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Orthosiphon stamineus and rosmarinic acid reduce heat stress in laying hens

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ABSTRACT

Heat stress is a great concern in the layer industry because it decreases egg production and egg quality. *Orthosiphon stamineus* extract (OE) has the potential to relieve heat stress, and rosmarinic acid (RA) is the main effective constituent of OE. However, the effects and mechanisms of OE and RA on heat stress in commercial laying hens remain unclear. In the current study, four hundred eighty 56 wk old hens were divided into 2 hen houses, one maintained at the low temperature and other maintained at a high temperature. Hens in each house were assigned to 3 dietary treatments (basal, basal + 0.4% OE, and basal + 0.02% RA) with 20 cages per treatment and 4 hens per cage. Performance traits were recorded, and 1 egg in 4 cages were collected randomly each day (35 eggs per treatment/wk) for egg quality assay. The excreta were collected by mixed every 5 adjacent cage at the end (28 d) of the experiment for apparent digestibility analysis, and then 1 hen from each 2 cages were sacrificed for blood and intestinal samples. The OE and RA supplementation increased the egg production rate ($P < 0.05$) and improved the eggshell strength and haugh unit ($P < 0.05$). The analysis of digestibility of basic nutrients showed OE or RA supplementation improved protein (OE: $P = 0.074$; RA: $P = 0.037$), fat, and Ca digestion ($P < 0.05$) in laying hens. To explore the mechanisms associated with the improvements of performance traits and egg quality, serum biochemical indices and related gene expression and intestinal villi integrity were measured. Supplementing the diet of the laying hens with OE or RA increased superoxide dismutase ($P < 0.05$) activities in both the serum and jejunum, decreased the malondialdehyde contents ($P < 0.05$), and also decreased the caspase-3 activity ($P < 0.05$) in jejunum of laying hens. Intestine sections showed RA supplementation improved the jejunum villus height ($P = 0.007$). The OE or RA supplements up-regulated nuclear factor erythroid 2-related factor 2 (*Nrf2)* and occludin gene expressions, and down-regulated caspase-3 gene expression in hens ($P < 0.05$). In conclusion, The OE and RA supplements may increase nutrient digestibility, stimulate the antioxidant defense mechanism system through the *Nrf2*-mediated pathway, decrease intestinal epithelial cell apoptosis, and protect the intestinal barrier. This study indicated that dietary supplementation with OE or RA is beneficial for laying hens under heat-stress conditions.

1. Introduction

Although the environments of chicken houses and pigpens have been greatly improved and enhanced over the past several decades, heat stress is still a great concern in livestock production (Nardone et al., 2010), especially in the layer industry. In laying hens, the effectiveness of egg production is severely affected by heat stress (Melesse et al., 2011; Karami et al., 2018), especially in the summers in tropical and subtropical regions.

Numerous environmental and feeding strategies have been employed to reduce heat stress in laying hens (Nawab et al., 2018). In recent years, researchers have focused on reactive oxygen species (ROS) imbalances and intestinal integrity decreases (Nawab et al., 2018) in hens in high-temperature environments. Heat exposure increases the ROS in hens (Lin et al., 2008; Cai et al., 2017), and the ROS increase induces animal intestinal barrier injuries in animals (Zhu et al., 2012; Tan et al., 2018). Intestinal barrier injuries lead to intestinal permeability increases, which cause inflammatory and other adverse reactions (Lambert, 2009; Nawab et al., 2018). These are major factors leading to the decreased performance of layers (Nawab et al., 2018).

Orthosiphon stamineus (*Clerodendranthus spicatus* Thunb.), more commonly known as “java tea”, is widely grown throughout

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southeastern Asia, Australia, and southern China. It has been consumed like tea for thousands of years in southern Asia, and in China, it is usually used as a traditional medicinal herb to treat kidney stones (Zhong et al., 2012b). *Orthosiphon stamineus* extract (OE) can ameliorate heat stress in laying hens (Cai et al., 2017), but the mechanism is unclear. A subsequent study using mice (Cai et al., 2019) showed that rosmarinic acid (RA) is the component of OE with the most effective antioxidant activity, and RA and OE protect animal intestines against oxidative stress by preventing intestinal epithelial cell apoptosis and subsequently protecting the intestinal barrier. The mechanism related to RA and OE activates the *Nrf2* pathway and increases downstream antioxidant enzyme activity levels (Cai et al., 2019).

Thus, we hypothesize that RA and OE may ameliorate heat stress in laying hens, using a mechanism similar to that in model animals. In this study, we compared the effects of RA and OE on laying hens exposed to heat stress conditions or a low temperature by analyzing the laying performance and egg quality. Then, the mechanisms were explored by comparing serum biochemical indices, the digestibility of basic nutrients, and the intestinal villi integrity of hens subjected to different treatments.

2. Materials and methods

2.1. Plant materials and extraction

Orthosiphon stamineus were picked in the early summer or late autumn in Yulin, Guangxi, China (22.5735N, 109.5906E). The extracts were obtained as previously described (Cai et al., 2019) with some modifications. In brief, dry powdered *O. stamineus* (20 kg) was extracted with 50% aqueous ethanol using an ultrasonic extracting tank at room temperature for 30 min. Then, the OE extracts were filtered and concentrated by spray-drying. The resulting powders were stored at −20 °C until use.

The extract power was analyzed to determine the main components and all analyses in triplicate. Proteins in the OE were measured using the Bradford (1976) method. Polysaccharides were analyzed using the phenol-sulfate method (Xi et al., 2010). The total polyphenol content in the extract was determined using the Folin–Ciocalteu method with gallic acid as the standard (Rana et al., 2015). Total flavonoids in the extract were determined with rutin as the standard (Taga et al., 1984). The main components were measured using kits (Congyi Bio., Shanghai, China). The OE content was 13.7% polysaccharide and 0.011% protein. The total polyphenol level in the OE was 197 mg/g gallic acid equivalent, and the total flavonoid content was 616 mg/g rutin equivalent. The antioxidant components were analyzed by HPLC method and the results showed in our previous studies (Cai et al., 2018, 2019).

2.2. Animals and management

The study protocol was approved by the Institutional Animal Care and Use Committee of Shanghai Academy of Agricultural Science (Shanghai, China). Maximum care was taken to minimize the suffering of the hens. In total, 480 laying hens (Hy-Line Sonia; Shanghai Liyuan Chicken Breeding Professional Cooperative, Shanghai, China) at 56 wk of age were obtained from a commercial hatchery. Hens were divided into 2 hen houses with 20 cages per treatment and 4 hens per cage. Hens in each house were assigned to 3 dietary treatments (basal, basal + 0.4% OE, and basal + 0.02% RA). One house was equipped with a wet-curtain cooling system and air conditioner to get a “low temperature” whereas the other was only equipped with a ventilation systems to get a “high temperature”. The temperatures and humidity levels were recorded using an electronic hygrometer every 2 h during the experimental period (Fig. 1). The dietary treatments consisted of soybean meal-based (basal) diet (Table 1) and 2 other diets containing 0.2% OE or 0.04% RA, and each treatment contained 80 hens. During the whole experimental period, the lights were on for 16 h/d, and the hens had free access to feed and water.

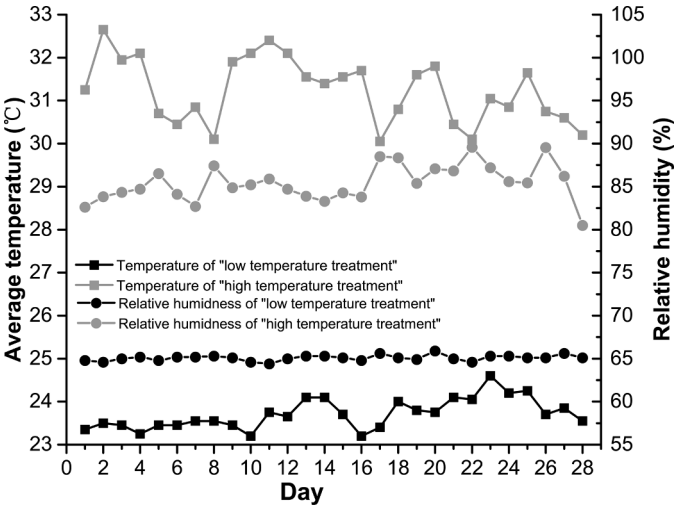

Fig. 1. Temperature and humidness of the 2 hen houses during the experimental period.

Table 1
Composition and nutrient content of the basal diet (as-fed).

Item	Content,%
Ingredients	
Corn	62.5
Soybean meal	23.0
Extruded soybean	3.00
Limestone	9.30
Premix[a]	2.20
Nutrient composition[b]	
Crude protein	17.2
Crude fiber	3.29
Ether extract	2.59
Ca	4.35
Available P	0.51
Lys	0.61
Met	0.38

[a] Supplied the following per kilogram of feed:vitamin A, 9900 IU; vitamin D3, 2625 IU; vitamin E, 49.5 mg; vitamin K3, 6 mg; vitamin B_1, 3 mg; vitamin B_2, 10.5 mg; niacin, 60 mg; vitamin B_6, 6 mg; vitamin B_{12}, 30 μg; biotin, 300 μg; pantothenic acid, 18 mg; folic acid, 3 mg; Fe, 120 mg; Cu, 9 mg; Mn, 140 mg; Zn, 120 mg; I, 1.12 mg; Se, 376 μg; ethoxyquin, 10 mg; Met 844 mg, choline chloride, 450 mg.

[b] Measured values. Sample was analyzed in triplicate.

2.3. Performance traits

Feed consumption levels were measured daily. All the eggs were collected daily, and egg weights were recorded. Hen body weights were measured every 2 wk during the experimental period. Average daily feed intake (ADFI), feed conversion ratio, and production performance were calculated based on 28 d intervals, and all the performance data were corrected for mortality.

2.4. Egg quality assay

To evaluate egg quality, 5 eggs per treatment were collected randomly each day and kept in a refrigerator at 4 °C until the anslysis (35 eggs were tested per wk for each treatment). Shell strength, albumen thickness, haugh unit, yolk color, and weight were determined using an egg quality tester (DET6000; NABEL Co., Ltd., Kyoto, Japan).

Table 2
Primers for quantitative RT-PCR.

Gene name	Primer(5′→3′)	GenBank No.	Refrence
β-actin	F:GAGAAATTGTGCGTGACATCA R:CCTGAACCTCTCATTGCCA	NM_205518	Lin et al., 2016
GAPDH	F:TGAAAGTCGGAGTCAACGGATT R:CCACTTGGACTTTGCCAGAGA	NM_204305	Ojano-Dirain et al., 2007
Caspase-3	F:TGGCCCTCTTGAACTGAAAG R:TCCACTGTCTGCTTCAATACC	NM_204725	Huang et al., 2013
BCL-2	F:CTAGGTGGTGAATATGCCAAAC R:TCCGAAATATGAAGAGGTGTTG	NM_205339	Lin et al., 2016
Nrf2	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	NM_205117	Lin et al., 2016
HSP70	F:GCAAGATCTGCTCCTGTTGGAT R:GTTACGCTTGATGAGAGCAGTCA	NM_001006685	Xue et al., 2016
Occludin	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	NM_205128	Shao et al., 2013
Claudin-1	F: GAGGATGACCAGGTGAAGAAG R: TGCCCAGCCAATGAAGAG	NM_001013611	Lin et al., 2016

2.5. Sample collection

Blood samples (2 mL) were taken at the end of the experiment from the brachial vein of 10 laying hens per treatment (one hen from each 2 cages). Blood samples were allocated in tubes without anticoagulant at 4 ˚C overnight and then centrifuged at 3000 × g at room temperature for 10 min to obtain serum. The serum was divided into five 1.5 mL tubes, and stored at −20 ˚C before analysis.

One hen in every two cages (10 hens per treatment) were randomly selected and sacrificed by decapitation. Carcasses were manually eviscerated. The entire small intestine was carefully removed and placed on ice. Pieces of the small intestine, approximately 2 cm in length, were resected from the middle portion of the jejunum and fixed in 4% neutral buffered formalin for the hematoxylin-eosin staining assay. Other segments of the jejunum were rinsed thoroughly with physiological saline, frozen in liquid N_2, and stored at −80 ˚C for further quantitative RT-PCR analyses and other biochemical analyses.

2.6. Apparent digestibility of nutrients

As described by Siyal et al. (2017), the excreta were collected from all the cages and every 5 adjacent cage were mixed on a tray covered with oven parchment paper after 3-d periods at the end (28 d) of the experiment. The feathers were carefully removed, and the excreta were pooled. Then, a few drops of dilute sulfuric acid were added, and the excreta were frozen at −20 ˚C until analysis. The pelleted diets were ground to pass through a 0.5-mm screen. Samples were dried in an air-dry oven at 60 ˚C for 48 h. The dry mass, crude protein, fat (ether extract), total Ca and P contents of the excreta and diet were determined according to methods 930.15, 984.13, 920.39, 927.02 and 965.05 of the AOAC (1990), respectively. Acid-insoluble ash was used as an indigestible marker, and it was analyzed using the method of Bu et al. (2015).

2.7. Serum biochemical analysis

Endotoxin and corticosterone in the sera were used to determine the intestinal barrier integrity of the hens. Endotoxin was assayed using the tachypleus amebocyte lysate method (Xiamen Bioendo Tech., Xiamen, China); corticosterone was assayed using an ELISA method (Cusabio, Wuhan, China). The activities of superoxide dismutase (SOD) and catalase (CAT) were determined to evaluate the antioxidant ability using assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The concentrations of total protein, albumin, triglyceride, and total cholesterol were also measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute).

2.8. Jejunum antioxidative capacity and caspase activity analysis

Frozen jejunum mucosal samples were homogenized in cold physiological saline and then centrifuged at 3500 × g for 15 min at 4 ˚C to collect the supernatant for analysis. The malondialdehyde (MDA) concentration and the superoxide dismutase (SOD) and catalase (CAT) activities were determined to evaluate the antioxidant abilities using assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). The activity of caspase-3 was measured with substrate peptide Ac-DEVD-pNA(N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide) using a caspase activity kit (Beyotime, Shanghai, China). All the operations were conducted according to the manufacturer's protocol. The absorbance was determined at 405 nm, and the activity of caspase 3 was determined by calculating the fold change of OE/RA-supplemented hens to control hens. The protein contents of the homogenates were measured using the Coomassie brilliant G-250 method (Congyi Bio., Shanghai, China).

2.9. Quantitative RT-PCR

Extraction of total RNA and its reverse transcription, and real-time PCR were performed according to our previous reports (Cai et al., 2019). Primers are listed in Table 2. The *β-actin* and *GAPDH* genes were used as internal controls for RNA template normalization. All the relative mRNA expression levels were calculated using the comparative Ct method (Livak and Schmittgen, 2001).

2.10. Hematoxylin-eosin staining

Samples of the jejunum were dehydrated, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin, and then photographed under a light microscope (Olympus, Tokyo, Japan). Villus height was calculated from the base of the villus, above the level of adjoining crypts, to the villus tip. The depth of a crypt was calculated from the boundaries between the crypt and myenteron to the base of the villus. At least five typical villi and crypts were measured for each hen, and the mean value was calculated.

2.11. Statistical analysis

Statistical analyses were completed using SPSS for Windows (IBM, Armonk, NY), and data analyzed using GLM procedure. The statistical model included temperature and extract. All data were verified to meet assumptions of normality. Multiple comparisons of the observed means were based on the Tukey's test. A P value < 0.050 was considered significant.

Table 3
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) on performance in hens[a].

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed Basal *vs* OE	Basal *vs* RA	OE *vs* RA
Egg production,%	81.0	81.3	81.3	70.1	74.2	74.0	0.43	<0.001	0.002	0.004	0.977
Egg mass, g	59.1	59.1	59.1	59.0	59.0	59.0	0.04	0.427	0.912	0.994	0.951
ADFI[c], g	109	109	108	101	102	101	0.78	<0.001	0.885	0.977	0.780
Feed: egg	2.27	2.26	2.26	2.45	2.33	2.32	0.02	0.001	0.106	0.065	0.964

[a] Data represent the mean of 20 cages (4 hens/cage) per treatment.
[b] SEM = standard error of the mean.
[c] ADFI = average daily feed intake.

3. Results

3.1. Performance of laying hens

As shown in Table 3, heat stress caused decreases in the egg production rate and ADFI, but increases in feed conversion ratio (eggs output divided by feed input) compared with the low temperature ($P < 0.050$). In this study, heat stress has no influence on average egg mass ($P = 0.912$) to hen mass. The supplementation of OE or RA improved egg production in high temperature environments (OE: $P < 0.001$; RA: $P < 0.001$). No differences in any of the performance indices were observed among all the performance indices between OE and RA supplementation hens.

3.2. Egg quality

During the 3 to 4 wk, the eggs from the OE and RA supplementation groups showed different eggshell strengths, albumen thicknesses, haugh unit, and yolk color (Table 4). Specifically, eggs of the hens feed with OE or RA supplementation increases in haugh unit compared with that feed with basal diet by wk 3 (OE: $P = 0.015$; RA: $P = 0.003$). Eggs of the OE or RA supplementation hens showed significant increases in eggshell strength (OE: $P = 0.016$; RA: $P = 0.002$) compared with the eggs of basal diet feed hens by wk 4. Supplementation of OE or RA also increased yolk color (wk4, OE: $P = 0.040$; wk3, RA: $P = 0.006$) of eggs. No difference was observed between the OE or RA supplementation groups in egg quality. Interestingly, unlike the performance indices, none of the egg quality showed difference between low and high temperature environments (except the "eggshell strength" in wk2).

3.3. Serum biochemical analyze

Both SOD and CAT are important antioxidases. In Table 5, the high temperature environment is induce decreases in the hens' serum SOD levels ($P = 0.033$). OE or RA supplementation increased the serum SOD levels (OE: $P = 0.027$; RA: $P = 0.014$). Corticosterone in serum is an indicator of stress. A higher serum corticosterone concentration signals more severe stress damage (Littin and Cockrem, 2001). As shown in Table 5, the RA supplemented diet reduced the corticosterone level ($P < 0.001$). Serum endotoxin increased rapidly with intestinal barrier injury, causing animal illness. In Table 5, the high temperature environment is induce increases in the hens' serum endotoxin levels ($P < 0.001$), whereas the levels in OE and RA supplemented hens decreased significantly ($P < 0.001$).

3.4. Antioxidative capacity and caspase-3 activity in the jejunum

As shown in Table 6, high temperature causing decrease in SOD

Table 4
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) on egg quality in Hy-Line Sonia hens[a].

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed Basal *vs* OE	Basal *vs* RA	OE *vs* RA
Eggshell strength, kgf											
wk 1	4.07	4.03	4.32	3.99	4.05	3.91	0.64	0.213	0.999	0.871	0.898
wk 2	4.06	4.41	4.37	3.98	3.99	3.99	0.06	0.009	0.449	0.441	1.00
wk 3	4.03	4.32	4.41	4.07	4.28	4.23	0.06	0.560	0.159	0.113	0.982
wk 4	4.01	4.49	4.41	4.06	4.36	4.64	0.06	0.068	0.016	0.002	0.082
Albumen height, mm											
wk 1	5.48	5.64	5.47	5.32	5.57	5.68	0.75	0.970	0.527	0.578	0.993
wk 2	5.44	5.58	5.36	5.60	5.53	5.84	0.08	0.264	0.984	0.927	0.980
wk 3	5.40	5.73	5.88	5.31	5.84	5.64	0.06	0.573	0.016	0.022	0.994
wk 4	5.93	6.28	6.51	5.99	6.53	6.18	0.07	0.946	0.031	0.053	0.968
Haugh unit											
wk 1	73.3	74.1	73.3	72.2	74.5	76.4	0.57	0.481	0.543	0.286	0.909
wk 2	74.0	74.5	73.6	74.6	73.8	77.4	0.66	0.369	0.991	0.763	0.695
wk 3	73.6	75.7	77.5	73.1	77.1	76.4	0.47	0.958	0.015	0.003	0.866
wk 4	76.2	78.3	79.8	76.7	80.4	78.4	0.47	0.681	0.035	0.045	0.991
Yolk color											
wk 1	5.29	5.26	5.58	5.76	5.33	5.43	0.08	0.391	0.441	0.992	0.517
wk 2	5.11	5.47	5.25	5.00	5.17	5.11	0.07	0.175	0.292	0.727	0.720
wk 3	5.29	5.10	5.66	4.94	5.39	5.62	0.07	0.795	0.710	0.006	0.054
wk 4	5.05	5.43	5.28	5.12	5.61	5.48	0.07	0.288	0.040	0.212	0.720

[a] One egg in 4 cages (20 cages/ treatment) were collected randomly each day (35 eggs per treatment/wk) for egg quality assay.
[b] SEM = standard error of the mean.

Table 5
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) on serum biochemical items in laying hens[a].

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed		
									Basal *vs* OE	Basal *vs* RA	OE *vs* RA
Total protein(TP),g/L	47.2	46.4	49.1	49.4	47.5	51.2	1.06	0.186	0.884	0.710	0.455
Albumin,g/L	21.8	22.2	25.2	24.7	22.0	23.7	0.51	0.718	0.634	0.598	0.153
Triglyceride (TG),mmol/L	12.0	11.3	12.2	16.1	11.1	13.2	0.62	0.188	0.136	0.607	0.586
Cholesterol,mmol/L	3.29	2.65	3.00	3.69	2.80	3.53	0.12	0.115	0.020	0.695	0.132
SOD[c],U/mL	284	291	293	247	286	289	4.65	0.033	0.027	0.014	0.936
CAT[c],U/mL	33.6	49.1	43.1	28.8	37.0	40.5	2.60	0.209	0.162	0.220	0.978
Corticosterone,ng/mL	313	300	296	361	298	255	7.35	0.004	0.164	<0.001	0.017
Endotoxin,U/mL	0.77	0.64	0.55	0.94	0.74	0.78	0.03	<0.001	<0.001	<0.001	0.624

[a] One hen from every 2 cages (20 cages/treatment), and 10 hens/treatment for serum biochemical parameters analysis.
[b] SEM = Standard error of the mean.
[c] SOD: superoxide dismutase; CAT: catalase.

($P = 0.002$) and increase ($P = 0.042$) in MDA level in jejunum of laying hens. Supplementation with OE or RA increased hens' jejunum SOD activity levels (OE: $P = 0.001$; RA: $P < 0.001$). This is consistent with the results of the serum analysis (Table 5). The jejunum MDA contents also showed similar trends: OE or RA supplementation decreased jejunum MDA content in high temperature (OE: $P = 0.001$; RA: $P = 0.002$). Caspase 3 is an important enzyme marker expressed during cell apoptosis (Cai et al., 2016). As shown in Table 6, the high temperature has a trend in induced hens' intestinal apoptosis ($P = 0.078$), whereas OE or RA supplementation reduced the apoptosis (OE: $P = 0.001$; RA: $P = 0.005$).

3.5. Apparent digestibility of nutrients

High temperature was resulted decrease in dry mass ($P = 0.002$), crude protein ($P < 0.001$), fat ($P < 0.001$), and total Ca ($P < 0.001$) in jejunum of laying hens. Supplementation with either OE or RA increased crude protein (OE: $P = 0.074$; RA: $P = 0.037$), ether extract (OE: $P = 0.022$; RA: $P = 0.003$), and calcium (OE: $P = 0.007$; RA: $P < 0.001$) apparent digestibility of nutrients by hens in the high temperature environment (Table 7).

3.6. Intestinal morphology

The effects of OE or RA supplements on the jejunum morphology in hens are shown in Table 8. The data showed both OE and RA supplements increased villi heights under heat-stress conditions (OE: $P = 0.002$; RA: $P < 0.001$). Heat stress remarkably reduced the villus height to crypt depth ratio, which is considered an important index for describing intestinal health (Song et al., 2014).

3.7. Tight junction protein and apoptosis-related genes' mRNA levels

Caspase-3 and *Bcl-2* are apoptosis-related genes, and Nrf2 is involved in the antioxidant signaling pathway (Cai et al., 2019). As shown in Table 9, heat stress result in upregulation in *Caspase-3* gene ($P < 0.001$) and downregulation in *Bcl-2* gene ($P < 0.001$), and the OE or RA supplements decreased the *caspase-3* mRNA level ($P < 0.001$). The OE or RA supplements strongly increased the *Nrf2* mRNA level ($P < 0.001$). Occludin and claudin-1 are important transmembrane proteins in animal intestinal epithelia (González-Mariscal et al., 2003). As shown in Table 9, heat stress downregulated the mRNA expression levels of *occludin* and *claudin-1* ($P < 0.001$) in hen intestinal epithelia. OE or RA supplements upregulated mRNA expression of *occludin* ($P < 0.001$), but had no effects on *claudin-1* in hens. There were no differences in the transcription levels of any genes tested between OE and RA supplementation groups.

4. Discussion

High environmental temperatures are injurious to animals, and modern commercial poultry produce more body heat owing to their rapid metabolism. This makes poultry more sensitive to the environmental temperature (Nawab et al., 2018). Heat stress causes high rates of morbidity and mortality. Payne (1966) showed that the performance level of laying hens decreased in temperatures greater than 32 °C, which exists universally in commercial hen houses. Our pervious monitoring data showed that in Shanghai, China, the greatest environmental temperatures in most of the commercial hen houses exceeded 32 °C in July and August. This study also showed that a

Table 6
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) on jejunum antioxidative capacity and caspase activity in Hy-Line Sonia hens[a].

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed		
									Basal *vs* OE	Basal *vs* RA	OE *vs* RA
SOD[c],U/mgprot	39.67	41.25	42.13	30.27	39.96	40.97	0.77	0.002	0.001	<0.001	0.793
CAT[c],U/mgprot	1.63	1.78	1.73	1.28	1.63	1.73	0.06	0.123	0.146	0.105	0.985
MDA[c],mmol/mgprot	0.56	0.45	0.41	0.85	0.42	0.49	0.03	0.042	0.001	0.002	0.966
Caspase3[d], fold change	1.00	0.95	0.92	1.16	0.98	1.04	0.02	0.078	0.001	0.005	0.876

[a] One hen from every 2 cages (20 cages/treatment), and 10 hens/treatment for biochemical parameters analysis.
[b] SEM = Standard error of the mean.
[c] SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde.
[d] The activity of Caspase3 of hens in normal temperature feed with normal diet were defined as 1.00.

Table 7
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) on nutrient apparent digestibility in Hy-Line Sonia hens[a].

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed		
									Basal *vs* OE	Basal *vs* RA	OE *vs* RA
DM[c],%	76.4	76.4	77.0	76.0	75.1	76.3	0.15	0.002	0.264	0.201	0.008
CP[c],%	66.4	66.0	66.7	62.6	64.6	64.1	0.32	<0.001	0.074	0.037	0.936
EE[c],%	72.0	72.3	73.1	67.7	69.6	69.4	0.42	<0.001	0.022	0.003	0.651
Ca,%	52.6	53.7	54.6	48.0	50.8	51.4	0.49	<0.001	0.007	<0.001	0.398
TP[c],%	48.2	49.8	50.9	48.0	48.4	48.6	0.35	0.055	0.444	0.123	0.687

[a] The excreta were collected from all cages (20 cages/ treatment), and every 5 adjacent cages were mixed on a tray covered, and 4 mixed samples were tested each treatment.
[b] SEM = Standard error of the mean.
[c] DM: dry mass; CP: crude protein; EE: ether extract (crude fat); TP: total P.

Table 8
Effect of *Orthosiphon stamineus* extract (OE) and rosmarinic acid (RA) supplementation on jejunum morphology in hens.[a]

Item	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed		
									Basal *vs* OE	Basal *vs* RA	OE *vs* RA
Villus height, μm	373	356	363	276	347	366	5.66	0.001	0.096	0.007	0.596
Crypt depth, μm	100	104	100	98.8	97.1	105	1.69	0.752	0.973	0.759	0.878
Villus wide, μm	80.3	82.4	80.5	76.1	79.3	78.7	0.43	<0.001	0.032	0.342	0.496
VH:CD[c], μm	3.73	3.44	3.63	2.79	3.58	3.50					

[a] One hen from every 2 cages (20 cages/treatment), and 10 hens/treatment for jejunum morphology analysis
[b] SEM = Standard error of the mean.
[c] VH:CD: Villus heights (VH) to crypt depth (CD) ratio, the values were calculated by average.

commercial hen house having only a common wetted pad and ventilation systems cannot be effectively cooled (Fig. 1).

The data of this study confirmed that a high environmental temperature caused decreased egg production and ADFI, and increased feed/egg ratios in laying hens, and also, high environmental temperatures cause a decrease in egg quality. This was consistent with our previous study in *Xinyang* laying hens (Cai et al., 2017) and that of Song et al. (2012) in 24 wk old Hy-line brown hens. Many hypotheses have been developed to explain the mechanisms of heat stress effects in laying hens (Lara and Rostagno, 2013). A popular hypothesis involved changes in the intestinal barrier function intact: heat stress induced oxidative stress (Lin et al., 2008; Akbarian et al., 2016), and oxidative stress induces cell apoptosis (Chandra et al., 2000; Cai et al., 2016), leading to intestinal barrier injury (Zhu et al., 2012). Injuries to the intestinal barrier result in enhanced uptakes of potentially noxious materials (*e.g.*, toxins and proinflammatory molecules) from the lumen (Söderholm and Perdue, 2001). This may lead to the decreased egg quality and performance caused by the high environmental temperature.

Orthosiphon stamineus is more commonly used as a traditional medicine or beverages (Ameer et al., 2012; Ashraf et al., 2018). It has an industrial base in the feed industry (Cai et al., 2018b); however, there are very limited reports on *O. stamineus* being used as a feed additive. The studies of Malahubban et al. (2013) showed that *O. stamineus* supplementation in diets was beneficial to broiler chickens. In addition, *O. stamineus* supplementation improved laying hens' performance and egg quality (Cai et al., 2017) and OE is more beneficial than *O. stamineus* powder. However, the hens used in the study were an endemic species called *Xinyang* laying hens. To establish a more general conclusion, this study was designed.

Here, the OE improved the laying performance and egg quality in heat stress-affected laying hens. However, the increase in the egg production rate was not as high as in a previous study on *Xinyang* laying hens (Cai et al., 2017). This may be because the egg production rate of

Table 9
Regulatory of tight junction proteins and apoptosis-related genes in hens[a].

Gene[d]	Low temperature			High temperature			SEM[b]	*P*-value			
	Basal diet	Basal diet + 0.2% OE[c]	Basal diet + 0.04% RA[c]	Basal diet	Basal diet + 0.2% OE	Basal diet + 0.04% RA		Temperature	Feed		
									Basal *vs* OE	Basal *vs* RA	OE *vs* RA
Caspase 3	1.04	0.92	0.89	1.77	1.45	1.54	0.046	<0.001	<0.001	<0.001	0.720
Bcl-2	1.02	1.04	1.11	0.88	0.95	0.94	0.017	<0.001	0.422	0.109	0.710
Nrf2	0.98	1.83	1.72	0.82	1.62	1.56	0.051	<0.001	<0.001	<0.001	0.056
Occlaudin	1.02	1.12	1.09	0.45	0.76	0.77	0.033	<0.001	<0.001	<0.001	0.939
Claudin-1	0.97	0.99	1.10	0.57	0.62	0.59	0.032	<0.001	0.621	0.131	0.570

[a] One hen from every 2 cages (20 cages/treatment), and 10 hens/treatment for qRT-PCR analysis.
[b] SEM = Standard error of the mean.
[c] OE: *Orthosiphon stamineus* extract; RA: rosmarinic acid.
[d] The expression of hens in normal temperature feed with normal diet were defined as 1.00, all data showed as fold change; The β-actin gene was used as the reference gene.

Hy-Line Sonia hens is much greater than that of *Xinyang* laying hens, and this limits space for further growth. Additionally, the environmental temperature in this study (Fig. 1) was lower than that in *Xinyang* laying hens (Cai et al., 2017).

In this study, we compared the hens raised at a low environmental temperature (23.5 ± 2.0 ˚C) and a high environmental temperature (continuously over 28 ˚C with a high temperature over 32 ˚C), and the data showed OE or RA supplementation did not significantly influence egg production, egg mass, ADFI, or the feed/egg ratio at a low environmental temperature. Data showed that the effects were correlated with the antioxidant ability of the OE or RA. This is consistent with previous studies (Lin et al., 2008; Cai et al., 2017). The formation of ROS is significantly augmented in laying hens by heat exposure (Lin et al., 2008). Many studies have confirmed that OE has a great antioxidant capability (Abdelwahab et al., 2011; Yam et al., 2013; Cai et al., 2018). The OE can activate the antioxidant system in animals through the *Nrf*-2-mediated pathway (Cai et al., 2019). This study also detected the *Nrf-2* mRNA level. Thus, this study implied that OE or RA supplementation alleviated the heat-stress effects on laying hens by improving the antioxidant capability.

Oxidative stress subsequently induced intestinal barrier injury as a mechanism of heat stress that causes decreases in egg quality and performance. The intestine acts as a barrier, eliminating toxins and infectious agents, and is composed of intestinal epithelial cells and intercellular junctions (Zhong et al., 2012a; Nawab et al., 2018). Occludin and claudin-1 are important transmembrane tight junction proteins that interact to form a complex protein network (Shin et al., 2006). Additionally, they are the molecular basis of the intestinal barrier. In this study, OE or RA supplementation could improve villi integrity in laying hens at the environmental temperature and increased the tight junction protein occludin mRNA levels. These results were similar to those of Zhang et al., 2017 achieved with probiotic mixture supplementation in laying hens or those of Jazideh et al. (2014) achieved with glutamine supplementation in broiler chickens. Research in piglets (Xiao et al., 2018) and rats (Sun et al., 1998) showed that intestinal barrier injury involved the apoptosis of intestinal epithelial cells. However, as far as we know, there are still no reports on the relationship between laying hens' intestinal villi and intestinal epithelial cell apoptosis. This study showed OE supplementation could decrease the *caspase-3* mRNA level. This was consistent with our previous study in high-fat-induced mice (Cai et al., 2019). A study by Abdelwahab et al. (2011) revealed that OE could reduce H_2O_2-induced cell apoptosis by increasing *Bcl-2* expression. But in this study, the data did not show a significant difference. The mechanisms behind these occurrences are still unknown. Briefly, OE protects the intestinal barrier by upregulating the tight junction protein occludin mRNA transcription levels, which may related to the anti-apoptotic effects of the OE.

The data showed that egg production increased in the OE supplementation hens. They also revealed that the apparent digestibility of protein, fat, and calcium increased significantly in Hy-Line Sonia hens fed the OE. This corresponded with that the hens supplement with OE increased egg production significantly, and it also revealed that the haugh unit increased by eggs of OE supplementation hens.

The OE contained 3 main types of phytochemicals: flavonoids, caffeic acid derivatives, and terpenoids (Ameer et al., 2012). RA is the main phenolic constituent of the alcohol (methanol)-water extract (Sumaryono et al., 1991; Cai et al., 2018; Zhang et al., 2019), and RA is the main effective constituent of the OE (Cai et al., 2018, 2019). Consequently, RA was also tested in this study. The data showed that there was no difference between the OE and RA supplementation treatments. This implied that RA may also be a potential additive that relieves the effects of heat stress.

5. Conclusion

In conclusion, the OE and RA supplements may increase nutrient digestibility, stimulate the antioxidant defense mechanism system through the *Nrf2*-mediated pathway, decrease intestinal epithelial cell apoptosis, and protect the intestinal barrier. This study indicated that dietary supplementation with OE or RA is beneficial for laying hens under heat-stress conditions. Overall, the supplementation of the laying hens' diet with OE or RA was beneficial under heat-stress conditions.

CRediT authorship contribution statement

Xuan Cai: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - original draft, Writing - review & editing. **Lu Zhang:** Methodology. **Xujie Chen:** Methodology. **Hongzhi Zhang:** Methodology. **Huiqin Xue:** Methodology. **Yang Lu:** Writing - review & editing, Validation. **Jianjun Tang:** Resources. **Yonghong Lu:** Conceptualization, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

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