgA of chicks challenged with *Salmonella* Enteritidis (7 D post-challenge).

Groups	sIgA $\mu\text{g}/\text{mL}$
NC	21 \pm 1.1
PC	27 \pm 1.8
ENR	26 \pm 3.0
TBA	28 \pm 3.8
CCA	22 \pm 1.2
<i>P</i> -value	0.223

NC, negative control group; PC, positive control group; ENR, antibiotic group; TBA, thymol-benzoic acid group; and CCA, cinnamylaldehyde-caproic acid group.

Data are expressed as mean \pm SEM.

on *S. Enteritidis* in chicks, in which the effect of TBA was much greater than CCA. This may be related to the fact that *Salmonella* is more sensitive to thymol (Helander et al., 1998). However, in the current study, the antibiotic group, ENR, consistently yielded the greatest bacteriostatic effect of all treatments.

When animals are in contact with pathogens, the natural immune response is to protect the animal from invasion and to destroy invaders. However, proinflammatory factors released during an immune response, such as IL-6 and TNF- α , result in inflammation and subsequent metabolism anomalies that often lead to tissue injury (Medzhitov and Janeway, 2000; Kim et al., 2013; Lee et al., 2013). The essential oils, thymol, carvacrol, and cinnamon oil, are all reported to have significant anti-inflammatory effects in rodents and to reduce tissue damage caused by inflammation (Guimaraes et al., 2012; Riella et al., 2012; Bujnakova et al., 2013). In terms of organic acids, Yang et al. (2016) reported that organic acids reduce the levels of IL-6 and TNF- α in serum of LPS-induced ICR mice, thereby reducing inflammatory injury. In our study, the addition of essential oil and organic acid complexes to the feed (groups TBA and CCA) decreased the release of TNF- α in serum, which may have reduce the inflammatory response. Additionally, although the serum IgG level in

the TBA group was higher than in the ENR group, the difference was not significant. However, the IgG level in the CCA group was significantly lower than TBA group, and this further indicates that humoral immunity in the TBA group was better than that of the CCA group.

The main defensive factor of intestinal mucosal immunity, sIgA, prevents pathogenic microorganisms from colonizing the intestine (Mantis et al., 2011). However, there were no significant differences among treatments in the current study, indicating that the EOA may not affect mucosal immunity.

Intestinal morphology is an indicator of intestinal health and integrity (Paiva et al., 2014). Intestinal villus height, crypt depth, and their ratio not only affect the function of intestinal digestion and absorption but also relate to the colonization of harmful bacteria. Dunsford et al. (1989) reported that a longer villus height reduced the colonization of harmful bacteria. An increased VCR provides an intestinal environment in favor of digestion and absorption, which is conducive to the digestion and absorption of nutrients (Pluske et al., 1996; Montagne et al., 2003). Xu et al. (2018) reported that the combined use of essential oils and organic acids did not alter intestinal morphology of piglets, including the villus height, crypt depth, and their ratios in the duodenum, jejunum, and ileum. However, the individual use of essential oils or organic acids increased the villus height of the duodenum. Consistent with this, in our study, the villus height, crypt depth, and their ratio in the duodenum and jejunum were not significantly different among the groups. On the other hand, the VCR of the ileum in the CCA group was significantly higher than that in the PC and ENR groups, indicating that this combination of essential oils and organic acids was even better for intestinal health than that of an antibiotic in our study.

In conclusion, the EOA had a bacteriostatic effect on *S. Enteritidis* in chickens. Overall, the bacteriostatic effect of the thymol-benzoic acid complex was

better than that of the cinnamaldehyde-caproic acid complex.

ACKNOWLEDGMENTS

This study was financially supported by the Agricultural Science and Technology Independent Innovation Fund Project of Jiangsu Province [CX(17)3033], Jiangsu Agriculture Research System (JATS[2018]248), and the Innovation Capacity Building Project of Jiangsu Province (BM2018026).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

- Amerah, A. M., G. Mathis, and C. L. Hofacre. 2012. Effect of xylanase and a blend of essential oils on performance and *Salmonella* colonization of broiler chickens challenged with *Salmonella* Heidelberg. *Poult. Sci.* 91:943–947.
- Basile, A., F. Senatore, R. Gargano, S. Sorbo, P. M. Del, A. Lavitola, A. Ritieni, M. Bruno, D. Spatuzzi, D. Rigano, and M. L. Vuotto. 2006. Antibacterial and antioxidant activities in *Sideritis italica* (Miller) Greuter et Burdet essential oils. *J. Ethnopharmacol.* 107:240–248.
- Basmacioglu-Malayoglu, H., P. Ozdemir, and H. A. Bagriyanik. 2016. Influence of an organic acid blend and essential oil blend, individually or in combination, on growth performance, carcass parameters, apparent digestibility, intestinal microflora and intestinal morphology of broilers. *Br. Poult. Sci.* 57:227–234.
- Beier, R. C., T. R. Callaway, K. Andrews, T. L. Poole, T. L. Crippen, R. C. Anderson, and D. J. Nisbet. 2017. Interactions of organic acids with *Salmonella* strains from feedlot water-sprinkled cattle. *J. Food. Chem. Nanotechnol.* 3:60–66.
- Bourassa, D. V., K. M. Wilson, C. R. Ritz, B. K. Kiepper, and R. J. Buhr. 2018. Evaluation of the addition of organic acids in the feed and/or water for broilers and the subsequent recovery of *Salmonella* Typhimurium from litter and ceca. *Poult. Sci.* 97:64–73.
- Bujnakova, D., S. Juhas, and S. Faix. 2013. The anti-translocation and anti-inflammatory effect of cinnamon oil in mice with tnb_s induced colitis. *Biologia.* 68:1000–1003.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food. Microbiol.* 94:223–253.
- Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. de la Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and *Salmonella* counts in experimentally challenged broilers. *Poult. Sci.* 93:599–606.
- Dunsford, B. R., D. A. Knabe, and W. E. Haensly. 1989. Effect of dietary soybean meal on the microscopic anatomy of the small intestine in the early-weaned pig. *J. Anim. Sci.* 67:1855–1863.
- Giannenas, I., C. P. Papanophytou, E. Tsalie, I. Pappas, E. Triantafyllou, D. Tontis, and G. A. Kontopidis. 2014. Dietary supplementation of benzoic acid and essential oil compounds affects buffering capacity of the feeds, performance of turkey poults and their antioxidant status, pH in the digestive tract, intestinal microbiota and morphology. *Asian Australas. J. Anim. Sci.* 27:225–236.
- Gong, J., J. Zhang, M. Xu, C. Zhu, Y. Yu, X. Liu, P. Kelly, B. Xu, and C. Wang. 2014. Prevalence and fimbrial genotype distribution of poultry *Salmonella* isolates in China (2006 to 2012). *Appl. Environ. Microbiol.* 80:687–693.
- Guimaraes, A. G., M. A. Xavier, M. T. de Santana, E. A. Camargo, C. A. Santos, F. A. Brito, E. O. Barreto, S. C. Cavalcanti, A. R. Antonioli, R. C. Oliveira, and L. J. Quintans-Junior. 2012. Carvacrol attenuates mechanical hypernociception and inflammatory response. *Naunyn Schmiedeberg's Arch. Pharmacol.* 385:253–263.
- Hafeez, A., K. Manner, C. Schieder, and J. Zentek. 2016. Effect of supplementation of phytogenic feed additives (powdered vs. encapsulated) on performance and nutrient digestibility in broiler chickens. *Poult. Sci.* 95:622–629.
- Helander, I. M., H. L. Alakomi, K. Latvakala, T. Mattilasandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Wright. 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. *J. Agr. Food. Chem.* 46:3590–3595.
- Kim, D. K., H. S. Lillehoj, S. H. Lee, S. I. Jang, E. P. Lillehoj, and D. Bravo. 2013. Dietary Curcuma longa enhances resistance against *Eimeria maxima* and *Eimeria tenella* infections in chickens. *Poult. Sci.* 92:2635–2643.
- Lee, K. W., H. Everts, H. J. Kappert, H. Wouterse, M. Frehner, and A. C. Beynen. 2004. Cinnamaldehyde, but not thymol, counteracts the carboxymethyl cellulose-induced growth depression in female broiler chickens. *Int. J. Poult. Sci.* 3:608–612.
- Lee, S. H., H. S. Lillehoj, S. I. Jang, E. P. Lillehoj, W. Min, and D. M. Bravo. 2013. Dietary supplementation of young broiler chickens with capsicum and turmeric oleoresins increases resistance to necrotic enteritis. *Br. J. Nutr.* 110:840–847.
- Liljebjelke, K. A., C. L. Hofacre, T. Liu, D. G. White, S. Ayers, S. Young, and J. J. Maurer. 2005. Vertical and horizontal transmission of salmonella within integrated broiler production system. *Foodborne. Pathog. Dis.* 2:90–102.
- Lin, D., M. Yan, S. Lin, and S. Chen. 2014. Increasing prevalence of hydrogen sulfide negative *Salmonella* in retail meats. *Food. Microbiol.* 43:1–4.
- Mantis, N. J., N. Rol, and B. Corthesy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal. Immunol.* 4:603–611.
- Medzhitov, R., and C. J. Janeway. 2000. Innate immunity. *N. Engl. J. Med.* 343:338–344.
- Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim. Feed Sci. Tech.* 108:95–117.
- Mueller, K., N. M. Blum, H. Kluge, and A. S. Mueller. 2012. Influence of broccoli extract and various essential oils on performance and expression of xenobiotic- and antioxidant enzymes in broiler chickens. *Br. J. Nutr.* 108:588–602.
- National Research Council. 1994. *Nutrient Requirements of Poultry*. 9th rev. ed. Natl. Acad. Press, Washington, DC.
- Nava, G. M., M. S. Attene-Ramos, H. R. Gaskins, and J. D. Richards. 2009. Molecular analysis of microbial community structure in the chicken ileum following organic acid supplementation. *Vet. Microbiol.* 137:345–353.
- Paiva, D., C. Walk, and A. McElroy. 2014. Dietary calcium, phosphorus, and phytase effects on bird performance, intestinal morphology, mineral digestibility, and bone ash during a natural necrotic enteritis episode. *Poult. Sci.* 93:2752–2762.
- Piddock, L. J. 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS. Microbiol. Rev.* 26:3–16.
- Piedrola, A. G. 2001. The active efflux pump in antimicrobial resistance. *An. R. Acad. Nac. Med. (Madr)* 118:343–357, 357–361.
- Pluske, J. R., M. J. Thompson, C. S. Atwood, P. H. Bird, I. H. Williams, and P. E. Hartmann. 1996. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *Br. J. Nutr.* 76:409–422.
- Riella, K. R., R. R. Marinho, J. S. Santos, R. N. Pereira-Filho, J. C. Cardoso, R. L. Albuquerque-Junior, and S. M. Thomazzi. 2012. Anti-inflammatory and cicatrizing activities of thymol, a monoterpene of the essential oil from *Lippia gracilis*, in rodents. *J. Ethnopharmacol.* 143:656–663.
- Sallam, K. I., M. A. Mohammed, M. A. Hassan, and T. Tamura. 2014. Prevalence, molecular identification and antimicrobial resistance profile of *Salmonella* serovars isolated from retail beef products in Mansoura, Egypt. *Food. Contr.* 38:209–214.

- Shah, D. H., X. Zhou, H. Y. Kim, D. R. Call, and J. Guard. 2012. Transposon mutagenesis of *Salmonella enterica* serovar Enteritidis identifies genes that contribute to invasiveness in human and chicken cells and survival in egg albumen. *Infect. Immun.* 80:4203–4215.
- Shao, X., S. Cheng, H. Wang, D. Yu, and C. Mungai. 2013. The possible mechanism of antifungal action of tea tree oil on *Botrytis cinerea*. *J. Appl. Microbiol.* 114:1642–1649.
- Wang, C. L., Y. C. Fan, C. Wang, H. J. Tsai, and C. H. Chou. 2016. The impact of *Salmonella* Enteritidis on lipid accumulation in chicken hepatocytes. *Avian. Pathol.* 45:450–457.
- WHO. 2018. Salmonella (non-typhoidal). Accessed Feb. 2018. <http://www.who.int/mediacentre/factsheets/fs139/en/>.
- Xu, Y. T., L. Liu, S. F. Long, L. Pan, and X. S. Piao. 2018. Effect of organic acids and essential oils on performance, intestinal health and digestive enzyme activities of weaned pigs. *Anim. Feed. Sci. Tech.* 235:110–119.
- Yang, N., C. Li, G. Tian, M. Zhu, W. Bu, J. Chen, X. Hou, L. Di, X. Jia, Z. Dong, and L. Feng. 2016. Organic acid component from *Taraxacum mongolicum* Hand.-Mazz alleviates inflammatory injury in lipopolysaccharide-induced acute tracheobronchitis of ICR mice through TLR4/NF-kappaB signaling pathway. *Int. Immunopharmacol.* 34:92–100.
- Zheng, L., Y. M. Bae, K. S. Jung, S. Heu, and S. Y. Lee. 2013. Antimicrobial activity of natural antimicrobial substances against spoilage bacteria isolated from fresh produce. *Food. Contr.* 32:665–672.
- Zhou, F., B. Ji, H. Zhang, H. Jiang, Z. Yang, J. Li, J. Li, Y. Ren, and W. Yan. 2007. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella* Typhimurium. *J. Food. Prot.* 70:1704–1709.