METABOLISM AND NUTRITION

Effects of star anise (*Illicium verum* Hook.f) oil on the nuclear factor E2–related factor 2 signaling pathway of chickens during subclinical *Escherichia coli* challenge

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ABSTRACT We characterized the mechanism underlying star anise (*Illicium verum* Hook.f) oil (**SAO**)–mediated antioxidant status during subclinical *Escherichia coli* (***E. coli***) challenge. A total of 512 male birds (White Leghorn) at 30 wk of age with similar body weight (2.14 ± 0.02 kg) were randomly divided into 2 groups with 1 group being orally challenged with *E. coli* (every other day from day 15 to day 27) during the experiment. Each group of birds was then randomly allocated to dietary treatment of SAO supplementation at 0, 200, 400, or 600 mg/kg of basal diet (8 replicate cages during each treatment). The treatments were arranged a 4 × 2 factorial arrangement. The experiment comprised 1 wk of adaptation and 3 wks of data collection. There was no interaction ($P > 0.05$) between SAO supplementation and *E. coli* challenge for final body weight and average daily feed intake of birds. However, *E. coli* challenge resulted in a significant decrease ($P < 0.001$) in final body weight of birds as compared with unchallenged birds. There were interactions between SAO supplementation and *E. coli* challenge for the activity of glutathione peroxidase (**GSH-Px**) and malondialdehyde (**MDA**) concentration in serum and for the activity of GSH-Px in the liver of birds. Supplementation of SAO enhanced the activities of antioxidant enzymes but decreased the MDA content in the serum and liver of birds, and it also enhanced the expression of genes including superoxide dismutase, catalase, and nuclear factor E2–related factor 2 (**Nrf2**) in the liver of the birds. Meanwhile, supplementation of SAO can also reduce *E. coli* challenge–induced oxidative stress in the serum and liver of birds, and the efficacy of SAO in birds during subclinical *E. coli* challenge is dose-dependent. In conclusion, the enhancement of antioxidant capacity by star anise or its effective compounds is through upregulation of Nrf2 signaling pathway. The optimum supplementation dose of SAO for protecting birds against *E. coli* challenge is 400 mg/kg.

Key words: SAO, *Escherichia coli*, antioxidant, enzyme, Nrf2

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INTRODUCTION

Poultry is exposed to many stress factors such as disease and enteric pathogens (Alhajj et al., 2017). The body's resistance to these factors depends mainly on the immune system and antioxidant capacity. Free radicals are continuously produced in aerobic organisms as a natural by-product of oxygen metabolism and may damage the cell if they are in excessive levels (Lee et al., 2015; Ding et al., 2017). Therefore, antioxidants to reduce free radicals have been proposed to prevent diseases associated with oxidative damage (Lee et al., 2015). Meanwhile, a high antioxidant status has been regarded as one of the major factors positively affecting birds' performance (Padmashree et al., 2007). Antioxidants from diet supplement have been used as the preventive or therapeutic mediator of oxidative damage caused by free radicals (Lee et al., 2015; Yu et al., 2018). Owing to the potential side effects of synthetic antioxidants, essential oils which are derived from organic products, can be served as an alternative source for the further improvement of synthetic antioxidants (Newberne et al., 1999; Wong et al., 2014).

Star anise (*Illicium verum* Hook.f) has long been used in traditional Chinese medicine and in the food industry

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Table 1. Analyzed concentrations of effective components in the experimental diets, mg/kg.

	Dietary star anise oil concentration, mg/kg			
Item	0	200	400	600
Anethole	ND	187.2	374.4	561.6
Estragole	ND	2.33	4.66	6.99
Anisaldehyde	ND	1.89	3.78	5.66

Abbreviation: ND, not detected.

(Yang et al., 2010; Wang et al., 2015) because of its antibacterial, antioxidant, anti-inflammatory, and anticancer properties (Huang et al., 2010; Yang et al., 2010). The essential oil extracted from star anise contained about 90% of trans-anethole [1-methoxy-4-(1-propenyl) benzene] and others such as estragole and anisaldehyde (Huang et al., 2010; Domiciano et al., 2013). Star anise and its essential oil are generally recognized as safe and used extensively in food, brewery, and health supplement industries (Ding et al., 2017).

More recently, it has been observed that star anise and its essential oil enhanced antioxidant ability in broilers and laying hens (Ding et al., 2017; Yu et al., 2018). However, the molecular mechanism by which supplementation of SAO **(SAO)** enhanced antioxidant ability of birds has not been fully elucidated. Nuclear factor E2–related factor 2 (**Nrf2**), which is a member of the NF-E2 family of the basic leucine zipper of redox-sensitive transcription factors, has been shown to play a critical role in deactivating or eliminating free radicals and carcinogens (Lee et al., 2015; Zhang et al., 2015). It regulates the expression of several phase II enzyme genes and antioxidants in response to oxidative stress. It is hypothesized that enhancement of antioxidant capacity by star anise or its effective compounds is through upregulation of Nrf2 signaling pathway. Animals, even of the same species, age, and sex, differ slightly in their metabolism. The male birds are preferred to female birds for evaluation of various kinds of actions of feed additives because of the low preparation cost involved in the process (McDonald et al., 1988). The present study used White Leghorn, as those used by Boa-Amponsem et al. (2001) and Vanpatten et al. (2004), which provide animal models for studying effects of star anise and its effective compounds. It may provide theoretical foundation for the subsequent research studies of star anise or its effective compounds on birds of other breeds.

The objective of this study was to assess the effects of SAO on antioxidant capacity and on Nrf2 signaling pathway in normal and *Escherichia coli* (***E. coli***)–challenged birds.

MATERIALS AND METHODS

Preparation of SAO

Fruits of star anise (*I. verum* Hook.f) were purchased from Wuma Market (Tai'an, Shandong, China) and authenticated based on the macroscopic characteristics described by the Chinese pharmacopoeia. The SAO was obtained using a traditional water steam distillation apparatus, and the chemical compositions of the oil were subjected to gas chromatographic analysis using the same procedure as described by Ding et al., (2017). The analyzed concentrations of effective components in SAO in the experimental diets are shown in Table 1.

E. coli *Challenge*

E. coli (O1K1) was obtained from Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Shandong Agricultural University (Tai'an, Shandong, China). *E. coli* challenge was based on the model described by Dahiya et al. (2005) and Liu et al. (2010), with slight modifications. Briefly, birds in the *E. coli*–challenged group were orally gavaged with 1.0 mL of actively growing culture of *E. coli* (0.5×10^4–0.8×10^4 cfu/mL) every other day from day 15 to day 27. Birds in the unchallenged groups received the same volume of sterile meat medium using the same procedure described previously.

Table 2. Ingredients and nutrient composition of experimental diets[1] (% as fed unless noted).

Ingredients	Composition	Nutrient levels	Content
Corn	60.0	ME, calculated (kcal/kg)	2,807
Soybean meal (44.2% CP)	21.5	Protein	16.20
Soy oil	3.0	Calcium	3.75
Fish meal	2.1	Total phosphorus	0.71
Wheat bran	2.0	Lys	0.90
Calcium hydrogen phosphate	1.5	Met	0.35
Limestone	8.5		
Sodium chloride	0.3		
DL-Met	0.1		
Premix[2]	1.0		
Total	100.0		

[1]The control group was fed the basal diet. The other treatment diets were the same basal diet supplemented.

[2]Supplied per kilogram of diet: vitamin A, 12,200 IU (retinol); cholecalciferol, 4,200 IU; vitamin E, 30 IU (dl-α-tocopherol); vitamin K_3, 4.5 mg; thiamin, 2.3 mg; riboflavin, 8.8 mg; pantothenic acid, 7 mg; pyridoxine, 4.0 mg; cobalamin, 0.016 mg; niacin, 30 mg; choline chloride, 500 mg; biotin, 0.20 mg; folic acid, 0.25 mg; Mn, 80 mg; Fe, 58 mg; Zn, 80 mg; Cu, 8 mg; I, 0.6 mg; Se, 0.3 mg.

Table 3. Gene-specific primers and GenBank numbers of chickens.

Gene	GenBank no.	Primer sequences (5′-3′)	Product size (bp)
Nrf2	NM_205117.1	F: CCTTGTCCTTTGATGACTGC R: TGGGTGGCTGAGTTTGATTA	153
GPX-4	AF498316	F: CATCACCAACGTGGCGTCCAA R: GCAGCCCCTTCTCAGCGTATC	92
SOD1	NM_205064	F: TTGTCTGATGGAGATCATGGCTTC R: TGCTTGCCTTCAGGATTAA AGTGAG	98
SOD2	NM_204211	F: CAGATAGCAGCCTGTGCAAATCA R: GCATGTTCCCATACATCGATTCC	86
CAT	NM_001031215.1	F: ACCAAGTACTGCAAGGCGAAAGT R: ACCCAGATTCTCCAGCAACAGTG	91
HO-1	NM_205344	F: ATCGCATGAAAACAGTCCAG R: CAAATAAGCCCACGGCGAC	78
GAPDH	NM_204305	F: GGTGAAAGTCGGAGTCAACGG R: CGATGAAGGGATCATTGATGGC	108

Abbreviations: F, forward; R, reverse.

Birds and Experimental Design

A total of 512 male birds (White Leghorn) at 30 wk of age with similar body weight (2.14 ± 0.02 kg) were randomly placed into 64 wire cages (60 × 90 × 40 cm) equipped with 2 nipple drinkers and 1 feeder. The cages were randomly divided into 2 groups with chickens in 1 group being orally challenged with *E. coli* during the experiment. Each group of birds was then randomly allocated to dietary treatment of SAO supplementation at 0, 200, 400, or 600 mg/kg of basal diet (8 replicate cages each treatment). The treatments were arranged a 4 × 2 factorial arrangement. The experiment comprised 1 wk of adaptation and 3 wks of data collection. All feeding conditions were the same between the 2 periods. The basal diet was formulated to meet or exceed the National Research Council (1994) requirements for chickens, and the diet compositions are shown in Table 2.

Birds were housed in an environmentally controlled room with the temperature between 21°C and 26°C and 16 h/d of illumination (10 to 20 lx) throughout the entire period of the experiment. All birds were fed ad libitum intake and had free access to water throughout the whole experiment. The animal care and use protocol was approved by the Shandong Agricultural University Animal Nutrition Research Institute.

Growth Performance and Sample Collection

Birds were fed twice daily ad libitum intake, and feed residue in each cage was weighed daily. The data were used to calculate ADFI. Birds in each cage were weighted at day 1 and day 28 of the experiment. Mortalities and health status were visually observed and recorded daily throughout the entire experimental period.

At the last day of the feeding trial, 1 bird per replication was randomly picked out and weighed after fasting for 8 h. Blood sample was taken from the wing vein into a nonheparinized tube. The samples were incubated at 37°C for 2 h, followed by centrifugation at 1,500 g for 10 min, and the supernatant was stored in 1.5-mL Eppendorf tubes at −20°C for further assay. The selected birds were then killed by cervical dislocation, and liver samples were collected. One part of the liver sample was frozen (−20°C) for analysis of enzyme activities, another part of the liver sample was immediately stored in an RNAfixer (BioTeke, Beijing, China) for analysis of relative mRNA levels, and the last part of the liver sample was frozen in liquid nitrogen for determination of protein expression.

Enzyme Activity

The activities of superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), and catalase (**CAT**) and malondialdehyde (**MDA**) concentration in serum and liver samples were determined using the same procedure as described by Zhang et al., (2009) with respective assay kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Liver samples were homogenized with ice-cold physiologic saline to obtain the homogenates to analyze the enzyme activities and MDA concentration. The activities of enzymes and the concentration of MDA were measured according to the spectrophotometric method and were expressed as units per milliliter for serum samples and units per milligram of protein for the liver.

Total RNA Extraction and Real-time Quantitative PCR Analysis

Total RNA of the liver was extracted using RNAiso Plus (Takara, Dalian, China) in accordance with the manufacturer's instructions. Concentrations and purity of RNA were determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany) by measuring the absorbance at 260 and 280 nm, and RNA quality was assessed via agarose gel electrophoresis. The total RNA sample was reverse transcribed using the PrimeScript RT Master Mix kit (Takara) in accordance with the manufacturer's instructions. Reverse

transcription was performed at 37°C for 15 min followed by heat inactivation for 5 s at 85°C. All of the cDNA preparations were stored frozen at −20°C until further use.

A real-time quantitative PCR was performed with the 7,500 Real Time PCR System (Applied Biosystems, Waltham, MA) in accordance with optimized PCR protocols using a SYBR Premix Ex Taq kit (Takara). The protocol consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C, 34 s at 60°C, 15 s at 95°C, and 60 s at 60°C, with a final step at 95°C for 15 s. The gene-specific primers for Nrf2, GPX-4, SOD1, SOD2, CAT, Heme Oxygenase 1 (**HO-1**), and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) are listed in Table 3. The ΔΔCt method was used to estimate mRNA abundance. GAPDH was used as the internal reference gene, and the mRNA expression of target genes was normalized to GAPDH mRNA expression.

Western Blotting

The total protein of the liver tissue was extracted using the radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) and detected using a bicinchoninic acid protein assay kit (Beyotime). After denaturation at 100°C for 4 min, protein extract (50 μg) per sample was subjected to SDS-PAGE. The separated proteins were transferred onto a polyvinylidene fluoride membranes at 250 mA for 60 min and blocked in a blocking buffer (5% nonfat milk in Tris-buffer saline with Tween-10 buffer) at 37°C for 1 h; then, the membranes were incubated with anti-CAT (1:2000, ab16731; Abcam, Cambridge, UK), anti-Nrf2 (1:1000, ab137550; Abcam), anti-SOD (1:500, ab13498; Abcam), and β-actin C4 antibody (**Actin**, 1:1500, SC-47778; Santa Cruz Biotechnology, Santa Cruz, CA) at 37°C for 1 h. After washing, the membranes were incubated with goat anti–rabbit IgG (H+L) secondary antibody (1:5000, 31,460; Thermo Fisher Scientific, Waltham, MA) at 37°C for 1 h. After washing, bands were visualized using an enhanced chemiluminescence substrate (SuperSignal West Pico Trial Kit, Pierce, Waltham, MA), and the signals were recorded using an imaging System (Bio-Rad ChemidocTM XRS+, Berkeley, CA) and analyzed using Image Lab software (Bio-Rad).

Statistical Analyses

Data were statistically analyzed using the two-way analysis of variance using general linear model procedure of SAS (version 8.2, SAS Institute Inc., Cary, NC). The statistical model included the main effects of SAO supplementation, *E. coli* challenge and their interactions and cage as a statistical unit. Polynomial contrasts were conducted to determine linear and quadratic responses of the birds (averaged between challenged and unchallenged groups) to dietary SAO dosages. The significance of differences among treatments was tested by Duncan's multiple range tests, with $P < 0.05$ being declared statistically significant.

RESULTS

Growth Performances

After challenging with *E. coli*, it was observed that most of the birds initially became dull and depressed

Table 4. Effects of star anise oil on growth performance of *E. coli*–challenged birds.[1]

Items	Body weight, kg	ADFI, g/d
Treatment		
Basal diet, unchallenged	2.11	63.63
200 mg/kg of star anise oil, unchallenged	2.14	65.44
400 mg/kg of star anise oil, unchallenged	2.11	65.13
600 mg/kg of star anise oil, unchallenged	2.10	63.13
Basal diet, challenged	2.04	64.08
200 mg/kg of star anise oil, challenged	2.07	66.09
400 mg/kg of star anise oil, challenged	2.04	64.89
600 mg/kg of star anise oil, challenged	1.95	61.20
SEM	0.034	1.374
Star anise oil		
0	2.08	63.86
200	2.10	65.77
400	2.08	65.01
600	2.02	62.16
E. coli challenge		
0	2.12[a]	64.33
+	2.02[b]	64.07
P values		
Star anise oil	0.133	0.065
Liner[2]	0.130	0.191
Quadratic[2]	0.110	0.020
E. coli	<0.001	0.785
Star anise oil × *E. coli*	0.652	0.779

[a,b]Means within a row with different letters differ significantly ($P < 0.05$).

[1]Data are means for 8 replicates per treatment.

[2]Liner and quadratic polynomial contrasts were performed on the means within the *E. coli*–challenged or unchallenged groups to star anise oil dosages.

Table 5. Effects of star anise oil on antioxidant enzymatic activities in serum of *E. coli*–challenged birds.[1]

Items	SOD	GSH-Px	CAT	MDA
Treatment				
Basal diet, unchallenged	666.8	1683.8[a,b]	1.861	4.984[b]
200 mg/kg of star anise oil, unchallenged	698.9	1682.1[a,b]	2.087	4.689[b]
400 mg/kg of star anise oil, unchallenged	688.2	1712.8[a,b]	2.202	4.727[b]
600 mg/kg of star anise oil, unchallenged	675.8	1743.6[a]	2.042	5.344[a]
Basal diet, challenged	670.3	1659.8[b]	1.762	5.656[a]
200 mg/kg of star anise oil, challenged	691.9	1665.0[b]	1.999	5.000[b]
400 mg/kg of star anise oil, challenged	676.8	1700.9[a,b]	2.273	5.697[a]
600 mg/kg of star anise oil, challenged	647.2	1528.2[c]	1.912	5.639[a]
SEM	16.352	24.192	0.077	0.086
Star anise oil				
0	668.5	1671.8	1.811[c]	5.320
200	695.4	1673.5	2.042[b]	4.844
400	682.5	1706.8	2.238[a]	5.212
600	661.5	1635.9	1.977[b]	5.492
E. coli challenge				
0	682.4	1705.6	2.048	4.936
+	671.5	1638.5	1.986	5.498
P values				
Star anise oil	0.184	0.048	<0.001	<0.001
Liner[2]	0.517	0.486	0.022	0.151
Quadratic[2]	0.093	0.241	<0.001	0.006
E. coli	0.353	<0.001	0.264	<0.001
Star anise oil × *E. coli*	0.801	<0.001	0.563	0.015

[a-c]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates per treatment.
[2]Liner and quadratic polynomial contrasts were performed on the means within the *E. coli*–challenged or unchallenged groups to star anise oil dosages.

and had abnormal droppings where undigested feed was detectable. There was no interaction between SAO supplementation and *E. coli* challenge for final body weight and ADFI of birds (Table 4). However, *E. coli* challenge resulted in a significant decrease ($P < 0.001$) in the final body weight of birds as compared with unchallenged birds.

Regardless of *E. coli* challenge, supplementation of SAO quadratically ($P = 0.020$) increased ADFI of the birds.

Antioxidant Enzymatic Activity in Serum

There were interactions between SAO supplementation and *E. coli* challenge for the activity of GSH-Px ($P < 0.001$) and MDA concentration ($P = 0.015$) but not for the activities of SOD and CAT in the serum of birds (Table 5).

Challenging birds with *E. coli* increased ($P = 0.015$) MDA concentration but had no effect on activities of SOD and CAT in the serum of the birds as compared with unchallenged birds. Supplementation of SAO at the concentrations up to 600 mg/kg of diet had no effect on serum activities of SOD irrespective of *E. coli* challenge. In contrast, *E. coli*–challenged birds supplemented 600 mg/kg of SAO diet had lower ($P < 0.001$) GSH-Px activity than other *E. coli*–challenged birds, but this effect of SAO on serum GSH-Px activity was not observed in unchallenged birds. Concentration of MDA was higher ($P = 0.015$) in the serum of birds supplemented with 600 mg/kg SAO diet than that of other unchallenged birds. On the contrary, for *E. coli*–challenged birds, supplementing 200 mg/kg of SAO diet decreased ($P = 0.015$) MDA concentration in the serum as compared with others.

Regardless of *E. coli* challenge, supplementation of SAO linearly ($P = 0.022$) and quadratically ($P < 0.001$) increased CAT activity in the serum, with the birds supplemented with 400 mg/kg of SAO diet having the highest ($P < 0.001$) CAT activity in the serum.

Antioxidant Enzymatic Activity in the Liver

There was an interaction between SAO supplementation and *E. coli* challenge for the activity of GSH-Px ($P < 0.001$) but not for the activities of SOD and CAT and MDA concentration in the liver of birds (Table 6).

Challenging birds with *E. coli* decreased ($P = 0.043$) the activity of CAT but had no effect on the activity of SOD and MDA concentration in the liver of the birds as compared with unchallenged birds. Supplementation of SAO at the concentrations up to 600 mg/kg of diet had significant effect ($P < 0.05$) on liver activities of SOD and CAT and MDA concentration irrespective of *E. coli* challenge. Meanwhile, *E. coli*–challenged birds supplemented 200 mg/kg and 400 mg/kg of SAO diet had higher ($P < 0.001$) GSH-Px activity than other *E. coli*–challenged birds, and this effect of SAO on liver GSH-Px activity was also observed in unchallenged birds. On the contrary, for *E. coli*–challenge birds, supplementing 600 mg/kg of SAO diet decreased ($P < 0.001$) the activity of GSH-Px in the liver as compared with others.

Table 6. Effects of star anise oil on antioxidant enzymatic activities in liver of *E. coli*–challenged birds.[1]

Items	SOD	GSH-Px	CAT	MDA
Treatment				
Basal diet, unchallenged	112.9	16.6^{e}	6.363	0.913
200 mg/kg of star anise oil, unchallenged	117.3	18.8^{b}	6.755	0.861
400 mg/kg of star anise oil, unchallenged	116.0	17.8^{c,d}	6.714	0.806
600 mg/kg of star anise oil, unchallenged	108.7	17.7^{d,e}	6.600	0.885
Basal diet, challenged	112.7	17.4^{d,e}	6.008	0.905
200 mg/kg of star anise oil, challenged	115.9	18.4^{b,c}	6.450	0.854
400 mg/kg of star anise oil, challenged	119.4	19.7^{a}	6.687	0.865
600 mg/kg of star anise oil, challenged	111.5	15.8^{f}	6.379	0.883
SEM	1.969	0.236	0.153	0.023
Star anise oil				
0	112.8^{b,c}	17.0	6.185^{b}	0.909^{a}
200	116.6^{a,b}	18.6	6.602^{a}	0.858^{b}
400	117.7^{a}	18.7	6.701^{a}	0.835^{b}
600	110.1^{c}	16.7	6.489^{a,b}	0.884^{a,b}
E. coli challenge				
0	113.7	17.7	6.608^{a}	0.866
+	114.9	17.8	6.381^{b}	0.877
P values				
Star anise oil	0.001	<0.001	0.011	0.017
Liner[2]	0.332	0.463	0.061	0.221
Quadratic[2]	<0.001	<0.001	0.004	0.006
E. coli	0.425	0.348	0.043	0.520
Star anise oil × *E. coli*	0.558	<0.001	0.723	0.400

[a-f]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates per treatment.
[2]Liner and quadratic polynomial contrasts were performed on the means within the *E. coli*–challenged or unchallenged groups to star anise oil dosages.

Regardless of *E. coli* challenge, supplementation of SAO quadratically increased ($P < 0.05$) activities of SOD and CAT but quadratically decreased ($P = 0.006$) MDA concentration in the liver.

Expressions of Genes in the Liver

The expressions of genes in the liver of birds are shown in Table 7. There were interactions ($P < 0.05$) between SAO supplementation and *E. coli* challenge for mRNA levels of SOD1, SOD2, HO-1, CAT, and GPX-4, but not for mRNA levels of Nrf2 in the liver of birds.

Challenging birds with *E. coli* decreased ($P < 0.001$) the expression of Nrf2 in the liver of the birds as compared with unchallenged birds. Supplementation of SAO at the concentrations up to 600 mg/kg of diet also had effect ($P < 0.001$) on the liver mRNA level of Nrf2 irrespective of *E. coli* challenge.

The expressions of SOD1 and SOD2 were higher ($P < 0.05$) in the liver of birds supplemented with SAO than those of other *E. coli*–challenged birds. On the contrary, for *E. coli*–unchallenged birds, supplementing SAO diet had no effect ($P > 0.05$) on mRNA level of SOD1 in the liver as compared with others. The expressions of CAT and GPX4 were higher ($P < 0.05$) in the liver of birds supplemented with 200 mg/kg and 400 mg/kg of SAO diet than that of other challenged birds. In contrast, *E. coli*–challenged birds supplemented 600 mg/kg of SAO diet had lower ($P < 0.001$) mRNA level of HO-1 than other *E. coli*–challenged birds, and this effects ($P < 0.05$) of SAO on the liver mRNA level of SOD2 and HO-1 were also observed in unchallenged birds. Moreover, supplementation of SAO at the concentrations up to 600 mg/kg of diet had significant effects ($P < 0.05$) on liver mRNA levels of CAT and GPX-4 irrespective of *E. coli* challenge.

Regardless of *E. coli* challenge, supplementation of SAO quadratically ($P < 0.001$) increased the expression of Nrf2 in the liver, with the birds supplemented with 400 mg/kg of SAO diet having the highest ($P < 0.001$) mRNA level of Nrf2 in the liver.

Relative Protein Expressions in the Liver

There was no interaction ($P > 0.05$) between SAO supplementation and *E. coli* challenge for the relative protein expressions of SOD, CAT, and Nrf2 in the liver of birds (Table 8 and Figure 1).

Challenging birds with *E. coli* decreased ($P < 0.001$) relative protein expressions of SOD and Nrf2 but had no effect on the protein expression of CAT in the liver of the birds as compared with unchallenged birds. Supplementation of SAO at the concentrations up to 600 mg/kg of diet had effects ($P < 0.05$) on liver relative protein expression of SOD, CAT, and Nrf2 irrespective of *E. coli* challenge.

Regardless of *E. coli* challenge, supplementation of SAO linearly ($P < 0.001$) and quadratically ($P < 0.001$) increased the relative protein expression of CAT in the liver. Meanwhile, supplementation of SAO quadratically ($P < 0.001$) increased the relative protein expression of Nrf2, with the birds supplemented with 400 mg/kg of SAO diet having the highest ($P < 0.001$) relative protein expression in the liver.

Table 7. Effects of star anise oil on expressions of genes in liver of *E. coli*–challenged birds.[1]

Items	SOD1	SOD2	HO-1	CAT	Nrf2	GPX-4
Treatment						
Basal diet, unchallenged	1.000^{a,b}	1.000^{a}	1.000^{b}	1.000^{a}	1.000	1.000^{b,c}
200 mg/kg of star anise oil, unchallenged	1.023^{a,b}	1.079^{a}	1.134^{a}	1.014^{a}	1.116	1.051^{b}
400 mg/kg of star anise oil, unchallenged	1.075^{a}	1.057^{a}	1.126^{a}	0.896^{b,c}	1.359	1.217^{a}
600 mg/kg of star anise oil, unchallenged	1.038^{a,b}	0.831^{b,c}	0.782^{d,e}	0.798^{d,e}	0.991	0.816^{d}
Basal diet, challenged	0.656^{e}	0.615^{e}	0.819^{c,d}	0.783^{e}	0.849	0.796^{d,e}
200 mg/kg of star anise oil, challenged	0.875^{c,d}	0.865^{b}	1.031^{b}	0.860^{c,d}	0.895	0.857^{d}
400 mg/kg of star anise oil, challenged	0.964^{b,c}	0.767^{c,d}	0.885^{c}	0.956^{a,b}	1.220	0.961^{c}
600 mg/kg of star anise oil, challenged	0.808^{d}	0.706^{d}	0.733^{e}	0.827^{c-e}	0.932	0.726^{e}
SEM	0.031	0.027	0.026	0.023	0.031	0.025
Star anise oil						
0	0.828	0.807	0.910	0.891	0.924^{c}	0.898
200	0.949	0.972	1.083	0.937	1.005^{b}	0.954
400	1.020	0.912	1.006	0.926	1.290^{a}	1.089
600	0.923	0.768	0.758	0.812	0.962^{b,c}	0.771
E. coli challenge						
0	1.034	0.992	1.011	0.927	1.117^{a}	1.021
+	0.826	0.738	0.867	0.856	0.974^{b}	0.835
P values						
Star anise oil	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Liner[2]	0.074	0.428	0.008	0.054	0.088	0.242
Quadratic[2]	0.005	0.004	<0.001	0.002	<0.001	<0.001
E. coli	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Star anise oil × *E. coli*	0.002	<0.001	0.004	<0.001	0.097	0.016

[a–e]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates per treatment.
[2]Liner and quadratic polynomial contrasts were performed on the means within the *E. coli*–challenged or unchallenged groups to star anise oil dosages.

DISCUSSION

Growth Performance

The final body weight and ADFI obtained in this study were within the range reported for this breed under similar feeding conditions (Antao et al., 2008). Although there was no interaction between SAO supplementation and *E. coli* challenge for the final body weight and ADFI, birds challenged with *E. coli* had decreased final body weight compared with those in the unchallenged group. As we all know, *E. coli* are the most common bacteria in commercial poultry production. Birds that survive *E. coli* challenges typically lose weight

Table 8. Effects of star anise oil on the relative protein expression in the liver of *E. coli*–challenged birds.[1]

Items	SOD	CAT	Nrf2
Treatment			
Basal diet, unchallenged	1.61	1.15	1.18
200 mg/kg of star anise oil, unchallenged	1.71	1.28	1.31
400 mg/kg of star anise oil, unchallenged	1.80	1.51	1.52
600 mg/kg of star anise oil, unchallenged	1.51	1.46	1.38
Basal diet, challenged	1.19	1.15	1.10
200 mg/kg of star anise oil, challenged	1.25	1.23	1.21
400 mg/kg of star anise oil, challenged	1.40	1.45	1.40
600 mg/kg of star anise oil, challenged	1.28	1.34	1.04
SEM	0.069	0.043	0.058
Star anise oil			
0	1.40^{b}	1.15^{c}	1.14^{b}
200	1.48^{a,b}	1.26^{b}	1.26^{b}
400	1.60^{a}	1.48^{a}	1.46^{a}
600	1.40^{b}	1.40^{a}	1.21^{b}
E. coli challenge			
0	1.66^{a}	1.35	1.35^{a}
+	1.28^{b}	1.30	1.19^{b}
P values			
Star anise oil	0.017	<0.001	<0.001
Liner[2]	0.761	<0.001	0.124
Quadratic[2]	0.175	<0.001	0.001
E. coli	<0.001	0.088	<0.001
Star anise oil × *E. coli*	0.397	0.609	0.097

[a–c]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates per treatment.
[2]Liner and quadratic polynomial contrasts were performed on the means within the *E. coli*–challenged or unchallenged groups to star anise oil dosages.

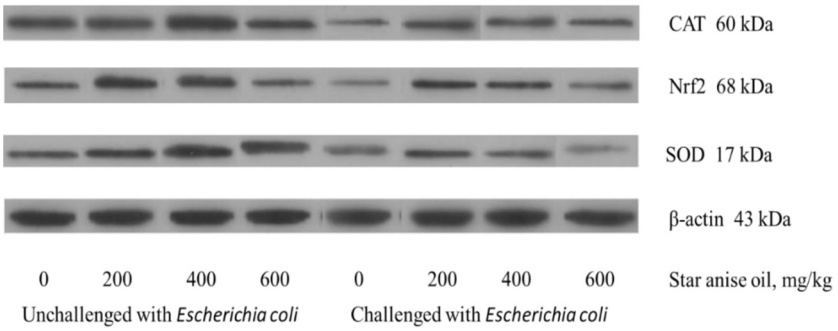

Figure 1. Western blot analysis in birds. Abbreviations: CAT, catalase; SOD, superoxide dismutase.

and have a poor feed-to-conversion ratio (Huff et al., 2006; Remus et al., 2014). However, the challenge did not result in *E. coli*–related morality in the present study. This phenomenon is likely associated with the dosage of *E. coli* we used in this study. We used *E. coli* challenge at a low dose in the experiment and tried to create a stress situation which finally resulted in weight loss of birds.

Supplementation of SAO at the concentrations up to 600 mg/kg of diet tended to change ($0.05 < P < 0.1$) the ADFI of birds irrespective of *E. coli* challenge. Meanwhile, the ADFI increased quadratically as the dietary SAO concentrations increased from 0 to 400 mg/kg; however, further increase of the concentration to 600 mg/kg resulted in an inferior effect even compared with those in control groups. This phenomenon is likely associated with the flavor characteristic in SAO. As an aromatic plants, star anise was rich in licorice flavor (Wang et al., 2011). The smell of the oil could stimulate appetite, which results in improvement of growth performance (Ertas et al., 2005; Ding et al., 2017). However, as the concentrations of star anise increased beyond a certain point, flavor becomes stronger. The factor also resulted in stress response of animals, thereby affecting the performance of birds. The result means that SAO shows a dose-dependent effect of promoting growth performance irrespective of *E. coli*. However, the mechanisms by which the SAO and *E. coli* challenge had no interactions with final body weight and ADFI of birds were not clear. This may be likely attributed to the age and species of the birds we used. In this study, we used White Leghorn, which provide animal models for studying effects of star anise and its effective compounds. It may provide theoretical foundation for the subsequent research studies of star anise or its effective compounds on birds of other breeds and ages.

Antioxidant Status

Our preliminary study showed that supplementation of star anise (*I. verum* Hook.f) essential oil at the rate of 200 mg/kg of diet enhanced antioxidant enzyme in broilers (Ding et al., 2017). Similarly, supplementation of SAO linearly increased the activities of antioxidant enzymes in the serum and liver of laying hens (Yu et al., 2018). The mechanisms by which the SAO enhances the antioxidant ability in birds, however, are not clear.

The present study's results show that the mRNA levels of SOD1, SOD2, HO-1, and Nrf2 in 400 mg/kg of SAO diet–supplemented groups were 1.23-, 1.13-, 1.11-, and 1.40-fold higher in the liver of birds, respectively, irrespective of *E. coli* challenge, as compared with those in the groups supplemented with no SAO. Meanwhile, the protein expression levels of SOD, CAT, and Nrf2 were 1.14-, 1.29-, and 1.28-fold higher in the liver of birds supplemented with 400 mg/kg of SAO diet relative to those of control birds irrespective of *E. coli* challenge. Other studies have demonstrated that phytochemicals, such as dieckol, resveratrol, epigallocatechin-3-gallate, and eckol, improved antioxidant status by enhancing Nrf2 signaling pathway–mediated antioxidant in *in vitro* and *in vivo* experiments. Dieckol induced the expression of antioxidant/detoxifying enzymes such as CAT, SOD, Glutathione S-transferase, HO-1, and NAD(P)H:quinone oxidoreductase in HepG2 cells which seemed to be associated with the ability of dieckol to activate transcriptional factor Nrf2 (Lee et al., 2015). Resveratrol augmented cellular antioxidant defense capacity through induction of HO-1 via Nrf2-ARE signaling, thereby protecting PC12 cells from oxidative stress (Chen et al., 2005). Polyphenolic ingredient (-)–epigallocatechin-3-gallate activated Akt and extracellular signal–regulated protein kinase1/2 (Antao et al., 2008). Eckol attenuated oxidative stress by activating Nrf2-mediated HO-1 induction via Erk and PI3K/Akt signaling (Kim et al., 2010). Our results were similar to those of other studies.

In recent years, the Nrf2 signaling pathway has attracted much attention because of its close relationship with antioxidants. As DNA-binding proteins recognize the antioxidant response element (**ARE**), Nrf2 regulates the expression of several phase II enzyme genes and antioxidants in response to noxious stimuli (Lee et al., 2015; Zhang et al., 2015). The activation of Nrf2–ARE signaling pathway leads to induction of antioxidant/phase II detoxifying enzymes such as HO-1, CAT, SOD, and GSH-Px (Zhang et al., 2015), which has been considered to be an adaptive and beneficial response to oxidative stress (Zhang et al., 2009; Ding et al., 2017). It is well recognized that SOD, CAT, and

GSH-Px are 3 main enzymes that regulate oxidative stress and that Nrf2 is critical in regulating the value of ROS. The observations that supplementation of SAO enhanced antioxidant capacity in the serum and liver of birds in this study demonstrated that SAO promoted the expression of SOD, CAT, and GSH-Px and activates the Nrf2 signaling pathway, which likely contributed to the enhanced antioxidant activity in birds.

SAO Supplementation and E. coli *Challenge*

E. coli is a ubiquitous, spore-forming anaerobic bacterium found in soil, dust, feces, feed, and poultry litter and in the intestinal tract of poultry and other species. Although most of *E. coli* strains are safe, some strains are virulent and could infect all types of birds at all ages in all types of poultry production (Guabiraba et al., 2015), causing economic threat to the poultry industry and food-borne disease in humans (Si et al., 2006; Antao et al., 2008). Birds suffered from *E. coli* use their immune system to eliminate *E. coli* but result in accumulation of free radicals (Rahman, 2007; Alhajj et al., 2017); then, the excessive free radicals resulted in stress response of animals. In this study, we used *E. coli* challenge in the experiment and tried to create a stress situation leading to birds' different antioxidant statuses.

The present results show that the activity of CAT, the mRNA level of Nrf2, and the relative protein expressions of SOD and Nrf2 in *E. coli*–challenged birds were 1.04-, 1.15-, 1.30-, and 1.13-fold lower in the liver, respectively, as compared with those of unchallenged birds. The decreased antioxidant status of birds by *E. coli* challenge is likely attributed to stimulate of virulent *E. coli*. However, an interesting finding of this study was that the activity of CAT and the expressions of CAT and GPX4 were 1.07-, 1.05-, and 1.06-fold higher in the liver of birds supplemented with 200 mg/kg of SAO diet relative to those of control birds, respectively. Results of the present study indicate that SAO reduces *E. coli* challenge–induced oxidative stress in the serum and liver of birds because of its antioxidant function. The supplementation of SAO may reduce the stress by *E. coli* overload. In other words, star anise not only can enhance the antioxidant ability of birds in normal conditions but also severed as an effective natural antioxidant in animal reproduction even in abnormal conditions. This is also consistent with the effects of SAO on ADFI obtained in this study. Furthermore, this study provided further insight into antioxidant mechanisms in birds fed SAO.

Surprisingly, further increase of the concentration to 600 mg/kg resulted in an inferior effect even compared with those of groups supplemented with no SAO. However, as the dietary SAO concentrations increased from 0 to 400 mg/kg irrespective of *E. coli* challenge, the growth performance and antioxidant status were increased. The result indicated that the efficacy of SAO in birds during subclinical *E. coli* challenge is dose-dependent. SAO has shown numerous pharmacological effects including a stimulating effect of digestion and antiparasitic, antibacterial, antioxidant, antifungal, and antipyretic effects (Huang et al., 2010; Yang et al., 2010). It has served as traditional medicine for a long time, and it has several effective compounds such as anthole, anisaldehyde, and estragole (Huang et al., 2010; Domiciano et al., 2013). All of these have good effects on animal productivity, which may have partially contributed to the increased antioxidant status of broilers in this study. As a coin has 2 sides, everything has advantages and disadvantages. The phenomena that the concentrations of star anise increased beyond a certain point may result in stress response of animals. However, the mechanisms of this phenomenon were not clear.

CONCLUSIONS

SAO shows the dose-dependent effect of promoting growth performance irrespective of *E. coli* challenge. However, there was no interaction between SAO supplementation and *E. coli* challenge for the final body weight and ADFI.

Supplementation of SAO can reduce *E. coli* challenge–induced oxidative stress in the serum and liver of birds. The mechanisms by which SAO exerts its antioxidant effect correlate with the activation of SOD, CAT, and Nrf2, via the activation of the Nrf2–ARE signaling pathway. Meanwhile, based on the results obtained in this study, the optimum supplementation dose of SAO for protecting birds against *E. coli* challenge is 400 mg/kg.

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