Evaluation of serum antioxidative status, immune status and intestinal condition of *Linwu* duck challenged by lipopolysaccharide with various dosages and replications

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ABSTRACT The present study investigated the dosage and replication effects of lipopolysaccharide challenges on the serum oxidative and immune status, and the intestinal morphology and permeability of *Linwu* ducks at the growing stage. A total of 500 54-day-old *Linwu* ducks were randomly assigned into 10 treatments, which included a factorial arrangement of 2 levels of LPS challenge replications (1 and 2 times) × 5 levels of lipopolysaccharide challenging dosages (0, 0.1, 0.2, 0.4, and 0.8 mg/kg). Each treatment consisted of 5 cages and 10 ducks per cage. The results showed significant replication effects of LPS on the body weight gain of ducks, that 2 replicates of LPS challenges significantly decreased the body weight gain than one challenge ($P = 0.036$). Regarding to the serum oxidative and immune status, dosage effects of lipopolysaccharide were found on the serum levels of superoxide dismutase ($P = 0.034$) and immunoglobulin A ($P = 0.007$), that 0.4 mg/kg lipopolysaccharides significantly increased the levels of these 2 parameters. Additionally, replication effects were found in the serum levels of interlukin 1β, that 2 replicates of LPS challenges significantly increased the interlukin 1β levels comparing to one challenge ($P = 0.010$). Regarding to the intestinal conditions, dosage effects of lipopolysaccharides were found on the ratio of villus height and crypt depth ($P = 0.005$) in duodenum, and the wall thickness of duodenum ($P = 0.010$) and jejunum ($P = 0.001$), that lipopolysaccharides at 0.1, 0.2, and 0.8 mg/kg significantly deteriorated the intestinal morphologies, especially in the duodenum and jejunum. Moreover, the dosage effects of lipopolysaccharides and the interactions of dosages and replications significantly influenced the permeabilities of the intestinal segments ($P < 0.05$). It appeared that 2 replicates of lipopolysaccharides at the dosage at 0.4 mg/kg could trigger oxidative and immunological stress, and damage the intestinal morphology and permeability of *Linwu* ducks at the growing stage.

Key words: duck, lipopolysaccharide, intestinal condition

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INTRODUCTION

Lipopolysaccharides (**LPS**) are the components of the outer membrane of gram-negative bacteria, which usually cause sickness syndromes in infected animals. LPS could be recognized by the toll like receptors and myeloid differential proteins at the membrane of the leukocytes, and triggered the immune and oxidative stresses by stimulating the secretions of the multiple cytokines, such as interleukin (**IL**)-6 and IL-1β (Byun et al., 2013). The stresses eventually led to the damages to the intestinal epithelial integrity and jeopardized the barrier functions of the gastrointestinal tract (Flaviana et al., 2019), and deteriorated the growth of the animals.

The effects of LPS on the health condition were extensively studied on mammals. Previous studies on rats and pigs illustrated that multiple inoculations of LPS at relatively low rates (≤ 0.1 mg/kg) could trigger the sickness symptoms including fever, reduction of weight gains, and changes in behaviors (Raetz and Christian, 1990; Rorato et al., 2017; Ahasan et al., 2018). Interestingly, a few studies also showed that broilers performed similar response patterns to rats after LPS challenges, but required greater

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dosages (≥ 0.5 mg/kg) (Hollis et al., 2011; Zheng et al., 2020). It seemed that poultry may be less sensitive to LPS than the mammals. However, there were few studies evaluating the dosage effects of LPS on ducks as compared with broilers.

On the other hand, animals that experienced repeated LPS exposures might acquire the endotoxin tolerance, resulting in reduced secretions of cytokines (West and Heagy, 2002). It was proved that endotoxin tolerance could be induced in pigs (Castegren et al., 2013), rats (Santos et al., 2020), and human monocyte and macrophage cells (Ghiboub et al., 2020). But whether birds were able to develop the endotoxin tolerance was still unknown.

Linwu duck is an important indigenous duck breed in South China, with the characteristics of fast growth, high feed efficiency and unique meat flavor and texture (Lin et al., 2016). The market ages for *Linwu* ducks are 70 d and the average body weights at the market age are about 1750 g. It was worrying that the manure polluted water with pathogenic bacteria and endotoxins, such as LPS, seriously deteriorated the health of the ducks. Therefore, it was highly important to understand the infectious endotoxin dosages and the ducks' responses to the endotoxin infections for a better management. In the present study, we used the *Linwu* duck as the experiment animal and stimulated the repeated LPS exposures by 2 replicates of LPS challenges. The aims of this study were, firstly to evaluate the dosage effects of LPS on the serum antioxidative and immunological status, as well as the intestinal conditions of the *Linwu* ducks; secondly to check the occurrence of LPS tolerance in *Linwu* ducks after 2 replicates of LPS inoculations.

MATERIALS AND METHODS

Animal and Treatments

This study was conducted after the approval of the Institutional Animal Care and Use Committee at Hunan Institute of Animal and Veterinary Science. *Escherichia coli* O55:B5 LPS used in this study were purchased from Sigma (St. Louis, MO, USA). A total of 500 54-day-old female *Linwu* ducks were obtained from Hunan Shunhua Duck Industrial Development Company (Linwu, China). The reason for choosing ducks at 54-day-old was because *Linwu* ducks at this age were in the maximum growing stage and suffered the most by LPS in the environment. Birds were housed in plastic plain netting cages with the dimension of 1.8 m × 1.2 m × 2 m (10 ducks/cage), and water and feed were provided ad libitum. The treatments included a factorial arrangement of 2 × 5, with replication (2 levels: 1 and 2 times) and dosage (5 levels: 0, 0.1, 0.2, 0.4 and 0.8 mg/kg) as the main factors. There were 5 cages of birds in each treatment as repeats. LPS were dissolved in saline and intraperitoneally injected into the birds either once (at 12 h before slaughtering), or twice (at 36 h and 12 h before slaughtering respectively) in accordance with the assigned dosage and replication. Saline was injected in the groups assigned with LPS dosage at 0. Diet for the birds met the National Research Council, 1994 for growing ducks, as showed in Table 1.

Table 1. Ingredients and nutrient composition of the basal diet.

Ingredients, %		Nutrient levels, %[2]	
Corn	50.68	Metabolic energy, MJ/kg	11.30
Soybean meal	24.50	Dry matter (DM)	87.3
Flour	10.00	Crude protein (CP)	17
Wheat middings	7.00	Calcium (Ca)	0.90
$CaHPO_4$	1.30	Total phosphorus (TP)	0.56
Salt	0.30	Available phosphorus (AP)	0.35
L-Lysine H_2SO_4	0.27	Salt	0.33
DL-Methionine	0.12	Lysine	0.9
Limestone	1.20	Methionine	0.4
Bentonite	3.63	Methionine and cystine	0.789
Premix[1]	1.00	Isoleucinese	0.732
		Threonine	0.6
		Tryptophane	0.264

[1]The premix provided the following nutrients per kg diet: vitamin A 12,000 IU; vitamin D_3 2,500 IU; vitamin E 20 mg; vitamin K_3 3 mg; vitamin B_1 3 mg; vitamin B2 8 mg; vitamin B6 7 mg; vitamin B12 0.03 mg; *D*-pantothenic acid 20 mg, nicotinic acid 50 mg, biotin 0.1 mg, folic acid 1.5 mg, Cu (as copper sulfate) 9 mg, Zn (as zinc sulfate) 110 mg, Fe (as ferrous sulfate) 100 mg, Mn (as manganese sulfate) 100 mg, Se (as sodium selenite) 0.16 mg, I (as potassium iodide) 0.6 mg.

[2]The nutrient levels were calculated values.

Sample Collection and Analysis

Growth Performance. Cage was taken as the unit of the BW measurement. BW of birds was recorded at 6 pm on 54 d (36 h before slaughtering) as the initial BW, and 6 am on 56 d (the time of slaughtering) as the final BW. All birds were fasted for 12 h before weighting. The BW gain (**BWG**) was calculated for the whole experimental period.

Sample Collection. One duck from each cage (5 ducks per treatment) was randomly selected for sampling. Serums were collected by centrifuging blood retrieved from the wing vein at 3,000 × g for 10 min, and placed at -20 °C for the following tests. After euthanasia, the mid-duodenum, jejunum, ileum and cecum (approximately 1 cm) were immediately taken and rinsed. Each tissue sample was carefully dissected into 2 pieces. One piece was preserved in 4% paraformaldehyde for intestinal morphology diagnoses, and the other was used for the tissue homogenate.

Serum Oxidant and Immune Status. The serum oxidant and immune status were represented by the levels of oxidative biomarkers including malonaldehyde (**MDA**), reduced glutathione (**GSH**), and superoxide dismutase (**SOD**), and the levels of inflammatory biomarkers including immunoglobulin A (**IgA**), IL-1β, and IL-6. A total of 50 serum samples were collected for oxidant and immune status estimation. The levels of these biomarkers were determined by the commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) with an automated fluorescence instrument (MultiskanSkyHigh, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' instructions.

Intestinal Morphology. Tissues of duodenum, ileum, jejunum, and cecum were cleaned and embedded in paraffin, and 5μm slices of the tissues were made by microtome (RM-2235, Leica microsystems AG., Hessen, Germany). A total of 200 samples were stained with hematoxylin and eosin, and observed under a microscope (Olympus Van-Ox S, Opelco, Washington, DC, USA) separately. 10 sections of the proper microscopic fields were chosen from each sample for the analysis of villus height (**VH**), crypt depth (**CD**), wall thickness (**WT**), and mucosa thickness (**MT**) under an image analysis system (Image-Pro, Media Cybernetics, Inc., Silver Springs, MD, USA). Briefly speaking, villus length was measured from the villous tip to the villous crypt junction; crypt depth was determined from the opening to basing of crypt; wall thickness was measured from the outer of the intestine to the submucosa and muscular layer junction, including the serosa layer and the muscular layer (Liao et al., 2020). The ratio of VH and CD (**VCR**) was calculated.

Intestinal Permeability. The intestinal permeability was represented by the levels of tight junction proteins (**TJPs**) in intestinal tissues and the activities of diamine oxidase (**DAO**) in the serums. A total of 200 intestinal tissue samples from 50 ducks were separately homogenized in PBS via a homogenizer (LabGEN 850, Cole-Parmer China, Shanghai, China). After centrifuged at 3000 r/min for 15 mins, the supernatants were collected for the examination of TJPs. The level of TJPs, including claudin (**CLDN**), occludin (**OCLN**) and zonula occludens 1 (**ZO-1**) were analyzed with commercially available kits (duck-specific antibodies) purchased from Jiangsu Yutong Biological Technology Co., Ltd (Nanjing, China). Activities of serum DAO were tested with assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical Analysis. The means of data were subjected to 2-way ANOVA, and the main factor effects and their interactions were evaluated. The level of significance was set at $P < 0.05$. Significant means were further compared by post hoc of Fisher's least significant difference test. Statistical Package for the Social Sciences 19.0 (IBM, Armonk, New York) was used for the data analysis.

RESULTS

Growth Performance

The growth performance was measured from d 54 to d 56 as it was the responding period of the birds receiving LPS challenges. During this period, the BWG of birds was influenced by the challenging replication ($P = 0.036$) (Table 2). Birds receiving 2 replicates of challenges had a significantly lower BWG than the ones having one challenge. Although the dosage effects of LPS were not significant, there were numerical decreases in BWG by LPS dosages at 0.1, 0.2, 0.4 and 0.8 mg/kg comparing to no LPS challenged group ($P = 0.227$).

Serum Antioxidative Status

Significant LPS dosage effects were observed in the SOD activities in the serums as showed in Table 3,

Table 2. Parameters related to the growth performance in accordance with the treatments.[1]

Item Treatment[2]	Dosage, mg/kg	Replication	Initial BW, g	Final BW, g	BWG, g
1	0	1	1479.6	1498.1	18.5
2	0.1	1	1454.7	1455.3	0.6
3	0.2	1	1491.0	1498.4	7.4
4	0.4	1	1487.3	1495.5	8.2
5	0.8	1	1485.9	1491.2	5.3
6	0	2	1479.6	1493.4	13.8
7	0.1	2	1501.4	1491.8	-9.6
8	0.2	2	1565.1	1549.8	-15.3
9	0.4	2	1590.3	1578.8	-11.5
10	0.8	2	1515.9	1457.2	-58.7
Main effect	Dosage				
	0		1479.60	1495.75	16.15
	0.1		1478.05	1473.55	-4.50
	0.2		1528.05	1524.10	-3.95
	0.4		1538.80	1537.15	-1.65
	0.8		1500.90	1474.20	-26.70
		Replication			
		1	1479.70	1487.70	8.00[a]
		2	1530.46	1514.20	-16.26[b]
Source of variance			*P*-value	*P*-value	*P*-value
Dosage effect			0.761	0.741	0.227
Replication effect			0.170	0.476	0.036
Dose × replication			0.915	0.865	0.491
SEM[3]			38.79	38.88	13.16

[a-c]Means in a column not sharing a same superscript letter are different ($P < 0.05$).

[1]Data is the mean of 6 replicates per treatment.

[2]Treatment 1-5 received one challenge of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, and 0.8 mg/kg LPS on d 54, respectively. Treatment 6-10 received 2 challenges of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, 0.8 mg/kg LPS on d 54 and 55, respectively.

[3]Standard error of the mean.Abbreviations: BW, body weight; BWG, body weight gain.

Table 3. Level of serum parameters related to antioxidative status in accordance with treatments.[1]

Item Treatment[2]	Dosage, mg/kg	Replication	SOD, U/mL	MDA, nmol/mL	GSH, nmol/mL
1	0	1	123.90	2.56	191.46
2	0.1	1	159.37	2.67	191.63
3	0.2	1	151.09	1.81	250.64
4	0.4	1	166.82	2.39	166.49
5	0.8	1	155.32	2.45	278.22
6	0	2	128.86	2.70	169.60
7	0.1	2	143.94	3.75	158.32
8	0.2	2	148.75	3.16	260.89
9	0.4	2	155.17	2.65	175.82
10	0.8	2	120.45	2.65	236.43
Main effect	Dosage				
	0		126.65[b]	2.62	179.32
	0.1		151.65[ab]	3.21	174.97
	0.2		149.92[ab]	2.48	255.20
	0.4		161.00[a]	2.52	171.15
	0.8		137.89[b]	2.55	257.33
		Replication			
		1	155.32	2.38	216.70
		2	139.44	2.99	197.68
Source of variance			*P*-value	*P*-value	*P*-value
Dosage effect			0.034	0.566	0.108
Replication effect			0.095	0.065	0.582
Dose × Replication			0.450	0.634	0.961
SEM[3]			8.37	0.35	30.50

[a-c]Means in a column not sharing a same superscript letter are different ($P < 0.05$).
[1]Data is the mean of 6 replicates per treatment.
[2]Treatment 1-5 received one challenge of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, and 0.8 mg/kg LPS on d 54, respectively. Treatment 6-10 received 2 challenges of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, 0.8 mg/kg LPS on d 54 and 55, respectively.
[3]Standard error of the mean.Abbreviations: GSH, reduced glutathione; MDA, malonaldehyde; SOD, superoxide dismutase.

that LPS at the dosage of 0.4 mg/kg significantly increased SOD activities comparing to no LPS treated groups and 0.8 mg/kg LPS treated groups ($P = 0.034$). No dosage effects, replication effects, or interactions of the 2 factors were found on the MDA ($P = 0.566$, 0.065, 0.064, respectively) or GSH ($P = 0.108$, 0.582, 0.961, respectively) levels in the serums.

Table 4. Level of serum parameters related to immune status in accordance with treatments.[1]

Item Treatment[2]	Dosage, mg/kg	Replication	IgA, mg/mL	IL-6, mg/mL	IL-1β, mg/mL
1	0	1	2.87	79.28	198.50
2	0.1	1	2.41	76.33	207.20
3	0.2	1	2.53	85.91	194.28
4	0.4	1	2.15	67.56	181.14
5	0.8	1	1.36	69.55	162.14
6	0	2	2.74	85.09	204.63
7	0.1	2	2.80	78.71	205.91
8	0.2	2	2.04	82.26	211.19
9	0.4	2	1.93	68.74	196.82
10	0.8	2	2.20	89.06	223.12
Main effect	Dosage				
	0		2.81[a]	82.51	201.91
	0.1		2.61[ab]	77.52	206.56
	0.2		2.29[abc]	84.08	202.74
	0.4		2.04[bc]	68.15	188.98
	0.8		1.78[c]	79.31	192.63
		Replication			
		1	2.27	75.58	188.24[b]
		2	2.33	80.77	208.33[a]
Source of variance			*P*-value	*P*-value	*P*-value
Dosage effect			0.007	0.359	0.507
Replication effect			0.668	0.343	0.010
Dose × replication			0.157	0.685	0.079
SEM[3]			0.23	5.73	8.89

[a-c]Means in a column not sharing a same superscript letter are different ($P < 0.05$).
[1]Data is the mean of 6 replicates per treatment.
[2]Treatment 1-5 received one challenge of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, and 0.8 mg/kg LPS on d 54, respectively. Treatment 6-10 received 2 challenges of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, 0.8 mg/kg LPS on d 54 and 55, respectively.
[3]Standard error of the mean.Abbreviations: IgA, immunoglobulin A; IL-6, interleukin 6; IL-1β, interleukin 1β.

Serum Immune Status

As showed in Table 4, the LPS dosage effects were observed in serum levels of IgA, and replication effects were observed in serum levels of IL-1β. For IgA levels, 0.4 mg/kg LPS significantly decreased the IgA levels comparing to no LPS treated groups, and 0.8mg/kg LPS significantly lowered the IgA levels comparing to no LPS treated groups and 0.1 mg/kg LPS treated groups ($P = 0.007$).

For IL-1β levels, no interactions were noticed among groups ($P = 0.079$). However, there were replication effects that 2 replicates of challenges significantly increased the serum levels of IL-1β comparing to the groups receiving one challenge ($P = 0.010$).

Intestinal Morphology

As showed in Table 5, significant dosage effects were found on the VCR and WT in duodenum, and the WT in jejunum. Compared to no LPS treated groups, LPS at all tested dosages significantly decreased the VCR in duodenum ($P = 0.005$) and the WT in jejunum ($P = 0.001$), but no differences were noticed among LPS treated groups. Additionally, LPS at dosages of 0.1, 0.4, and 0.8 mg/kg significantly decreased the WT in duodenum comparing to no LPS treated groups and 0.2 mg/kg LPS treated groups ($P = 0.010$).

Representative images of H&E-stained duodenal and jejunal tissues under 0, 0.1, 0.2, 0.4 and 0.8 mg/kg LPS challenges were showed in Figure 1. It was noticeable that the intestinal wall thicknesses were thinner in LPS treated groups than no LPS treated groups.

Intestinal Permeability

As showed in Table 6, effects of LPS dosage and the interactions of dosage and replication were observed in the majority of the intestinal permeability related parameters, such as the DAO activities in serum, and the levels of TJPs in intestinal segments. Replication effects were only seen on the OCLN levels in duodenum and cecum, and CLDN levels in ileum. For DAO, LPS at all tested dosages significantly increased the DAO activities in serum comparing to no LPS treated group. 0.1 and 0.4 mg/kg LPS treated groups showed significantly higher DAO activities than 0.2 and 0.8 mg/kg LPS treated groups ($P < 0.001$).

For TJPs, compared to no LPS treated groups, LPS at dosages of 0.1 and 0.4 mg/kg increased levels of the ZO-1, OCLN, and CLDN in duodenum ($P = 0.011$, $P < 0.001$, $P = 0.011$, respectively), jejunum ($P < 0.001$, $P = 0.059$, $P < 0.001$, respectively), ileum ($P = 0.001$, $P = 0.001$, $P = 0.008$, respectively), and cecum ($P = 0.001$, $P = 0.011$, $P < 0.001$, respectively). Differences of TJP levels among LPS treated groups were not detected except the following parameters: the OCLN levels in duodenum were significantly higher with 0.8 mg/kg LPS than 0.1 mg/kg LPS; the CLDN levels in jejunum were significantly higher with 0.2 mg/kg LPS than 0.4 mg/kg LPS; the ZO-1 levels in cecum were significantly higher with 0.8 mg/kg LPS than 0.4 mg/kg LPS; the OCLN levels in cecum were significantly higher with 0.2 and 0.8 mg/kg LPS than 0.4 mg/kg LPS; and the CLDN levels in cecum were significantly higher with 0.2 mg/kg LPS than 0.1 and 0.4 mg/kg LPS.

Additionally, compared to the groups receiving one LPS challenge, 2 replicates of LPS challenges significantly increased the levels of OCLN in duodenum ($P = 0.015$) and cecum ($P = 0.034$), along with CLDN in ileum ($P = 0.014$).

DISCUSSION

LPS were able to trigger stress related acute responses in animals, and they were usually utilized as stimuli for the animal stresses. Depending on animal species and breeds, LPS dosages for the stimulation of the responses varied. Mammals required much lower LPS dosages than the poultry did for the deterioration in growth performance (Nogueira et al., 2019). It was reported that LPS at the dosage of 0.25 mg/kg were able to induce the body weight loss in mice (Kamdi et al., 2021), and 0.025 mg/kg LPS could influence the growth performance of weanling pigs (Gu et al., 2017). On the other hand, the minimum dosage of LPS showing negative effects on the poultry was uncertain. Some studies on broilers found that LPS over 0.5 mg/kg could trigger the declines in the growth, immune and oxidative stresses, along with the damages in the intestines (Csernus et al., 2020; Han et al., 2020; Chen and Yu, 2021). However, few studies were available associated with the dosage effects of LPS on ducks. Moreover, endotoxin tolerance was found in mammals and fish with repeated exposure of the same endotoxin (Novoa et al., 2009; Castegren et al., 2013; Santos et al., 2020), which alleviated or even bolished the negative responses to the stimulus. Though (Marais et al., 2011) reported that Pekin ducks established the tolerance after 5 injections of LPS, whose levels of corticosterone in the plasma increased, and hypothalamo-pituitary-adrenal responses blunted, the information about the integreted influences of repeated exposure and dosages of LPS on ducks were still scarece.

In the present study, we evaluated the dosage as well as the replication effects of LPS challenges on the growth performance, the oxidative and immune status, and the intestinal conditions of *Linwu* ducks. We took 2 replicates of LPS or saline challenges as a model of repeated exposure to the endotoxin. Our results showed that during the 3-d experimental period, no dosage effects of LPS were noticed associated with the BWG in the ducks ($P = 0.227$), but the replication effects of LPS played a significant role, that 2 replicates of challenges significantly decreased the BWG of birds comparing to the ones receiving one challenge ($P = 0.036$). Previous study reported that 3 injections of LPS reduced the BWG of birds comparing to a single LPS challenge

Table 5. Parameters related to intestinal morphology in accordance with treatments.[1]

Item			Duodenum					Jejunum					Ileum					Cecum				
Treatment[2]	Dosage,mg/kg	Replication	VH, μm	CD, μm	VCR	WT, μm	MT, μm	VH, μm	CD, μm	VCR	WT, μm	MT, μm	VH, μm	CD, μm	VCR	WT, μm	MT, μm	VH, μm	CD, μm	VCR	WT, μm	MT, μm
1	0	1	470.14	93.47	4.81	259.51	653.28	424.05	88.46	4.49	245.77	602.76	381.93	67.92	5.46	156.88	487.17	147.96	43.43	3.37	149.59	225.92
2	0.1	1	440.59	104.34	4.23	187.92	600.36	401.02	85.87	4.74	199367	574.06	363.97	67.27	5.49	143.02	497.58	188.64	45.95	4.21	177.05	278.13
3	0.2	1	401.08	93.39	4.29	250.23	565.41	397.28	81.23	4.98	195.08	566.75	390.54	68.07	5.75	163.37	511.28	184.71	43.58	4.28	165.13	237.28
4	0.4	1	413.95	97.84	3.91	218.28	556.12	347.86	74.07	4.93	165.12	519.71	376.27	64.49	5.88	187.83	534.52	173.76	44.32	3.92	144.53	234.35
5	0.8	1	434.10	102.05	4.28	201.45	612.74	376.30	87.65	4.27	186.20	523.54	353.50	76.81	4.62	161.80	469.50	157.40	48.08	3.34	149.91	228.91
6	0	2	457.13	97.24	4.72	252.42	635.76	402.38	90.63	4.30	228.86	584.62	357.48	69.71	5.14	151.54	472.59	142.20	44.57	3.20	142.39	215.32
7	0.1	2	389.77	107.94	3.62	233.97	552.61	407.25	89.57	4.61	173.76	571.45	360.61	72.68	4.95	168.99	517.65	153.06	46.31	3.30	135.04	230.15
8	0.2	2	412.99	100.85	4.09	217.46	574.02	380.95	80.69	4.75	164.44	526.72	338.22	62.72	5.42	154.57	482.20	083.68	47.48	3.89	162.62	240.51
9	0.4	2	437.68	103.05	4.25	230.35	611.31	383.24	84.73	4.66	188.46	550.24	355.97	66.08	5.45	151.21	498.24	161.41	43.21	3.72	145.62	246.41
10	0.8	2	413.90	99.79	4.20	210.08	560.80	388.32	95.26	4.09	193.94	545.84	365.17	68.54	5.44	158.93	489.40	167.91	45.08	3.70	169.67	253.87
Main effect	Dosage																					
	0		462.91	95.57	4.76[a]	255.57[a]	643.54	412.01	89.67	4.38	236.37[a]	592.68	368.35	68.91	5.28	153.91	479.07	144.76	44.06	3.28	145.59	220.03
	0.1		415.18	106.14	3.93[b]	210.95[b]	576.49	404.13	87.72	4.67	186.72[b]	559.26	362.29	69.98	5.22	156.00	507.62	170.85	46.13	3.76	153.71	254.14
	0.2		407.04	91.12	4.19[b]	233.85[ab]	569.72	389.12	80.96	4.87	179.76[b]	546.74	364.38	65.40	5.58	158.48	496.74	184.19	45.53	4.08	163.87	238.89
	0.4		425.81	100.44	4.10[b]	223.65[b]	583.71	365.55	79.99	4.80	176.79[b]	534.98	366.12	65.29	5.67	169.52	516.38	167.59	43.77	3.82	145.07	240.38
	0.8		424.00	100.92	4.24[b]	205.77[b]	586.77	382.31	91.46	4.18	190.07[b]	534.69	359.33	72.67	5.03	160.37	479.45	162.66	46.58	3.52	159.79	241.29
		Replication																				
		1	430.38	98.41	4.30	221.98	595.26	387.85	83.64	4.69	196.39	549.85	372.88	68.95	5.44	162.58	500.54	171.43	45.14	3.84	156.33	241.54
		2	422.29	101.77	4.18	228.80	586.90	392.43	88.18	4.48	190.07	555.78	355.49	67.95	5.28	156.68	492.02	161.65	45.33	3.56	150.59	237.25
Source of variance																						
Dosage effect			0.108	0.258	0.005	0.010	0.145	0.503	0.090	0.159	0.001	0.498	0.997	0.233	0.603	0.558	0.707	0.123	0.823	0.092	0.491	0.502
Replication effect			0.469	0.263	0.338	0.555	0.582	0.863	0.151	0.318	0.325	0.869	0.331	0.684	0.583	0.366	0.692	0.329	0.889	0.161	0.444	0.749
Dose × replication			0.422	0.895	0.283	0.097	0.377	0.859	0.825	1.000	0.218	0.828	0.832	0.341	0.545	0.054	0.832	0.563	0.806	0.293	0.190	0.311
SEM[3]			15.13	3.42	0.16	11.33	21.85	18.91	3.62	0.22	11.40	24.28	18.51	2.65	0.30	6.56	20.88	10.17	1.90	0.22	8.64	12.50

[a-b]Means in a column not sharing a same superscript letter are different ($P < 0.05$).

[1]Data is the mean of 6 replicates per treatment.

[2]Treatment 1-5 received one challenge of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, and 0.8 mg/kg LPS on d 54, respectively. Treatment 6-10 received 2 challenges of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, 0.8 mg/kg LPS on d 54 and 55, respectively.

[3]Standard error of the mean.Abbreviations: CD, crypt depth; MT, mucosa thickness; VCR, ratio of villus height and crypt depth; VH, villus height; WT, wall thickness.

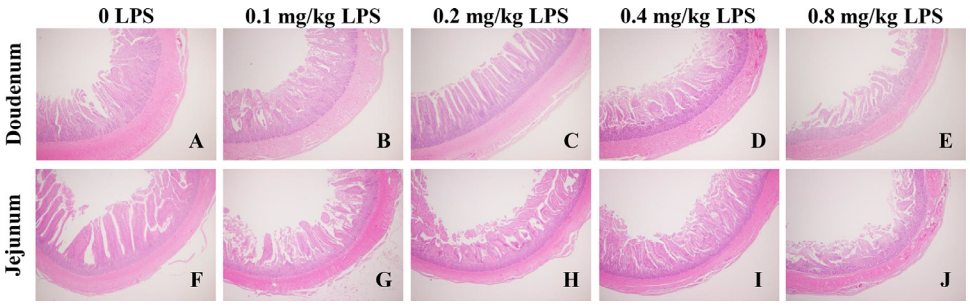

Figure 1. Representative images of H&E-stained duodenum and jejunum sections (40 × magnification).
(A-G): Duodenum sections treated with 0, 0.1, 0.2, 0.4, and 0.8 mg/kg LPS, respectively; (F-J): Jejunum sections treated with 0, 0.1, 0.2, 0.4, and 0.8 mg/kg LPS, respectively.

(Klasing et al., 1987), which was in line with our finding. However, Takahashi et al. (1995) reported that no changes in the growth or feed utilization were found in broilers under repeated injections of LPS. According to (Marais et al., 2011), both the dosage and the number of consecutive injections of LPS would elevate the levels of plasma corticosterone in birds. The corticosterone directedly or indirectly inhibited the synthesis and/or release of the proinflammatory cytokines and promoted the production of antioxidative cytokines, causing the development of LPS tolerance. However, ducks may have a delayed tolerance establishment for the fact that they continued to develop stress-related responses such as fever even after couple of injections of LPS (Marais et al., 2011). In the present study, the BWG of ducks decreased after 2 LPS injections regardless of the LPS dosages. The possible explanation might be that the experimental time frame of the present study was not long enough to stimulate the LPS tolerance; therefore, the continuous productions of cytokines took up energies and caused the decreases in the body weight gain.

In order to further understand the dosage effects of LPS in ducks, we continued to test the parameters related to the serum antioxidative and immune statuses, including the activities of SOD, and the concentrations of MDA, GSH. IgA, IL-6, and IL-1β in serums. It was confirmed that the tolerance was not established by one or 2 replicates of LPS challenges in ducks, as the replication effects were not noticed on any tested parameters relating to the oxidative status. Significant dosage effects of LPS were found on the activities of SOD ($P = 0.034$), and 0.4 mg/kg LPS treated groups showed the highest SOD levels among all groups, which demonstrated distinct oxidative stress responses. Our results were consistent with previous study that both one and 2 replications of 0.5 mg/kg LPS injections increased the SOD activities in the peripheral blood of 10-week-old layers (Perez et al., 2017). The mechanism of the dosage-dependent manner of LPS inducing the oxidative stress might be that the accumulation of LPS increased the production of reactive oxygen species in the neutrophils and mitochondria, which caused the redox imbalance in the organism (Gessner et al., 2016). On the other hand, serum IgA levels were affected by LPS dosages ($P = 0.007$), and it seemed that higher LPS dosages resulted in lower IgA levels. It could be explained that LPS were able to modulate the cytidine deaminase and influence the IgA production in the B cells (Park, 2005), and IgA level would decline under the severe immune stress caused by LPS infections (Awad et al., 2013). Interestingly, serum IL-1β levels were not influenced by the LPS dosages ($P = 0.507$) but by the challenge replications, that 2 replications of challenges increased the serum levels of IL-1β comparing to the groups receiving one challenge ($P = 0.010$). It was reported that in macrophages activated by LPS, lots of biosynthetic precursors were needed for the LPS stimulated modulations, including the proinflammatory cytokines productions. And these precursors were provided by the tricarboxylic acid cycle in a time dependent manner (Palsson-Mcdermott et al., 2015). In the present study, thought the 2 replicates of LPS did not establish the tolerance in the ducks, it provided time for the preparation of the precursors and possibly promoted the production of the IL-1β. However, further studies were needed to confirm the hypothesis.

Considering the gastrointestinal tract was important for the nutrient utilization and immune function of the animal (Choct, 2009), we speculated that the dosage and/or replication effects of LPS might influence the intestinal morphology and permeability. In the present study, significant dosage effects of LPS were noticed on the VCR and WT in the duodenum ($P = 0.005$ and 0.010, respectively), and WT in the jejunum ($P = 0.001$). Besides, all tested LPS dosages lowered the measured parameters comparing to no LPS treated groups. Similar results were reported previously that deteriorating effects of LPS on the intestinal morphologies of the birds and mice were dosage dependent (Zhang et al., 2013; Chao et al., 2020; Li et al., 2020). It was also reported that LPS destroyed the function of the goblet cells in the intestine, which led to an obvious decrease in the mucus thickness and increased the permeability of the intestinal barrier (Loonen et al., 2014). Data showed that significant dosage effects and interaction of dosage and replication of LPS on all measured parameters relating to the intestinal permeability ($P < 0.050$), except for OCLN levels in the jejunum. Specifically, compared to no LPS treated groups, LPS demonstrated significant harms to the intestinal permeabilities. However, inconsistent differences were

Table 6. Parameters related to intestinal permeability in accordance with treatments.[1]

Item				Duodenum			Jejunum			Ileum			Cecum		
Treatment[2]	Dosage,mg/kg	Replication	DAO, U/mL	ZO-1, ng/g	OCLN, ng/g	CLDN, ng/g	ZO-1, ng/g	OCLN, ng/g	CLDN, ng/g	ZO-1, ng/g	OCLN, ng/g	CLDN, ng/g	ZO-1, ng/g	OCLN, ng/g	CLDN, ng/g
1	0	1	12.19^{d}	84.87^{a}	199.45^{a}	289.86^{a}	95.16^{a}	167.79^{ab}	328.28^{a}	93.13^{a}	190.25^{a}	299.72^{a}	84.49^{a}	168.70^{a}	316.90^{a}
2	0.1	1	22.55^{a}	60.43^{e}	106.66^{e}	189.79^{e}	58.87^{d}	117.52^{d}	194.46^{d}	60.63^{d}	107.62^{e}	183.69^{e}	55.99^{d}	123.02^{b}	169.82^{d}
3	0.2	1	14.66^{cd}	70.82^{abcde}	169.16^{bc}	267.72^{abc}	80.58^{abc}	150.66^{bc}	295.74^{ab}	85.69^{ab}	153.54^{bcd}	257.29^{abcd}	73.35^{abc}	165.68^{a}	297.07^{ab}
4	0.4	1	19.18^{b}	66.09^{de}	135.46^{d}	208.77^{de}	69.25^{cd}	143.78^{bcd}	243.56^{cd}	65.65^{cd}	134.09^{de}	220.65^{de}	64.86^{cd}	120.20^{b}	236.49^{c}
5	0.8	1	17.35^{bc}	70.46^{bcde}	136.34^{d}	251.92^{abcd}	72.45^{cd}	138.27^{cd}	221.94^{cd}	68.37^{cd}	144.10^{cd}	239.53^{cd}	73.20^{abc}	151.26^{a}	250.53^{bc}
6	0	2	12.49^{d}	81.27^{abc}	196.29^{a}	278.53^{abc}	91.28^{ab}	162.05^{abc}	313.45^{ab}	88.54^{ab}	179.41^{ab}	289.38^{ab}	80.97^{a}	164.71^{a}	301.12^{ab}
7	0.1	2	15.16^{c}	82.89^{ab}	166.53^{bc}	284.83^{ab}	87.05^{ab}	179.30^{a}	268.73^{bcd}	82.32^{ab}	167.35^{abc}	286.92^{abc}	80.47^{a}	160.62^{a}	270.77^{abc}
8	0.2	2	19.41^{b}	62.40^{e}	139.75^{d}	228.94^{cde}	69.04^{cd}	120.15^{d}	234.42^{cd}	64.90^{d}	137.25^{cd}	233.54^{d}	68.21^{bc}	149.81^{a}	250.39^{bc}
9	0.4	2	18.92^{b}	68.91^{cde}	144.58^{cd}	235.38^{bcde}	69.64^{cd}	141.26^{bcd}	212.51^{d}	78.16^{bcd}	142.03^{cd}	246.21^{bcd}	68.68^{bc}	153.26^{a}	229.47^{c}
10	0.8	2	15.10^{c}	77.87^{abcd}	174.55^{ab}	287.01^{a}	79.48^{bc}	167.13^{ab}	296.60^{ab}	86.89^{ab}	164.18^{abcd}	287.96^{abc}	75.77^{ab}	161.17^{a}	258.14^{bc}
Main effect	Dosage														
	0		12.35^{c}	82.87^{a}	197.69^{a}	273.56^{a}	93.00^{a}	164.60	320.04^{a}	90.58^{a}	184.23^{a}	293.98^{a}	82.53^{a}	166.48^{a}	308.13^{a}
	0.1		18.85^{a}	71.66^{b}	136.60^{c}	237.31^{b}	72.96^{b}	148.41	231.60^{bc}	71.47^{b}	137.48^{b}	235.30^{b}	68.23^{bc}	143.91^{bc}	220.29^{c}
	0.2		17.03^{b}	66.61^{b}	154.46^{bc}	248.33^{ab}	74.81^{b}	135.41	265.08^{b}	75.30^{b}	145.40^{b}	245.41^{b}	70.78^{bc}	157.75^{ab}	273.73^{ab}
	0.4		19.05^{a}	67.50^{b}	140.02^{bc}	222.08^{b}	69.45^{b}	142.52	228.03^{c}	71.90^{b}	138.06^{b}	233.43^{b}	66.77^{c}	138.56^{c}	232.98^{c}
	0.8		16.22^{b}	74.16^{ab}	155.45^{b}	269.47^{ab}	75.96^{b}	152.70	259.27^{bc}	77.63^{b}	154.14^{b}	263.74^{ab}	74.49^{b}	156.22^{ab}	254.33^{bc}
		Replication													
		1	17.39	66.94	147.33^{b}	239.60	74.43	142.60	253.82	73.93	144.87	237.70^{b}	69.79	146.93^{b}	251.55
		2	16.21	74.67	164.34^{a}	262.94	79.30	153.98	265.14	80.16	158.04	268.80^{a}	74.82	157.91^{a}	261.98
Source of variance															
Dosage effect			< 0.001	0.011	< 0.001	0.011	< 0.001	0.059	< 0.001	0.001	0.001	0.008	0.001	0.011	< 0.001
Replication effect			0.069	0.172	0.015	0.067	0.197	0.102	0.454	0.062	0.093	0.014	0.071	0.034	0.510
Dose × replication			< 0.001	0.022	< 0.001	0.007	0.003	< 0.001	< 0.001	< 0.001	0.011	0.006	0.003	0.016	0.004
SEM[3]			1.13	3.93	10.08	15.53	4.58	8.76	17.61	4.49	10.04	15.69	3.49	7.25	17.26

[a-c]Means in a column not sharing a same superscript letter are different ($P < 0.05$).

[1]Data is the mean of 6 replicates per treatment.

[2]Treatment 1-5 received one challenge of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, and 0.8 mg/kg LPS on d 54, respectively. Treatment 6-10 received 2 challenges of LPS at dosage of 0 (saline), 0.1, 0.2, 0.4, 0.8 mg/kg LPS on d 54 and 55, respectively.

[3]Standard error of the mean.Abbreviations: CLDN, claudin; DAO, diamine oxidase; OCLD, occludin; ZO-1, zonula occludens 1.

noticed among LPS treated groups with uncertain mechanisms. These results partially confirmed with the changes in the intestinal morphology associated with LPS dosages. And similar outcomes were reported in rats (Bein et al., 2017) and broilers (Wu et al., 2013). Interestingly, replications of LPS challenge also affected some parameters, that OCLN levels in the duodenum ($P = 0.015$) and the cecum ($P = 0.034$), and CLDN levels in the ileum ($P = 0.014$) increased with 2 replicates of LPS challenges comparing to the ones receiving one challenge. These results were opposite to our expectations. Chen et al. (2018) also stated increased CLDN levels in the intestinal tissue of broilers with multiple LPS challenges. TJPs were a multiprotein complexes composed of transmembrane proteins, peripheral membrane proteins and regulatory molecules, of which CLDN and OCLN belonged to the transmembrane proteins (Turner, 2009). The possible reason for the increased CLDN and OCLN levels with 2 replicates of LPS challenges could be associated with the compensatory reaction, whereas the decreased levels of other TJPs were due to the direct loss by the LPS challenges (Chen et al., 2018).

In conclusion, this study indicated significant effects of dosage and/or replications of LPS challenge on the parameters associated with the body weight gain, serum antioxidative and immune status, and intestinal morphology and permeability of *Linwu* ducks. We concluded that 2 replicates of LPS challenges at the dosage of 0.4 mg/kg would not induce the LPS tolerance, but were able to deteriorate the growth performance, induce oxidative and immunological stress and damage the intestinal morphology and permeability of *Linwu* ducks at the growing stage.

DISCLOSURES

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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