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Dietary resveratrol supplementation on growth performance, immune function and intestinal barrier function in broilers challenged with lipopolysaccharide

Lei-Zheng Zhang,* Jian-Gang Gong ,† Jia-Hui Li,* Yan-Shuang Hao,* Hong-Jian Xu,* Yan-Ci Liu,‡ and Zhi-Hua Feng*,[1]

*College of Animal Science and Technology, Hebei Agricultural University, Baoding, Hebei 071001, China; †College of Food Science and Technology, Hebei Agricultural University, Baoding, Hebei 071001, China; and ‡Baoding Vocational and Technical College, Baoding, Hebei 071001, China

ABSTRACT This study discusses the effects of resveratrol (**RES**) on the productive performance, immune function and intestinal barrier function of broiler chickens challenged with lipopolysaccharide (**LPS**). Two hundred and forty 1-day-old male Arbor Acres broilers were randomly divided into 4 groups of 6 replicates each, with 10 broilers per replicate. This experiment used a 2 × 2 factorial design with dietary factors (basal diets or basal diets supplemented with 400 mg/kg RES were administered from d 1 to 21) and stress factors (intraperitoneal injection of 0.5 mg/kg BW of saline or LPS at 16, 18 and 20 d of age). The results showed that LPS challenge had a significant adverse effect on average daily gain (**ADG**) in broilers at 16 to 21 d of age ($P < 0.05$), whereas the addition of RES to the diet inhibited the LPS-induced decrease in ADG ($P < 0.05$). RES also alleviated LPS-induced immune function damage in broilers, which was manifested by the decrease of spleen index ($P < 0.05$) and the recovery of serum immunoglobulin M and ileal secretory immunoglobulin A content ($P < 0.05$). The LPS challenge also disrupts intestinal barrier function and inflammation, and RES mitigates these adverse effects in different ways. RES attenuated LPS-induced reduction of villus height in the jejunum and ileum of broilers ($P < 0.05$). LPS also caused an abnormal increase in plasma D-lactic acid levels in broilers ($P < 0.05$), which was effectively mitigated by RES ($P < 0.05$). LPS challenge resulted in a significant decrease in mRNA expression of occludin in the intestinal mucosa ($P < 0.05$), which was mitigated by the addition of RES ($P < 0.05$). RES significantly decreased the mRNA expression of toll-like receptor 4, nuclear factor kappa-B and tumor necrosis factor alpha in the ileum tissue stimulated by LPS ($P < 0.05$). Taken together, this study shows that RES exerts its beneficial effect on broilers challenged with LPS by alleviating immune function damage, relieving intestinal inflammation and barrier damage, and thus improving growth performance.

Key words: resveratrol, broiler, lipopolysaccharide, inflammation, intestine

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INTRODUCTION

With the increased intensification and scale of farming worldwide, the immune function of poultry has been disrupted under high production pressure, which makes them more susceptible to external harmful factors that produce different stress responses represented by immune stress (Li et al., 2015). Immune stress can lead to damage to the intestinal barrier and digestive disorders in poultry, triggering various inflammatory diseases, reducing their production performance and even leading to death, resulting in huge economic losses (Zhang et al., 2020). Traditionally, antibiotics have been used extensively in the livestock industry as an effective means of treating infections and resisting external environmental stresses. However, the long-term use of large amounts of antibiotics has created problems such as resistance and residues that threaten human health, and countries are taking antibiotics more seriously (Wang et al., 2020). Therefore, in the context of restricting the use of antibiotics, the search for natural and efficient alternatives to antibiotics to alleviate animal stress, protect intestinal health and maintain production in a safer way has become the focus of current research.

Today, plant polyphenols are considered a promising feed additive because they have antibiotic-like effects without causing microbial resistance (Yahfoufi et al., 2018; Pérez-Burillo et al., 2021; Erinle et al., 2022). As

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[1]Corresponding author: fzhauh@163.com

the star compound of plant polyphenols, resveratrol (**RES**, 3,4,5-trihydroxystilbene) is a phytocide secreted by plants in response to stress or pathogen attack and is a very versatile exogenous additive that is found in high levels in human foods such as grapes, mulberries and peanuts (Jin et al., 2021). In vitro and animal studies have demonstrated the role of RES in maintaining intestinal barrier function (Borges et al., 2018; Chen et al., 2021; Yang et al., 2022), alleviating inflammation (Chen et al., 2019; Tong et al., 2020), and resisting heat stress (Liu et al., 2014; Zhang et al., 2017; He et al., 2019; Wang et al., 2021; Meng et al., 2023), among others. Furthermore, there has been evidence of the potential of RES to alleviate inflammation, improve hepatic antioxidant capacity and optimize intestinal flora in poultry under biological (Mohebodini et al., 2019) and chemical (Zhu et al., 2023) models of immune stress. Although RES biological functions have been partially demonstrated in models of immune stress in poultry, the variables studied have not systematically focused on the relationship between intestinal barrier function and maintenance of production performance. Therefore, we speculate that RES continues to exert a stable anti-inflammatory and intestinal protective effect in broiler models of immune stress, which helps to maintain normal growth in broilers. In this study, lipopolysaccharide (**LPS**), which is easy to control and has good reproducibility, was used as an immune system activator to induce immune stress in broilers (Takahashi et al., 1997; Xie et al., 2000). By evaluating the effects of RES on growth performance, immune status, and intestinal barrier of broilers under LPS-induced immune stress, it provides reference and basis for the rational utilization of RES in broiler production.

MATERIALS AND METHODS

Ethics Statement

The protocols for the animal study were approved by the Institutional Animal Care and Use Committee of Hebei Agricultural University and carried out under the Guidelines for the Care and Use of Laboratory Animals of China. (University Identification Number: HB/2019/03)

Experiment Design, Diets and Management

A total of 240 one-day-old male Arbor Acres broilers of similar body weight (42.20 g ± 0.30 g) were randomly divided into 4 treatment groups according to a 2 × 2 factorial design, with 6 replicates per group and 10 broilers per replicate. The experimental period was 21 d. The were 4 treatments: 1) CON group (fed basal diet followed by saline injection); 2) RES group (fed basal diet with 400 mg/kg of RES followed by saline injection); 3) LPS group (fed basal diet followed by LPS injection); and 4) L-RES group (fed basal diet with 400 mg/kg of RES followed by LPS injection). The experimental diets (Table 1) were formulated according to the NRC (1994) requirements for broilers, and the dose of RES (purity ≥ 99%, Aladdin, Shanghai, China) in the diets was selected based on our unpublished pre-experimental data combined with previous studies (Liu et al., 2016; Wang et al., 2021). All broilers were fed and watered ad libitum throughout the trial period. The temperature remained at 34°C for the first week, then gradually decreased to 24°C at a rate of 3°C to 4°C `per week, and remained unchanged thereafter. The light was maintained throughout the day during the experimental period.

Table 1. Composition and nutrient levels of the basal diets (air-dry basis).

Ingredients, %		Nutrient level [2]	
Corn	57.80	ME/(Kcal/kg)	3,023.35
Soybean meal	35.16	CP %	21.55
Oil	2.00	Ca %	0.92
Limestone	1.20	Total phosphorus %	0.68
Salt	0.30	Available P %	0.43
CaHPO4	1.68	Lysine %	1.12
L-Lysine	0.36	Methionine %	0.52
DL-Methionine	0.22	Threonine %	0.81
L-Threonine	0.18		
Choline chloride	0.10		
Premix [1]	1.00		
Total	100.00		

[1]Provided per kg of complete diet: vitamin A, 15,000 IU; vitamin D_3, 3,450 IU; vitamin E, 22.50 mg; vitamin K_3, 2.25 mg; vitamin B_1, 2.7 mg; vitamin B_2, 8.40 mg; vitamin B_6, 4.86 mg; vitamin B_{12}, 0.03 mg; niacin, 44.55 mg; folic acid, 1.47 mg; biotin, 0.18 mg; pantothenic acid, 16.56 mg; Fe, 102 mg; Cu 8.5 mg; Mn, 97.34 mg; Zn, 72.76 mg; I, 0.48 mg; Se, 0.30 mg.

[2]Nutrient level: Lysine, Methionine, and Threonine were calculated values, whereas others were analyzed values.

At 16, 18, and 20 d of age, broilers in the LPS and L-RES groups were injected intraperitoneally with 0.5 mL of LPS (0.5 mg/kg BW; *E. coli* O55: B5; Sigma-Aldrich, Shanghai, China), whereas the other 2 groups were injected with the same amount of normal saline. The administration and dose of LPS were chosen according to a previous study (Li et al., 2015). Each intraperitoneal injection was preceded by a 12-h fast to ensure consistent absorption efficiency of LPS in broilers, during which time only adequate water was provided.

Sample Collection

On the morning of d 21, after weighing, 1 broiler from each replicate with near-average body weight was selected and euthanized by cervical dislocation after taking a 10 mL blood sample from a wing vein. The sacrificed experimental chickens were dissected, and the thymus, spleen and bursa of Fabricius were removed and weighed. Blood samples were centrifuged at 3,000 × *g* for 20 min at 4°C, and serum was collected. Rapid fine separation of jejunum and ileum was performed, and 2 cm mid-section samples of jejunum and ileum were placed in 4% paraformaldehyde to carry out intestinal morphology measurements. The ileal mucosa was scraped with a sterile scraper, the sample was collected into 2 mL cryotubes and placed in liquid N_2 for rapid freezing. The serum and frozen mucosa were stored at -80°C for further analysis.

Measurement of Growth Performance

Broilers were weighed on an empty stomach at 1, 15, and 21 d of age in each replicate. The amount of feed remaining for each repetition was recorded daily, and the feed consumption was calculated accordingly. The average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed-to-gain ratio (**F/G**) of broilers in each group during the nonstress period (1−15 d of age) and stress period (16−21 d of age) were counted at the end of the experiment.

Determination of Immune Organ Index and Serum Immunoglobulin Content

The immune organ index is the ratio of the weight of immune organs (g) to the live weight before slaughter (kg) at the end of the experiment (21 d of age).

The levels of the serum immunoglobulins (immunoglobulin G [**IgG**], immunoglobulin M [**IgM**] and immunoglobulin A [**IgA**]) in question were determined by ELISA kits. The kits were purchased from the Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

Secretory Immunoglobulin A Content in Ileal Mucosa

The ileal mucosa (about 200 mg) was weighed and then homogenized in physiological saline. The homogenate was centrifuged at 5,000 × *g* for 15 min in a centrifuge at 4°C, and the supernatant was collected and stored rapidly at -20°C. Secretory immunoglobulin A (**SIgA**) content was analyzed by ELISA kits from the Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

Analyses of Intestinal Morphology and Histology

Jejunum and ileum samples were fixed, dehydrated, embedded in paraffin, cut into 5 μm slices and stained with hematoxylin-eosin (**H&E**). Villus height (**VH**) and crypt depth (**CD**) were measured using a light microscope (Olympus Optical Co., LTD., Beijing, China) in combination with Image J analysis software and the ratio of the 2 (**V/C**) was calculated from the results. Sections were stained with a thickness of 5 μm with periodic acid-Schiff according to the procedure described by Liu et al. (2016). The number of goblet cells in 5 different intestinal villous areas of 1 section of each broiler was counted under Olympus light microscopy (Olympus Optical Co., Beijing, China) at a final magnification of 400×. The number obtained from the counting for statistical analysis was expressed as the cell counts per 100 columnar epithelial cells. The average of 5 structures per chicken was used for statistical analysis of the data.

Determination of Serum Diamine Oxidase Activity and D- lactic Acid Contents

The activity of diamine oxidase (**DAO**) activity and D-lactic acid (**D-LA**) content in serum samples were analyzed by commercial kits obtained from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China).

RNA Extraction and qRT-PCR

Total RNA was extracted from the ileum according to the RNAiso Plus (Takara, Beijing, China) instructions and then reverse-transcribed into cDNA using the Prime Script RT kit (Takara, Beijing, China). All primers (Table 2) are designed by Oligo 7, and synthesized by Personalbio Technology (Shanghai, China) Co., Ltd after verification of primer specificity. Real-Time PCR amplification of cDNA was performed using the SYBR Premix Ex Taq II kit (Takara, Beijing, China). The PCR reaction volume was 20 μL, including 10 μL of SYBR premix Ex Taq (2×), 2 μL of cDNA template, 0.4 μL of forward and reverse primers, 0.4 μL of ROX reference dye (50×), 6.8 μL of RNase-free water. PCR amplification was performed at 95°C for 300 s, followed by 95°C for 5 s, 60°C for 30 s and 72°C for 20 s, for a total of 40 cycles. The relative mRNA expression of each target gene was calculated by the $2^{-\Delta\Delta Ct}$ method using β-actin as an internal reference.

Statistical Analyses

All data were subjected to 2-way ANOVA using the general linear model procedure of SPSS 26.0 statistical software (version 22.0 for Windows, SPSS Inc., Chicago, IL), with model main effects including LPS, RES, and the reciprocal effects of both. When the reciprocal effects were significant, multiple comparisons were performed using Duncan's method. Experimental results are expressed as mean ± standard error of the mean, and statistical differences between groups are indicated at $P < 0.05$.

RESULTS

Growth Performance

As presented in Table 3, before the LPS challenge, broilers exhibited similar growth performance (ADG, ADFI, and F/G) among groups ($P > 0.05$). During the LPS challenge period, the LPS challenge adversely affected the ADG, ADFI, and F/G of broilers compared to nonchallenged broilers ($P < 0.05$). RES supplementation significantly increased ADG in broilers compared to diets without RES supplementation ($P = 0.002$). There was a significant interaction between LPS and RES on ADG ($P = 0.046$). RES supplementation could significantly alleviate the decrease of ADG induced by LPS ($P < 0.05$), whereas RES had no significant effect on ADG of broilers injected with saline ($P > 0.05$).

Table 2. Gene primers sequence.

Genes[1]	Primer sequence (5′−3′) [2]	Product size/bp	GenBank accession no.
β-actin	F: GTCCACCGCAAATGCTTCTA R: GTCCACCGCAAATGCTTCTA	104	NM_205518.2
ZO-1	F: ATGTTGAGCACGCATTTG R: TTTGGCATTCTTCCCACT	57	XM_040706827.1
Occludin	F: GCTCTGCCTCATCTGCTTC R: CCACGTTCTTCACCCACTC	134	NM_205128.1
Claudin-1	F: CTTCAGCACCTTCCTTTCC R: CGCTACATCTTCTGTTGGC	110	NM_001013611.2
MUC2	F: CTGCTGTGCTCCACCATTAAGTCC R: GCTTGACACGCTCGGAGTATAACG	127	XM_001234581.3
TLR4	F: CCAGATGAGTTTCCTGTCG R: ATCCTGCTTTTCCTTGACC	136	KU235329.1
MyD88	F: GGAGGATGGTGGTCGTCATT R: GGTCTTGCACTTGACCGGAA	128	NM_001030962.5
NF-κB	F: TCAACGCAGGACCTAAAGACAT R: GCAGATAGCCAAGTTCAGGATG	162	NM_001396396.1
IL-1β	F: GCCATGACCAAACTGCTG R: AAGGTGACGGGCTCAAAA	95	NM_204524.2
IL-6	F: AATCCCTCCTCGCCAATCTG R: CAAATAGCGAACGGCCCTCA	118	NM_204628.2
IL-10	F: TGCGAGAAGAGGAGCAAAGC R: AACTCCCCCATGGCTTTGTAG	89	XM_015885136.2
TNF-α	F: GCCCTTCCTGTAACCAGATG R: ACACGACAGCCAAGTCAAC	71	NM_204267.2

[1]ZO-1 = zonula occludens 1; MUC2 = mucin 2; TLR4 = Toll-like receptor 4; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor kappa-B; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; IL-10 = interleukin 10; TNF-α = tumor necrosis factor alpha.
[2]F = forward primer; R = reverse primer.

Table 3. Effects of dietary resveratrol supplementation on growth performance of lipopolysaccharide-challenged broiler chickens.

	Treatments					*P*-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
Before LPS challenge (1−15 d of age)								
ADG/g	24.11	24.38	23.81	24.21	0.53		0.519	
ADFI/g	30.70	30.87	30.16	30.55	0.36		0.448	
F/G	1.28	1.27	1.27	1.26	0.02		0.689	
During LPS challenge (16−21 d of age)								
ADG/g	26.46[a]	27.05[a]	20.84[c]	23.21[b]	0.42	<0.001	0.002	0.046
ADFI/g	44.14	44.80	37.49	39.20	0.58	<0.001	0.054	0.375
F/G	1.67	1.66	1.80	1.72	0.03	0.023	0.079	0.164

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed-to-gain ratio.
[a,b,c]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Table 4. Effects of dietary resveratrol supplementation on immune organ index of lipopolysaccharide-challenged broiler chickens.

	Treatments					*P*-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
Thymus index	1.75	1.84	1.90	2.04	0.10	0.105	0.263	0.825
Spleen index	0.81[b]	0.93[b]	1.29[a]	0.98[b]	0.07	0.002	0.175	0.006
Bursa of Fabricius index	2.45	2.60	2.83	2.74	0.16	0.119	0.850	0.433

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection.
[a,b]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Immune Organ Index

As shown in Table 4, compared with the unchallenged group, LPS significantly improved the spleen index of challenged broilers ($P = 0.002$), but had no significant effect on broiler thymus index and bursa of Fabricius index ($P > 0.05$). In contrast, the RES intervention alone had no significant effect on the immune organ index of the broilers ($P > 0.05$). There was a significant interaction between LPS and RES on spleen index of broilers ($P = 0.006$). RES supplementation in the diet could significantly alleviate the increase of spleen index

Table 5. Effects of dietary resveratrol supplementation on serum immunoglobulin and ileal mucosa SIgA content of lipopolysaccharide-challenged broiler chickens.

	Treatments					*P*-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
IgA (g/L)	2.13	2.22	2.34	2.25	0.10	0.267	0.987	0.389
IgG (g/L)	4.23	4.48	4.65	4.70	0.15	0.046	0.329	0.522
IgM (g/L)	1.38[c]	1.53[b,c]	1.75[a]	1.58[b]	0.05	0.001	0.771	0.004
SIgA/(μmol/L)	14.96[c]	16.83[b,c]	20.05[a]	17.88[b]	0.64	<0.001	0.826	0.005

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; SIgA = secretory immunoglobulin A.

[a,b,c]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Table 6. Effect of dietary resveratrol supplementation on intestinal morphology and goblet cells count of lipopolysaccharide-challenged broiler chickens.

	Treatments					*P*-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
Jejunum								
VH/μm	975.50[a]	1023.77[a]	753.88[c]	877.31[b]	17.58	<0.001	0.001	0.045
CD/μm	137.59	130.11	150.31	145.75	7.15	0.061	0.410	0.841
V/C	7.26	8.02	5.08	6.09	0.45	<0.001	0.066	0.782
Goblet cell	32.75	37.59	21.33	27.50	1.51	<0.001	0.002	0.668
Ileum								
VH/μm	835.63[a]	863.81[a]	634.54[c]	730.70[b]	16.07	0.001	0.001	0.047
CD/μm	129.56	122.47	138.65	133.57	6.43	0.132	0.356	0.877
V/C	6.53	7.14	4.62	5.54	0.32	<0.001	0.027	0.631
Goblet cell	28.55	34.35	23.40	26.55	1.64	<0.001	0.013	0.428

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; VH= villus height; CD= crypt depth; V/C = villus height/crypt depth.

[a,b,c]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

caused by LPS ($P < 0.05$), whereas RES supplementation had no significant effect on spleen index of broilers injected with saline ($P > 0.05$).

Serum Immunoglobulin and Ileal Mucosa SIgA Content

As shown in Table 5, the LPS challenge resulted in a marked increase of serum IgG, IgM and ileal mucosal SIgA levels in broilers ($P < 0.05$). Dietary addition of RES had no significant effect on serum immunoglobulin and ileal SIgA levels ($P > 0.05$). There was a significant interaction between LPS and RES on serum IgM level ($P = 0.004$) and ileal mucosal SIgA level ($P = 0.005$) in broilers. RES supplementation significantly alleviated the increase of serum IgM and ileal mucosal SIgA levels caused by LPS challenge ($P < 0.05$), whereas RES had no significant effect on serum IgM and ileal mucosal SIgA levels of broilers injected with saline ($P > 0.05$).

Intestinal Morphology and Goblet Cells Count

As shown in Table 6, the LPS challenge significantly reduced the VH, V/C and the number of goblet cells in the jejunum and ileum of broiler chickens ($P < 0.05$). Dietary RES significantly increased the jejunum and ileum VH and the number of goblet cells ($P < 0.05$) and decreased ileal V/C ($P = 0.027$). There was a significant interaction between LPS and RES on the VH of jejunum ($P = 0.045$) and ileum ($P = 0.047$) of broilers. RES could improve the reduction of VH of jejunum and ileum under LPS challenge ($P < 0.05$), whereas RES had no significant effect on the VH of broilers injected with normal saline ($P > 0.05$).

Serum DAO Activity and D-LA Content

As shown in Table 7, serum DAO activity ($P < 0.05$) and D-LA concentrations ($P < 0.05$) were significantly higher in broilers challenged with LPS compared to the nonchallenged group. Conversely, RES supplementation significantly reduced serum DAO activity ($P = 0.016$) and D-LA concentrations ($P = 0.002$) in broilers. A significant interaction between LPS and RES on serum D-LA levels was observed ($P = 0.038$). RES alleviated the abnormal increase of serum D-LA level caused by LPS challenge ($P < 0.05$), whereas RES had no significant effect on serum D-LA level in broilers injected with normal saline ($P > 0.05$).

Table 7. Effect of dietary resveratrol supplementation on serum diamine oxidase activity and D-lactic acid content of lipopolysaccharide-challenged broiler chickens.

						P-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
DAO/(U/mL)	0.28	0.26	0.36	0.32	0.01	<0.001	0.016	0.519
D-LA/(μmol/L)	33.93[c]	32.57[c]	42.96[a]	37.38[b]	0.95	<0.001	0.002	0.038

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; DAO = diamine oxidase; D-LA = D-lactic acid.

[a,b,c]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Table 8. Effect of dietary resveratrol supplementation on ileal tight junction protein and *MUC2* mRNA expression in lipopolysaccharide-challenged broiler chickens.

						P-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
ZO-1	1.00	1.34	0.61	0.91	0.06	<0.001	0.001	0.732
Occludin	1.00[b]	1.36[a]	0.70[d]	0.85[c]	0.05	<0.001	0.001	0.029
Claudin-1	1.00	1.15	0.73	0.86	0.05	<0.001	0.017	0.872
MUC2	1.00	1.25	0.69	0.80	0.05	<0.001	0.002	0.197

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; ZO-1 = zonula occludens 1; MUC2 = mucin 2.

[a,b,c,d]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Table 9. Effect of dietary resveratrol supplementation on mRNA expression of genes related to the *TLR4/NF-κB* signaling pathway in the ileum of lipopolysaccharide-challenged broiler chickens.

						P-values		
Items[1]	CON	RES	LPS	L-RES	SEM	LPS	RES	Interaction
TLR4	1.00[c]	0.90[c]	1.81[a]	1.55[b]	0.04	<0.001	0.001	0.042
MyD88	1.00	0.92	1.48	1.34	0.07	<0.001	0.121	0.690
NF-κB	1.00[c]	0.93[c]	1.65[a]	1.31[b]	0.07	<0.001	0.003	0.045
IL-1β	1.00	0.87	2.28	1.66	0.15	<0.001	0.018	0.116
IL-6	1.00	0.84	1.78	1.50	0.07	<0.001	0.003	0.400
IL-10	1.00	1.52	0.91	1.34	0.07	<0.001	0.059	0.534
TNF-α	1.00[c]	0.90[c]	2.39[a]	1.81[b]	0.11	<0.001	0.005	0.040

[1]CON = fed basal diet followed by saline injection; RES = fed basal diet with 400 mg/kg of resveratrol followed by saline injection; LPS = fed basal diet followed by lipopolysaccharide injection; L-RES = fed basal diet with 400 mg/kg of resveratrol followed by lipopolysaccharide injection; TLR4 = toll-like receptor 4; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor kappa-B; IL-1β= interleukin 1 beta; IL-6 = interleukin 6; IL-10 = interleukin 10; TNF-α = tumor necrosis factor alpha.

[a,b,c]In the same row, different lowercase letters represent significant differences ($P < 0.05$). Values are the mean and standard error of the mean, with the mean being the result of 6 replications of each treatment (n = 6).

Ileal Tight Junction Protein and Mucin 2 Gene mRNA Expression

As shown in Table 8, LPS-challenged chickens had significantly lower mRNA levels of tight junction protein genes and mucin 2 (***MUC2***) in the ileum than nonchallenged broilers ($P < 0.05$). However, the RES supplementation significantly increased zonula occludens 1 (***ZO-1***), occludin, claudin-1 and *MUC2* mRNA levels ($P < 0.05$). A significant interaction was observed between LPS and RES on occludin mRNA levels in ileum mucosa of broilers ($P = 0.029$). RES supplementation significantly increased occludin mRNA level in the ileum mucosa of broilers challenged with LPS ($P < 0.05$), and RES significantly increased occludin mRNA level in the ileum mucosa of broilers injected with saline ($P < 0.05$).

Ileal TLR4/NF-κB Signaling Pathway Related Gene mRNA Expression

As shown in Table 9, the LPS challenge significantly increased the mRNA levels of Toll-like receptor 4 (***TLR4***), myeloid differentiation factor 88 (***MyD88***), nuclear factor kappa-B (***NF-κB***), interleukin 1 beta (***IL-1β***), interleukin 6 (***IL-6***), and tumor necrosis factor alpha (***TNF-α***) mRNA levels in the broiler ileum tissue ($P < 0.05$) and interleukin 10 (***IL-10***) mRNA levels were significantly lower ($P < 0.05$). In contrast, RES supplementation significantly decreased the mRNA abundance of the above genes ($P < 0.05$), except for *MyD88* and *IL-10* ($P > 0.05$). Significant interactions were observed between LPS and RES on *TLR4* ($P = 0.042$), *NF-κB* ($P = 0.045$) and *TNF-α* ($P = 0.040$)

mRNA expression. RES could alleviate the abnormal increase of *TLR4*, *NF-κB,* and *TNF-α* mRNA expression in the ileum mucosa of broilers challenged with LPS ($P < 0.05$), but RES had no significant effect on the abundance of *TLR4*, *NF-κB,* and *TNF-α* mRNA in the ileum mucosa of broilers injected with normal saline ($P > 0.05$).

DISCUSSION

It has been shown that broilers' reduced growth rates and limited production potential are typical outward signs caused by LPS damage (Li et al., 2015; Chen et al., 2018). In the present study, LPS stimulation significantly reduced ADG and ADFI of broilers during the stress period. It increased F/G, which again confirmed the harmful effects of LPS on the growth performance of broilers, indicating the success of the immune stress model of broilers constructed by LPS injection in this experiment. The decrease in productivity is mainly due to the diversion of nutrients and energy needed to maintain growth to processes associated with the inflammatory immune response (Zhang et al., 2022). Among other explanations, the overproduction of inflammatory cytokines under immune stress reduces 5-hydroxytryptamine levels in the cerebral cortex and hippocampus through feedback messages, which in turn leads to behavioral changes, such as decreased appetite and depression, resulting in reduced food intake and contributing to decreased productive performance (Lu et al., 2021). We also found that RES could reverse the ADG decline of broilers during the immune stress period (16−21 d of age), implying that RES effectively alleviated the decline in performance of broilers during the early stage of LPS challenge. In a similar study, Hong et al. (2022) reported that RES reverses the decline in piglet growth performance caused by deoxynivalenol by alleviating mitochondrial damage and maintaining intestinal morphology. Another study showed that dietary supplementation of 400 mg/kg RES could protect the intestinal morphology of yellow-feathered broilers, reduce intestinal inflammation and intestinal mucosal damage, and alleviate LPS-induced slowing of weight gain and weight loss (He et al., 2022). In addition, RES has been interpreted as a potent phytoestrogen that acts to improve nutrient digestion and absorption by upregulating epidermal growth factor and its receptors, activating phosphorylated extracellular regulatory kinase signaling cascades, promoting proliferation and differentiation of gastrointestinal epithelial cells, and repairing damaged mucosa (Serrero and Lu, 2001; Penner et al., 2011). In other words, the positive effect on growth performance shown in this study can be explained by the fact that RES uses the intestine as a target organ to counteract the impairment of the digestive and absorptive capacity of broilers by LPS by maintaining its barrier function.

T and B cells are derived from bone marrow pluripotent stem cells, which also need to differentiate and mature in the thymus, spleen and bursa of Fabricius before being transported to peripheral immune organs or the body as a whole to function as cellular and humoral immunity (Zhang et al., 2021). Therefore, the relative developmental status of immune organs is relevant for determining the strength of immune function in poultry. In the present study, the LPS challenge caused a significant increase in relative spleen weight in broilers, similar to the findings of Li et al. (2018a). LPS-induced compensatory splenomegaly may be associated with a high production of inflammatory factors due to the overactivation of the splenic immune system (Gao et al., 2022). Differently, LPS stimulation did not significantly affect the bursal index in broiler chickens in this experiment, which differs from the results of Wang et al. (2016), which may be related to the species of animal tested, the frequency of LPS injections and the timing of sampling. In this experiment, the addition of 400 mg/kg RES significantly alleviated the elevated spleen index in broilers caused by LPS, suggesting that RES may positively affect maintaining a routine and moderate immune system response under conditions of immune stress. RES enhances the immune function of lymphocytes by regulating the expression and secretion of lymphocyte cytokines (*IL-3, IL-4, IL-12, IFN-γ*) and is considered an essential way of correcting the abnormal development of immune organs (Schwager et al., 2021). In addition, RES activates the cytotoxic activity of cytotoxic T cells and NK cells to kill the target cells of specific reactions and reduce the damage to internal organs by adverse factors (Falchetti et al., 2001). Anyhow, RES attenuated the compensatory hyperplasia of the broiler spleen under immune stress, which may facilitate the efficiency of the immune system to clear inflammatory necrotic intestinal epithelial cells and maintain the integrity of the physical barrier of the intestinal mucosa.

To further understand the mitigating effect of RES on inflammatory injury in immune-stressed broilers, we selected immunoglobulins as target bioactive molecules. Undoubtedly, in addition to the immune organ index, the quantity and activity of immunoglobulins are also critical indicators to evaluate the immune function status of birds. As a key active molecule in the body's immune system, immunoglobulins recognize and clear specific antigens and reflect the humoral immune status of animals (Yuan et al., 2023). In contrast, SIgA is secreted by plasma mother cells and is a major effector in the intestinal immune response system (Li et al., 2022). This is the same as previously reported by Cui et al. (2021), where we found that IgG, IgM, and SIgA content in broiler serum and ileum could be increased in LPS. This indicates that after LPS stimulation induces an immune stress response, the body's immune response is activated, causing plasma cells to rapidly secrete large amounts of IgM and IgG, and mucosal epithelial cells to secrete SIgA for defense against undesirable attacks (Ramadan et al., 2020). Polyphenolic compounds from medicinal plants have been reported to have immunoprotective properties and immunomodulatory potential in poultry organisms (Darmawan et al., 2022). As an

active polyphenol, RES can benefit the immune response by binding IgG through an additional ligand to the Fc receptor (Nimmerjahn and Ravetch, 2010). A study by Fu et al. (2018) found that RES modulates the humoral immune response of piglets by improving lymphocyte differentiation in piglets' immune organs, increasing *INF-γ* levels, and decreasing *TNF-α* levels. Likewise, our data showed that RES supplementation alleviates the elevated serum IgM levels in broilers under immune stress and normalizes SIgA levels in the ileum. And the positive changes in broiler immunoglobulins were consistent with the trend in spleen indices, suggesting that RES ameliorated the LPS-induced impairment of intestinal barrier function at the immunological level.

The small intestine is the leading site of digestion and absorption in animals, and changes in its shape and structure can, to some extent, reflect the body's ability to digest and absorb nutrients (Li et al., 2015; Dalia et al., 2020). In this study, LPS disrupted the structural integrity of the broiler intestine, as evidenced by a significant decrease in VH and V/C, which is in convergence with the findings of Feng et al. (2023). The lower VH led to a reduction in the surface area of food contact with the villi, which directly led to a reduction in the absorption capacity and growth rate of the broiler organism. In addition, plant-based polyphenols, represented by RES, have been found to optimize intestinal morphology and improve intestinal function. For example, the addition of RES to heat-stressed broiler diets can increase jejunal and ileal VH and V/C and reduce crypt depth (Liu et al., 2016; Zhang et al., 2017; Wang et al., 2021). And this improved effect on intestinal morphology is also present in mice and piglets (Meng et al., 2019; Zhuang et al., 2021). In addition, grape seed and grape skin-derived polyphenols have also been shown to have significant positive effects on VH and V/C in pigs (Gessner et al., 2013). Among the data that could be collected, the mechanism of action of RES on intestinal morphology involves many immune responses, and signaling pathways, of which regulate intestinal flora balance, increasing the expression of proteins associated with tight junctions between intestinal epithelial cells, reducing the production of inflammatory factors and cascade inhibition of the *TLR4/NF-κBp65/MAPKs* pathway are some of the mainstream views (Cheng et al., 2014; Na et al., 2016; Fu et al., 2018). Our observations suggest that intestinal digestion and absorption are improved due to RES intake and indicate that RES promotes nutrient absorption through positive intestinal histological changes, thus improving growth performance.

The structure and function of intestinal mucosa constitute a mucosal solid immune system, which makes it difficult for foreign bacteria and viruses to break through this defense line and cause harm to organisms. There is substantial evidence that intraperitoneal injection of LPS leads to increased intestine permeability, disrupting its barrier function (Zhang et al., 2020; Yu et al., 2022). The DAO and D-LA are blood indicators for assessing intestinal mucosal damage (Fukudome et al., 2014). DAO is widespread in the mucosal villi of the mammalian small intestine. Its activity is closely related to nucleic acid and protein synthesis in mucosal cells, reflecting the integrity and degree of damage to the mechanical barrier of the intestine (Liu et al., 2020). D-LA is a metabolite of *Escherichia coli, Lactobacillus,* and other bacteria under ischemic and hypoxic conditions. Animals do not have the enzyme system to metabolize D-LA rapidly, so it can be used to indicate damage to the intestinal mucosa, permeability changes and bacterial translocation (Chen et al., 2017). In this study, DAO activity and D-LA levels in broiler serum were significantly increased after LPS injection, which corresponded to mucosal damage in the small intestine. However, RES reversed the abnormal increase in D-LA concentrations in broilers challenged by LPS, which was consistent with an increase in jejunal and ileal VH and a trend toward improved mRNA abundance of mucosal occludin. It indicates that RES as a protective agent could attenuate intestinal mucosal damage and protect intestinal morphological structures in broiler chickens under immune stress environment.

Tight junctions, mucins and goblet cells constitute critical components of intestinal epithelial barrier integrity (Gallo and Hooper, 2012; Lu et al., 2014; Nakamura et al., 2018). Among them, tight junctions are an essential form of intercellular junctions, mainly composed of structural proteins such as claudin and occludin and various types of connexin molecules, which are the essential structures of the mucosal mechanical barrier (Zeisel et al., 2019). The mucus layer is composed of core proteins such as *MUC2* and other secretory substances secreted by cupped cells, and impaired protection of the mucus layer is an essential cause of infections, many chronic inflammatory diseases and infestation by intestinal bacteria (Kim and Ho, 2010; Liu et al., 2016). Growing evidence showed that LPS interferes with the expression level of intestinal tight junctions (Lee et al., 2017). Again, the present study supports the adverse effect of LPS on the abundance of tight junction gene mRNA in broiler ileum as a potential mechanism for the elevated levels of DAO and D-LA. It also showed that the mRNA abundance of the *MUC2* was significantly reduced in the broiler ileum due to the LPS challenge, which is consistent with the reduction mentioned above in the number of goblet cells in the jejunum and ileum due to LPS. This finding is similar to the results of Li et al. (2018b). In contrast, RES significantly enhanced the number of cupped cells and the mRNA abundance of tight junction proteins and *MUC2* (Etxeberria et al., 2015; Zhang et al., 2017). One experiment found that cyclophosphamide-induced immunosuppressed mice treated with RES showed increased mRNA and protein levels of tight junction proteins (*ZO-1*, claudin-1 and occludin) involved in intestinal physical barrier function (Song et al., 2022). Similarly, RES in this assay demonstrated a positive effect on the tight junction system in intestinal epithelial cells, which effectively corroborates these results. Combined with the above results, RES significantly reversed the morphological damage of the

ileum and jejunum induced by immune stress and correspondingly reduced serum D-LA content. We speculate that RES can reduce intestinal mucosal damage by restoring LPS-stimulated tight junction protein levels in broilers, thereby keeping the physical barrier of the intestine intact.

Mechanistically, damage to the intestinal physical barrier leads to increased intestinal permeability to bacterial toxins and undesirable factors, which induces various inflammatory diseases (Zhang et al., 2020). *TLR4*, an important member of the Toll-like receptors (**TLRs**) family, acts as a pattern recognition receptor for LPS and activates the *NF-κB* signaling pathway through a series of cell membrane and intracellular cascade reactions in response to LPS stimulation, regulating the expression of genes related to the inflammatory immune response and inducing an inflammatory response, which in turn induces immune stress in animals (Zhu et al., 2018; Kayisoglu et al., 2021). In this study, intraperitoneal injection of LPS mediated the development of intestinal inflammation in broiler chickens, as evidenced by altered mRNA abundance of 7 representative members of the *TLR4/NF-κB* pathway family in the ileal mucosa, whereas down-regulation of the expression of tight junction-related genes was also a valid corroboration of the development of intestinal inflammation. And RES showed excellent anti-inflammatory potential in model assays of inflammation induced by different stressors (Meng et al., 2023). A study showed that RES supplementation reversed the elevated intestinal *TNF-α* and *IL-1β* mRNA abundance in piglets caused by deoxynivalenol, indicating that RES can alleviate gut inflammation in piglets caused by deoxynivalenol (Qiu et al., 2021). Similarly, in a cyclophosphamide-induced immunosuppression model in mice, RES intervention reduced protein levels of *TLR4* and *NF-κB* and decreased inflammation-associated cytokine gene expression (Song et al., 2022). A study by Tong et al. (2020) on cells found that RES alleviated LPS-induced inflammation by inhibiting the *TLR4-NF-κB/MAPKs/IRF3* signaling cascade. In our study, 400 mg/kg RES can reduce the expression levels of *TLR4, My D88, NF-κB, IL-1β, IL-6,* and *TNF-α*, and increase the expression of *IL-10*, thus inhibiting intestinal inflammation. Combined with the previously mentioned results, RES significantly alleviated the LPS-induced reduction in ileal tight junction protein expression and elevated serum D-LA levels. This suggests that RES maintains the integrity of the intestinal mucosal barrier by inhibiting the expression of *TLR4/NF-κB* pathway and inflammatory factors. Finally, LPS-induced nutrient absorption disorders and growth performance of broilers were improved.

In conclusion, this study reports that in an LPS-challenged broiler model of immune stress, RES improved immune function in broilers and alleviated intestinal barrier damage and inflammation, which is vital to maintaining broiler production performance. This effect may have important implications for the use of RES in poultry production, and for the development of promising immunomodulators.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

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