Effects of dietary pyrroloquinoline quinone disodium supplementation on inflammatory responses, oxidative stress, and intestinal morphology in broiler chickens challenged with lipopolysaccharide

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ABSTRACT This study was conducted to investigate the effects of pyrroloquinoline quinone disodium (**PQQ·Na$_2$**) on inflammatory responses, oxidative stress, and intestinal morphology of broiler chickens challenged with lipopolysaccharide (**LPS**). A 2 × 2 factorial arrangement in a complete randomized design experiment was used to study the effect of dietary PQQ·Na$_2$ (0 or 1 mg/kg) on broiler chickens with or without a challenge with LPS. A total of two hundred eighty-eight 1-day-old Arbor Acre broiler chickens were randomly assigned to 4 treatments with 6 replicate cages of 12 birds per cage. All experimental broilers were injected intraperitoneally with 0.5 mg/kg body weight of either *Escherichia coli* LPS or sterile saline at 16, 18, and 20 d of age. Results showed that injecting LPS significantly increased the concentrations of interleukin-1beta (**IL-1β**) in serum of birds on day 20 and day 21. Meanwhile, LPS injection increased ($P < 0.05$) the relative mRNA expression of interleukin-6 (**IL-6**) in the duodenal mucosa of broilers on day 21. However, dietary supplementation with PQQ·Na$_2$ decreased ($P < 0.05$) the concentration of IL-6 in serum of birds on day 20 and the levels of IL-1β, IL-6, and interleukin-10 (**IL-10**) in serum of broiler chickens on day 21. Besides, supplementation of PQQ·Na$_2$ within diet decreased ($P < 0.05$) the mRNA expressions of IL-1β and IL-10 in the duodenal mucosa of birds on day 20. Relative to saline injection, the activity of glutathione peroxidase (**GSH-Px**) in serum and the activities of total superoxide dismutase (**T-SOD**) and catalase (**CAT**) in liver were found to be lower ($P < 0.05$) in broilers after LPS challenge on day 21. However, birds fed with PQQ·Na$_2$ showed higher ($P < 0.05$) GSH-Px activity in serum and higher ($P < 0.05$) T-SOD activities in liver on day 21 and day 42. Pyrroloquinoline quinone disodium also significantly attenuated the LPS-induced decreases in villus height to crypt depth ratio in the duodenum of broilers. In conclusion, dietary PQQ·Na$_2$ supplementation significantly exerted protective effects on inflammation damage and oxidant stress of broilers under LPS challenge by regulating the expression of inflammatory cytokines (IL-1β, IL-6, and IL-10) and activities of antioxidant enzymes (GSH-Px, T-SOD, and CAT). Moreover, dietary PQQ·Na$_2$ supplementation significantly ameliorated the LPS-impaired intestinal morphology in broilers. Therefore, it has been considered that PQQ·Na$_2$ can be used as a potential feed additive in broiler production.

Key words: pyrroloquinoline quinone disodium, broiler, inflammatory responses, oxidative stress, intestinal morphology

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INTRODUCTION

A variety of factors such as infection, pathogenic microorganisms, and environmental pollution can result in immunological stress and oxidative stress in poultry (Yang et al., 2011; Li et al., 2015). Even in normal condition, birds are ineluctably confronted with the immunological challenges or oxidative stress related to bacteria or its products like lipopolysaccharide (**LPS**) that could threaten poultry health status (Coble et al., 2011). Lipopolysaccharide, a membrane glycolipid produced by gram-negative bacteria, is an endotoxin that can cause an acute systemic inflammatory responses and an imbalance between the oxidation and antioxidant defense systems (Roura et al., 1992; Hagir et al.,

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2004; Takahashi et al., 2008). Immunological stress reaction induced by LPS can affect the physiological and biochemical processes of animals and interfere with their normal metabolism and functions in gut (Klasing, 1998; Huff et al., 2008; Shini et al., 2008). Therefore, injection of LPS in chickens has been used as a good model to study systemic inflammation and oxidative stress in poultry industry (Leshchinsky and Klasing, 2001; De Boever et al., 2009). Previous studies showed that LPS-induced negative responsiveness in broilers can be modulated by dietary supplementation of feed additive, such as vitamins, synthetic antioxidants, natural plant extracts, and so on (Fylaktakidou et al., 2004; Shen et al., 2010; Jang et al., 2014). Thus, it is of great interest to identify a novel and effective feed additive that could modulate the immune and antioxidant system to protect chicks from immunological stress.

Pyrroloquinoline quinone (**PQQ**) is a water-soluble, anionic, and quinonoid substance that has been shown to be a vitamin-like redox cofactor and an essential nutrient in animals (Killgore et al., 1989; Steinberg et al., 1994; Stites et al., 2000; Kasahara and Kato, 2003; Zhang et al., 2006). Because of its versatile functions, PQQ disodium salt (**PQQ·Na$_2$**; the most widely used PQQ commercial product) has been authorized as a natural health product in Canada and a novel type of food and dietary supplement in the European commission (Health Canada, 2012; EFSA NDA Panel et al., 2017). Pyrroloquinoline quinone has attracted considerable attention, as it is an effective antioxidant which can catalyze the conversion of superoxide to dioxygen and scavenge-free radicals (Akagawa et al., 2015; Hwang and Willoughby, 2018). Pyrroloquinoline quinone disodium has been found to help maintain redox status in fast-growing broilers (Samuel et al., 2015; Wang et al., 2015). Pyrroloquinoline quinone also has been increasingly studied with its role in mitigating inflammation in recent years. For example, PQQ in human breast milk could serve as an intriguing dietary therapeutic for ameliorating inflammation caused by exposure to an overabundance of toxic lipids during development (Mitchell et al., 1999). With respect to dietary regulation, PQQ can inhibit LPS-induced inflammatory responses through downregulating the NF-kB and p38/JNK activation in microglial cells of mice (Yang et al., 2014). In addition, previous study showed that dietary supplementation of PQQ·Na$_2$ has the ability to protect gut health of the piglets (Yin et al., 2019). Therefore, it is plausible that supplemental PQQ·Na$_2$ could provide a protective effect when chickens are challenged with LPS, but the literature is nonexistent.

To the best of our knowledge, the usefulness of PQQ·Na$_2$ has not yet been fully demonstrated in poultry industry. The objective of this study was to investigate the effects of dietary PQQ·Na$_2$ on the inflammatory responses, oxidative stress, and intestinal morphology in broilers subjected to acute LPS challenge. The results of this study could lay a foundation for the further application of PQQ·Na$_2$ in the poultry industry.

MATERIALS AND METHODS

Materials

The PQQ·Na$_2$ used in this study was provided by Shanghai Medical Life Sciences Research Center Co. Ltd. (Shanghai, China). *Escherichia coli* 055:B5 LPS (L2880) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO).

Feeding Experiment Design and Bird Management

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of China Agricultural University. The experiment was designed as a 2 × 2 factorial arrangement with dietary PQQ·Na$_2$ supplementation (0 or 1 mg/kg PQQ·Na$_2$) and LPS challenges (injection with LPS or sterile saline). A total of two hundred eighty-eight 1-day-old commercial Arbor Acres male broilers were randomly distributed into 4 treatment groups containing 6 replicate cages of 12 birds/cage. At 16, 18 and 20 d of age, birds were injected intraperitoneally with either 0.5 mg/kg body weight (**BW**) of *E. coli* LPS or sterile saline. Birds were housed in an environmentally controlled room maintained at 35°C from 1 to 7 d, which was then gradually reduced to 24°C at the rate of 2°C per week and then kept constantly. Continuous light was provided for the entire period of experiment. Feed and fresh water were available ad libitum. The compositions of basal diets and nutrients level are presented in Table 1. The basal diet was formulated to meet or exceed the Chinese Feeding Standard of Chickens (Ministry of Agriculture of People's Republic of China, 2004) of broilers.

Growth Performance

The BW and feed intake for each pen were recorded on day 1, 21, and 42 to determine the average daily gain, average daily feed intake (**ADFI**), and ratio of feed to gain, and these parameters were corrected for mortality.

Sample Collection

On day 20, at 2 h after injecting LPS or saline, blood samples were collected from the anterior vena cava into test tubes, and serum was separated by centrifugation at 3,000 × *g* for 20 min at 4°C. Then, the serum samples were frozen at −20°C until analysis.

On day 21 and day 42, 2 birds from each replicate, close to the average BW, were selected for samples collection. Serum samples were collected and stored in the same way as described previously. Birds were then sacrificed via exsanguination of the left jugular artery for the collection of tissue samples. Two-centimeter segments from the median sections of the duodenum were collected and preserved in 10% neutral buffered formalin for further morphological measurements. The rest

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

Ingredient	Starter diet (%, from day 1 to day 21)	Grower diet (%, from day 22 to day 42)
Corn	57.76	60.77
Soybean meal	35.5	31.9
Calcium hydrogen phosphate	1.8	1.7
Limestone	1.3	1.1
Salt	0.3	0.3
DL-methionine	0.21	0.1
Poultry vit mix[1]	0.3	0.3
Poultry mineral mix[2]	0.03	0.03
Choline chloride	0.1	0.1
Corn oil	2.7	3.7
Total	100.00	100.00
Nutrient level		
Crude protein %	21.51	20.00
Metabolizable energy MJ/kg	3.00	3.10
Calcium %	1.00	0.90
Total phosphorus %	0.68	0.65
Nonphytate phosphorus %	0.44	0.42
Methionine %	0.54	0.41
Cystine %	0.90	0.76
Lysine %	1.16	1.06
Tryptophan %	0.26	0.24
Threonine %	0.80	0.75
Arginine %	1.39	1.29
Histidine %	0.54	0.50
Isoleucine %	0.86	0.79
Leucine %	1.75	1.65
Phenylalanine %	1.03	0.96
Phenylalanine tyrosine %	1.75	1.64
Valine %	0.99	0.92
Glycyl-DL-serine %	1.80	1.68
Na %	0.14	0.14
Chlorine %	0.22	0.22

[1]Vitamin mix provided the following (per kg of diet): vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E, 20 mg; vitamin K_3, 3 mg; vitamin B_2, 2.5 mg; vitamin B_6, 0.4 mg; vitamin B_{12}, 0.015 mg; vitamin B_5, 8 mg; nicotinic acid, 25 mg; folic acid, 1.2 mg; choline chloride 450 mg.

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

portion of the small intestine was opened longitudinally, and the mucosa from the middle portion of the duodenum was gently scraped off the underlying muscular tissue by using a glass slide and stored at deactivation centrifuges tube, quickly frozen in liquid nitrogen, and stored at −80°C for analysis.

Determination of Inflammatory Cytokine Content in Serum

The concentrations of interleukin-1beta (**IL-1β**), interleukin-6 (**IL-6**), and interleukin-10 (**IL-10**) in serum were determined with commercially available chicken cytokine ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocol.

Real-Time Quantitative PCR Analysis for Duodenal Mucosa

Total RNA was isolated from the duodenal mucosa samples using Trizol Reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. The purity and concentration of the total RNA were measured in a NanoDrop-2000 spectrophotometer (ThermoFisher Scientific Co., Waltham, MA) using the 260/280 nm absorbance ratio. Reverse transcription was done using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd. Life Science Department, Osaka, Japan) following the manufacturer's protocol, and the cDNA was stored at −20°C. Real-time quantitative PCR was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR Premix Ex Taq II kit (ThermoFisher Scientific Co.), as a detector on a Bio-Rad CFX Connect Real-Time PCR Detection System (BioRad Laboratories, Mississauga, ON, Canada). Pairs of primers were designed and checked for target identity using GenBank from the National Center for Biotechnology Information. Forward and reverse sequences of primers are summarized in Table 2. Each sample was analyzed in triplicate under the following PCR conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s, with a final extension at 72°C for 5 min. Specificity of the PCR products was evaluated by the analysis of the melting curve. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), which normalized to the reference mRNA level of GAPDH. The values of saline treated broilers fed the basal diet were used as a calibrator.

Table 2. Primers used for RT-qPCR amplification of chicken cytokines in this study.

Primer	F/R	Nucleotide sequence (5′- 3′)	Accession No.
GAPDH	F	CAACACAGTGCTGTCTGGTGGTA	NM_204305
	R	ATCGTACTCCTGCTTGCTGATCC	
IL-1β	F	5′ CCTGTCTCTGTCCCTACCCCCTA 3′	HQ739080
	R	5′ GTCAACGGGTGTGCTGCAGGAAC 3′	
IL-6	F	5′ GCATTCACTGAGTTTCCACCATT 3′	AJ309540
	R	5′ GTAGCACTGAGGGACATGGTAAG 3′	
IL-10	F	5′ GATTCAGATAGAAGTTCTGTGCC 3′	AJ621254.1
	R	5′ GGTAACTTCTCTAAACACAGCAG 3′	

Abbreviations: IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10.

Determination of Oxidative Stress Parameters in Serum/Liver

The contents of total superoxide dismutase (**T-SOD**), catalase (**CAT**), glutathione peroxidase (**GSH-Px**) and malondialdehyde (**MDA**) in serum were measured using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Approximately 1 g of chicken liver was homogenized with 9 mL of 0.9% sodium chloride buffer (w/v, 1:9) on ice, and then centrifuged at 1,000 × *g* at 4°C for 10 min to obtain the supernatant. The supernatant was collected for the total protein concentration determination using a BCA Protein Assay Kit (P0010S, Beyotime Biotechnology, Shanghai, China) and stored at −80°C for analysis. The enzymatic activities of T-SOD, CAT, and GSH-Px and the level of MDA in the supernatant of the liver homogenate were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute). All procedures were performed according to the manufacturer's instructions.

Morphological Measurements of Duodenum

To carry out a morphological analysis of the duodenal epithelium, formalin-fixed tissue samples were dehydrated, washed with physiological saline solution, treated in tissue-processor apparatus, and embedded in paraffin wax. Transverse sections were cut (6 μm thickness) using a rotary microtome (Leica RM 2145, Leica Instruments GmbH, Nussloch, Germany) and stained with hematoxylin and eosin. The cross-sections were viewed and photographed using an Olympus IX81 microscope and analyzed using CellSens Imaging software (Olympus America Inc., Center Valley, PA) to determine the villi height (**VH**) and crypt depth (**CD**). The ratio of villus height to crypt depth (**VH**/**CD**) can be finally calculated. At least 3 sections with 3 observations for each sample were viewed.

Statistical Analysis

The data are presented as the mean values with a pooled standard error of the mean and were analyzed by 2-way analysis of variance using the general linear model procedure of SAS 9.0 (SAS Institute Inc., Cary, NC) as a 2 × 2 factorial arrangement with dietary $PQQ{\cdot}Na_2$ and LPS challenge as main effects as well as their interactions. When interactions were significant ($P < 0.05$), differences between means were determined using Tukey's procedure.

RESULTS

Growth Performance

As shown in Table 3, LPS injection had no effect on growth performance ($P > 0.05$) in broiler. Meanwhile, dietary $PQQ{\cdot}Na_2$ supplementation had no significant effects on BW, average daily gain, ADFI, and ratio of feed to gain during the entire experimental period.

Inflammatory Cytokines Contents in Serum

Inflammatory cytokine contents in serum are listed in Table 4. On day 20, relative to saline injection, LPS challenge resulted in an increase ($P < 0.05$) in the levels of serum IL-1β, IL-6, and IL-10 levels; dietary $PQQ{\cdot}Na_2$ supplementation led to a lower level of serum IL-6 compared with basal diet without $PQQ{\cdot}Na_2$. On day 21, the level of serum IL-1β was elevated ($P < 0.05$) after LPS challenge; the concentrations of serum IL-1β, IL-6, and IL-10 were significantly decreased after dietary $PQQ{\cdot}Na_2$ supplementation. Interaction ($P < 0.05$) between dietary $PQQ{\cdot}Na_2$ and LPS challenge was found in broiler on day 42, as reflected by the result that serum IL-1β was the lowest in LPS-challenged broilers fed diet containing $PQQ{\cdot}Na_2$.

mRNA Expression of Cytokines in Duodenal Mucosa

As shown in Table 5, at the age of 21 d, LPS challenge increased ($P < 0.05$) the mRNA expressions of IL-1β and IL-6 in the duodenal mucosa of broiler chicks compared with the un-challenged chickens; dietary supplementation with $PQQ{\cdot}Na_2$ significantly reduced the mRNA expressions of IL-1β and IL-10 compared to the group without $PQQ{\cdot}Na_2$ supplementation. Additionally, at the age of 42 d, LPS challenge up-regulated ($P < 0.05$) the mRNA expression of IL-1β in duodenal mucosa of broilers. Interaction ($P < 0.05$) between dietary $PQQ{\cdot}Na_2$ and LPS challenge was found in the mRNA expression of IL-1β in duodenal mucosa of chickens on day 42.

Table 3. Effect of dietary PQQ·Na_2 supplementation on growth performance in broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 21								
BW, g	549.5	538.6	531.4	520.6	28.85	0.805	0.631	0.788
ADG, g	24.40	24.20	24.00	23.70	1.62	0.934	0.462	0.653
ADFI, g	42.50	40.00	41.28	39.82	2.48	0.407	0.802	0.515
F/G, g/g	1.74	1.66	1.72	1.68	0.14	0.572	0.368	0.981
Day 42								
BW, g	2,256	2,181	2,186	2,134	96.25	0.140	0.183	0.779
ADG, g	85.76	82.32	82.75	79.94	4.66	0.134	0.197	0.874
ADFI, g	131.7	131.8	133.6	127.0	7.56	0.332	0.591	0.317
F/G, g/g	1.54	1.61	1.61	1.59	0.10	0.610	0.523	0.294

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge.

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; F/G, ratio of feed to gain; LPS, lipopolysaccharide; SEM, pooled standard error of the mean.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

Serum Antioxidant Indices

As summarized in Table 6, LPS challenge significantly increased ($P < 0.05$) the GSH-Px activity in serum of birds on day 21. Meanwhile, broilers fed diet with PQQ·Na_2 supplementation showed significantly higher GSH-Px activity in serum both on day 20 and day 21. Interaction between dietary PQQ·Na_2 and LPS challenge in the levels of T-SOD, CAT, GSH-Px, and MDA in serum were not significant throughout the experiment.

Liver Antioxidant Status

As indicated in Table 7, LPS challenge decreased ($P < 0.05$) T-SOD and CAT activities in liver of birds on day 21. However, PQQ·Na_2 diet increased ($P < 0.05$) the T-SOD activity in liver of chickens on day 21 and the activities of T-SOD and CAT in liver of broilers on day 42. Moreover, dietary supplementation with PQQ·Na_2 significantly reduced the content of MDA in liver of birds on day 42. No interaction ($P > 0.05$) was found in liver antioxidant indices between dietary PQQ·Na_2 and LPS challenge.

Duodenal Morphology

Some data on duodenal morphology for the broilers are shown in Table 8. On day 21, the duodenal VH/CD of birds challenged with LPS was lower ($P < 0.05$) than the birds unchallenged with LPS. However, dietary

Table 4. Effect of dietary PQQ·Na_2 supplementation on inflammatory cytokines content in serum of broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items (pg/mL)	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 20								
IL-1β	41.36	59.76	47.56	54.39	11.37	0.024	0.972	0.277
IL-6	23.43	41.87	42.74	51.54	11.54	0.026	0.022	0.394
IL-10	43.78	55.60	36.37	60.46	14.07	0.013	0.804	0.359
Day 21								
IL-1β	35.46	36.76	37.2	43.56	2.74	0.011	0.003	0.072
IL-6	16.83	20.43	22.56	27.17	5.49	0.138	0.029	0.848
IL-10	28.93	28.52	35.86	34.86	5.72	0.790	0.028	0.916
Day 42								
IL-1β	50.17^{a,b}	45.27^{b}	48.47^{b}	61.74^{a}	8.71	0.328	0.060	0.045
IL-6	35.69	39.03	44.93	45.33	10.18	0.661	0.145	0.771
IL-10	35.31	37.97	38.7	38.96	5.07	0.553	0.403	0.627

[a,b]Means values with different superscripts within each row are significantly different ($P < 0.05$).

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge.

Abbreviations: IL-1β, interleukin-1beta; IL-6, interleukin-6; IL-10, interleukin-10; LPS, lipopolysaccharide; SEM, pooled standard error of the mean.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

Table 5. Effect of dietary PQQ·Na_2 supplementation on mRNA expression of cytokines in duodenal mucosa of broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 21								
IL-1β	0.56	0.69	1.01	1.23	0.14	0.033	<0.01	0.544
IL-6	0.86	1.13	1.01	1.95	0.52	0.038	0.086	0.229
IL-10	0.25	0.59	1.04	1.19	0.26	0.089	<0.01	0.467
Day 42								
IL-1β	1.07[b]	1.21[b]	1.01[b]	2.04[a]	0.38	0.010	0.065	0.037
IL-6	1.18	1.08	1.00	1.22	1.27	0.703	0.927	0.284
IL-10	1.33	0.99	1.14	1.30	0.66	0.779	0.860	0.466

[a,b]Means with no common superscript within each row are significantly ($P < 0.05$) different.

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge.

Abbreviations: IL-1β, interleukin-1beta; IL-6, interleukin-6; IL-10, interleukin-10; LPS, lipopolysaccharide; SEM, pooled standard error of the mean.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

PQQ·Na_2 supplementation led to increased ($P < 0.05$) VH/CD in duodenum of broilers. On day 42, VH and VH/CD were significantly lower ($P < 0.05$) in the duodenum of broilers injected with LPS compared with broilers injected with saline. Meanwhile, higher VH/CD ($P < 0.05$) was observed in the duodenum of chickens fed with PQQ·Na_2 compared to chickens fed without PQQ·Na_2.

DISCUSSION

Immunological stress, oxidative stress and gastrointestinal health are viewed as the critical issue in poultry production as these factors influence the health status of birds (Roura et al., 1992; Xie et al., 2000). This study was undertaken to examine the effects of dietary supplementation with PQQ·Na_2 on inflammatory responses, antioxidant stress and intestinal morphology in broilers challenged with *E. coli* LPS.

Our results demonstrated that chickens injected with LPS exhibited no significant difference on growth performance compared to chickens injected with saline, which are in agreement with the study of Wu et al. (2013). In contrast, negative effects on growth performance were reported by Klasing and Barnes (1988) and Webel et al. (1998), where LPS injection significantly reduced the weight gain and ADFI of chickens. Though PQQ has been recognized as a growth hormone-like factor in microorganisms (Shimao et al., 1984) and a growth promoter in rodents (Steinberg et al., 1994, 2003), no significant increase was observed in growth performance of broiler chickens fed with PQQ·Na_2 in this study. To

Table 6. Effect of dietary PQQ·Na_2 supplementation on antioxidant indices in serum of broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 20								
T-SOD, U/mL	141.62	140.44	145.11	145.59	14.86	0.924	0.389	0.871
CAT, U/mL	7.29	7.21	7.60	7.31	1.10	0.612	0.580	0.768
GSH-Px, U/mL	508.71	515.69	461.76	438.04	80.87	0.766	0.020	0.565
MDA, nmol/mL	4.17	3.77	3.42	3.58	0.85	0.783	0.144	0.358
Day 21								
T-SOD, U/mL	171.00	167.03	167.53	171.92	8.21	0.933	0.389	0.871
CAT, U/mL	7.42	7.32	7.73	7.96	1.28	0.939	0.255	0.690
GSH-Px, U/mL	590.57	465.8	476.47	444.53	93.61	0.011	0.046	0.144
MDA, nmol/mL	2.59	2.60	2.60	2.59	0.49	0.997	0.992	0.939
Day 42								
T-SOD, U/mL	180.88	178.07	180.88	177.71	8.42	0.248	0.938	0.943
CAT, U/mL	8.27	8.47	8.23	8.24	1.20	0.777	0.694	0.805
GSH-Px, U/mL	651.23	677.36	693.73	663.53	111.86	0.911	0.740	0.440
MDA, nmol/mL	2.58	2.60	2.55	2.56	0.29	0.852	0.726	0.928

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge.

Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; LPS, lipopolysaccharide; MDA malondialdehyde; SEM, pooled standard error of the mean; T-SOD, total super-oxide dismutase.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

Table 7. Effect of dietary PQQ·Na_2 supplementation on antioxidant indices in liver of broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 21								
T-SOD,U/mg protein	83.49	77.78	75.88	70.45	7.73	0.044	0.006	0.956
CAT, U/mg protein	7.62	6.02	6.27	5.58	1.51	0.026	0.072	0.406
GSH-Px, U/mg protein	762.38	746.75	786.35	777.45	117.96	0.805	0.495	0.934
MDA, nmol/mg protein	4.32	4.34	4.57	4.86	0.86	0.595	0.150	0.633
Day 42								
T-SOD,U/mg protein	80.98	72.23	70.81	67.62	8.57	0.050	0.018	0.341
CAT, U/mg protein	7.54	7.34	5.60	5.68	2.02	0.937	0.008	0.824
GSH-Px, U/mg protein	923.30	870.60	902.50	841.90	217.79	0.433	0.726	0.957
MDA, nmol/mg protein	3.90	4.71	4.87	4.96	0.80	0.098	0.048	0.209

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge

Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; LPS, lipopolysaccharide; MDA malondialdehyde; SEM, pooled standard error of the mean; T-SOD, total super-oxide dismutase.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

some extent, the growth performance of broiler chickens may be associated with many factors, such as age, health status of body, environmental hygiene, dietary ingredient, the dosage of feed additive and experiment protocols so on (Yang et al., 2009).

Inflammatory cytokines play a key role as communication signals in the regulation of inflammation response (Crusz and Balkwill, 2015; Sun et al., 2017). IL-1β and IL-6 are produced by monocytes and macrophages and served as important pro-inflammatory cytokines with a relevant role in early phase of inflammation (Corwin, 2000; Oda et al., 2005; Dung et al., 2009; Waititu et al., 2014). IL-10 is a pivotal anti-inflammatory cytokine that can inhibit pro-inflammatory cytokines including IL-1β and IL-6 (Kambayashi et al., 1995; Groux and Powrie, 1999). When pro-inflammatory cytokines are expressed in large quantities, the body can regulate the inflammatory response by up-regulating the anti-inflammatory cytokines expression (Corwin, 2000). The current results showed that LPS challenge increased the contents of IL-1β, IL-6, and IL-10 in serum of broilers on day 20 or day 21 and the mRNA expressions of IL-1β, IL-6, and IL-10 in intestinal mucosa of broilers on day 21 or day 42. The gastrointestinal tract is the largest immune organ for systemic immunity in animals (Ziegler et al., 2003). So these results implied that the inflammation model in broilers was successfully established in this study. Consistent with our results, Yang et al. (2019) and Wu et al. (2013) stated that LPS stimulation could lead to the release of inflammatory cytokines in broilers. The releases of both pro-inflammatory (IL-1β and IL-6) and anti-inflammatory (IL-10) in broilers can be alleviated by dietary PQQ·Na_2 in our study. Similar to our findings, dietary PQQ supplementation could inhibit IL-6 content in human plasma by Harris et al. (2013). Furthermore, it has been found that PQQ exerted inhibitory effects on LPS-induced neuro-inflammatory by inhibiting the levels of NF-kB and MAPK pathways and then suppressing the expressions of IL-1β and IL-6 (Yang et al., 2014).

Table 8. Effect of dietary PQQ·Na_2 supplementation on duodenal morphology in broiler chickens challenged with LPS.

	Dietary PQQ·Na_2 levels, mg/kg[1]							
	1		0			*P*-values		
Items	Saline	LPS	Saline	LPS	SEM	LPS	PQQ·Na_2	Interaction
Day 21								
VH, μm	1302.72	1280.41	1253.80	1229.84	140.64	0.684	0.430	0.990
CD, μm	156.86	165.72	172.89	179.93	20.56	0.379	0.104	0.919
VH/CD	8.40	8.04	7.45	6.80	0.57	0.040	<0.01	0.567
Day 42								
VH, μm	1526.2	1364.7	1413.8	1251.8	168.44	0.037	0.144	0.997
CD, μm	195.62	199.66	205.34	217.23	39.30	0.617	0.433	0.823
VH/CD	8.01	7.09	7.17	6.00	1.05	0.029	0.049	0.791

P-values for main effect of LPS challenge, the main effect of dietary PQQ·Na_2, and the interaction between the dietary PQQ·Na_2 and LPS challenge.

Abbreviations: CD, crypt depth; LPS, lipopolysaccharide; SEM, pooled standard error of the mean; VH, villus height; VH/CD, the ratio of villus height to crypt depth.

[1]PQQ•Na_2, dietary pyrroloquinoline quinone disodium supplementation; LPS, dietary treatment was injected LPS; saline, dietary treatment was injected saline.

These findings indicated that the anti-inflammation effects of PQQ·Na_2 in broiler may be achieved by regulating both the pro-inflammatory cytokines and the anti-inflammatory cytokines. Moreover, the interaction between dietary PQQ·Na_2 and LPS challenge suggested that the PQQ·Na_2 may play a long-term beneficial role to birds that are exposed to immunological stress. Previous study also showed that early PQQ·Na_2 supplementation had persistently protective effects on the developmental programming of hepatic inflammation in obese mice (Karen and Michael, 2019). Therefore, this study may provide a new evidence for developing PQQ·Na_2 as a novel dietary supplement adding to the broiler basal diet to prevent broilers from inflammatory response.

Recent studies revealed that LPS could disturb the balance between pro-oxidant and antioxidant systems that leads to tissue oxidative damage in broilers (Wu et al., 2013). While the oxidative damage can be largely counteracted by a sophisticated antioxidant defense system including the enzymatic T-SOD, GSH-Px and CAT (Mates et al., 2000; Chen et al., 2009). Thus, the levels of these enzymes in body are considered to be sensitive indicators reflecting the status of oxidative stress in animals. Our experiment found that LPS significantly induced oxidative stress of chickens, characterized the decreased activities of T-SOD, CAT and GSH-Px in serum and liver. Whereas, the dietary supplementation of PQQ·Na_2 increases the activities of antioxidant enzymatic, such as T-SOD, CAT and GSH-Px and decreases the MDA content in broilers. Previous studies found the similar results, which indicated that PQQ could modulate the delicate balance between oxidants and antioxidants in broilers by regulating the activity of antioxidant enzymes (Wang et al., 2015, 2016). The level of MDA could be used as a biomarker for evaluating the degree of lipid peroxidation (Satoshi et al., 1989; Kotunia et al., 2004). The increase of antioxidant enzyme activity reflect an effective antioxidant defense system in animals (Jos et al., 1999). Based on these results, we can conclude that PQQ exhibited protective ability on oxidative stress not only by direct neutralization of lipid peroxidation but also by induction of antioxidant enzymes (Misra et al., 2004). Thus, it can be concluded that PQQ could act as an effective antioxidant applied in broilers diet to alleviate oxidant stress induced by some pathogen.

The intestinal morphology is an important indicator that reflects the health of the digestive tract and the response of the intestine to certain feed substances (Boguslawska-Tryk et al., 2012; Cao et al., 2015). In the present study, injecting LPS produced a shorter VH and a lower VH/CD ratio of duodenum, which suggests that LPS caused the increase of the permeability of intestinal epithelial layer, the inflammatory response and the motor dysfunction of intestine in broilers (Collins, 2001). Previous study indicated that many factors such as microbial challenges and the composition of animal feed have effects on the VH/CD ratio of the intestine in animals (Sayan et al., 2018). As expected, the current study showed that dietary PQQ·Na_2 supplementation significantly increased VH/CD ratio of the duodenum. Similar result was reported that the PQQ·Na_2 supplementation increases the villus height and decreases the crypt depth in small intestine of pigs (Yin et al., 2019). It is commonly recognized that a higher VH/CD is positively correlated with the digestive and absorptive functions in the gastrointestinal tract of the birds (Boguslawska-Tryk et al., 2012; Munyaka et al., 2012). Therefore, PQQ·Na_2 can be regard as a potentially effective feed additive which can promote broilers intestinal health by enhancing the ratio of VH/CD in duodenum.

In conclusion, dietary PQQ·Na_2 supplementation significantly exerted protective effects on inflammation damage and oxidant stress of broilers under LPS challenge by regulating the expression of inflammatory cytokines and activities of antioxidant enzymes. Moreover, dietary PQQ·Na_2 supplementation significantly ameliorated the LPS-impaired intestinal morphology. Therefore, it has been believed that PQQ·Na_2 is a potential feed additive with beneficial efficacy and should be considered to apply to broiler production.

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