



Effects of dietary gallic acid on growth performance, diarrhea incidence, intestinal morphology, plasma antioxidant indices, and immune response in weaned piglets

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ARTICLE INFO

Keywords:

Diarrhea incidence
Gallic acid
Inflammatory cytokine
Intestinal morphology
Lipopolysaccharide
Weaned piglets

ABSTRACT

This study was conducted to evaluate the effects of dietary gallic acid (GA) at 100, 200, or 400 mg/kg on growth performance, diarrhea incidence, intestinal morphology, plasma antioxidant indices, and immune response in weaned piglets. A total of 96 male weaned piglets (25 ± 1 days of age) with an initial body weight of 8.40 kg were allocated into 6 replicated pens (4 piglets per pen) for each of the 4 experimental treatments. On day 21, 24 piglets (1 piglet/pen) received an intraperitoneal lipopolysaccharide (LPS) injection. The blood samples were collected before (0 h) and 4 h after LPS challenge, and the piglets were slaughtered 4 h postchallenge to collect small intestinal samples. Dietary GA supplementation did not affect the growth performance of piglets ($P > 0.05$), but reduced diarrhea incidence was observed in piglets administered 400 mg/kg dietary GA compared with piglets fed a control diet ($P < 0.01$). In the jejunum, dietary GA at 100–400 mg/kg increased the ratio of villus height to crypt depth ($P < 0.01$), while dietary GA at 200 and 400 mg/kg reduced crypt depth ($P < 0.01$), and dietary GA at 100 mg/kg increased jejunum villus height ($P < 0.01$). In the ileum, dietary GA at 100 mg/kg increased the V:C ratio ($P = 0.029$). LPS injection reduced the activities of catalase, glutathione peroxidase and superoxide dismutase ($P < 0.01$) and increased the production of malondialdehyde ($P = 0.054$) and the contents of immunoglobulin (Ig) A, IgM, tumor necrosis factor (TNF)- α , and interleukin (IL)-8 ($P < 0.01$) in the plasma of piglets. However, there was no effect of diet on plasma antioxidant and immune indices ($P > 0.05$). Dietary GA linearly increased ileal secretory IgA content ($P = 0.053$). Moreover, dietary GA supplementation showed a linear effect on the expression *TNF- α* ($P < 0.01$); supplementation of 400 mg/kg GA significantly downregulated the expression of *TNF- α* in the ileal mucosa ($P < 0.01$). The trend analysis showed a linear effect of dietary GA supplementation on the ileal expression of *IL-6* ($P = 0.063$), *NF- κ B1* ($P = 0.045$), and *RelA* ($P = 0.062$). In conclusion, dietary supplementation with GA in the basal diet contained 3000 mg ZnO /kg attenuated postweaning diarrhea and protected intestinal integrity, which may be attributed to the modulation of the intestinal local immune response by GA. Our results have implications for developing new strategies to enhance gut health and to decrease diarrhea in weaned piglets.

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; C, crypt depth; CAT, catalase; GA, gallic acid; G:F, gain-to-feed ratio; GSH-Px, glutathione peroxidase; IL, interleukin; I.P., intraperitoneal; LPS, lipopolysaccharide; MDA, malondialdehyde; sIgA, secretory IgA; SOD, superoxide dismutase; TNF- α , tumor necrosis factor- α ; V, villus height; V:C, villus height-to-crypt depth ratio

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<https://doi.org/10.1016/j.anifeedsci.2020.114391>

Received 9 February 2019; Received in revised form 31 December 2019; Accepted 3 January 2020
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1. Introduction

Weaning is characterized as a stressful period in swine production, which is associated with marked changes in gut morphology and increased proinflammatory cytokines in the gastrointestinal tract (Pluske et al., 1996; Pié et al., 2004), which consequently results in increased infection susceptibility and increased incidence of diarrhea (Moraes et al., 2012). Postweaning diarrhea causes serious economic losses in the swine industry due to the increased mortality and morbidity and the reduced growth performance of surviving pigs (Jiang et al., 2015a).

It has long been known that phenolic compounds are major bioactive phytochemicals and are widely distributed in plants, exhibiting antioxidant and antimicrobial properties (Bravo, 1998; Kim et al., 2009; Zhang et al., 2014) and have health benefits (Nour et al., 2012). Gallic acid (GA) is a type of phenolic compound present in persimmon, wine, and herbs (Hogan et al., 2009). Previous studies have demonstrated the pharmacological and health benefits of GA, including antioxidant, anti-inflammation, antibacterial, and anticancer properties (Yen et al., 2002; Kim, 2007; Kang et al., 2008; Kaur et al., 2009; Borges et al., 2013; Verma et al., 2013; Jiang et al., 2015; Pandurangan et al., 2015; Shao et al., 2015). In addition, Samuel et al. (2017) observed decreased plasma MDA (lipid peroxidation byproduct) content in broiler chicks with GA supplementation. Dietary GA has been reported to regulate growth and to improve systemic health when used as a supplement to broiler chick and ruminant diets (Wei et al., 2016; Samuel et al., 2017); however, there is limited information concerning the effects of GA on the production performance and health of weaned piglets.

As an effective immune challenge tool, *E. coli* LPS inflammation models have been used to assess acute-phase response, antioxidant and anti-inflammatory properties in pigs (Bissonnette et al., 2016). We hypothesized that dietary GA supplementation enhances systemic and/or intestinal defense and therefore improves growth performance and reduces diarrhea incidence in weaned piglets. Therefore, the current study was conducted to investigate the efficacy of dietary supplementation with GA on growth performance, diarrhea incidence, and intestinal morphology in weaned piglets. In addition, antioxidant indices and immune responses in the plasma and the gene expression of inflammatory mediators in the ileum were also determined under an i.p. LPS challenge. Our results may potentially help to inform the management of piglets in the weaning period, in which stressful agents are especially common.

2. Materials and methods

The animal protocol for this research was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences.

2.1. Experimental design and animal management

The experiment was carried out at the Langfang Experimental Station of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. A total of 96 healthy weaned crossbred barrows (Duroc × Landrace × Yorkshire), with similar initial body weight (BW, 8.40 ± 0.09 kg) and age (25 ± 1 days), were randomly assigned to 4 treatment groups that were fed basal diets of corn-soybean meal supplemented with 0, 100, 200, or 400 mg/kg of GA. The control group (CTR) was fed a basal diet without GA. The GA used in the experiment was a synthetic product (purity ≥ 900 g/kg) was purchased from Kemin Industries (Zhuhai) Co., Ltd. (Zhuhai, Guangdong, China). There were 6 replicate pens per treatment and 4 piglets per replicate. During the experiment, the piglets were allowed *ad libitum* access to water and feed, and the pens were regularly cleaned. A combination of daylight and artificial light was used, and ventilation was achieved by using variable-speed fans. The starting temperature of 28 °C was adjusted weekly to reach a final temperature of 26 °C. Piglets were housed in pens (4 piglets/pen), located beside an 80 cm walkway with 12 pens (2.00×2.00 m²) on each side, with a slatted floor. Each pen was equipped with two water nipples and a feed trough. The diet for the piglets was formulated according to the National Research Council (2012) nutrient requirements, and the composition and nutrient levels of the basal diets are shown in Table 1. The diet for the piglets was manufactured 1 week before the start of the trial, without the inclusion of any antibiotic growth promoters, and 3000 mg/kg zinc oxide was included in the diets. The levels of dry matter, crude protein, ash, NDF, ADF, calcium and phosphorus in the feed were determined as described by Jiang et al. (2015b).

2.2. Growth performance and diarrhea incidence measurements

Body weight was recorded on the weaning day (day 1) and at the end of the trial (day 21). Feed intake was recorded daily, and the residual feed was measured at the end of the trial. Growth performance was evaluated by calculating the average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F) for each pen.

To determine the incidence of diarrhea, fecal scores were monitored daily by visually appraising each subject using the following five-point fecal consistency scoring system: 1 = hard, dry pellet; 2 = firm, formed stool; 3 = soft, moist stool that retains its shape; 4 = soft, unformed stool; and 5 = watery liquid that can be poured. A liquid consistency (score 4–5) was considered indicative of diarrhea (Jiang et al., 2015a,b). The incidence of diarrhea (%) was calculated as a percentage of the number of piglets with diarrhea divided by the total number of weaned piglets.

2.3. LPS challenge and sample collection

At the end of the trial (day 21), 1 piglet per pen (total number = 24) with a body weight close to the pen average was selected and

Table 1
Ingredient and nutrient composition of the basal diet (as fed basis).

Item	Amount, g/kg
Ingredients	
Corn	223.7
Extruded corn	235.8
Soybean meal	150.0
Extruded soybean	145.0
Whey	150.0
Fish meal	55.0
Soybean oil	5.0
Dicalcium phosphate	5.5
Limestone (CaCO ₃)	6.5
Salt	2.5
Choline chloride (60 %)	0.8
L-Lysine HCl	5.5
DL-Methionine	0.9
Threonine	0.8
Vitamin and mineral premix ¹	10.0
Zinc oxide	3.0
Analyzed nutrient content	
Dry matter, g/kg	910.7
Crude protein, g/kg	195.6
Calcium, g/kg	8.5
Phosphorus, g/kg	6.8
Ash, g/kg	61.5
NDF, g/kg	155.5
ADF, g/kg	22.2
Calculated nutrient content	
Metabolizable energy, MJ/kg	14.23
Lysine, g/kg	13.0
Methionine, g/kg	4.0
Threonine, g/kg	7.3
Tryptophan, g/kg	2.0

¹ Premix supplied per kg of diet: vitamin A, 35.2 mg; vitamin D₃, 7.68 mg; vitamin E, 128 mg; vitamin K₃, 8.16 mg; vitamin B₁, 4 mg; vitamin B₂, 12 mg; vitamin B₆, 8.32 mg; vitamin B₁₂, 4.8 mg; niacin, 38.4 mg; calcium pantothenate, 25 mg; folic acid, 1.68 mg; biotin, 0.16 mg; iron (FeSO₄ · H₂O), 171 mg; manganese (MnSO₄·H₂O), 42.31 mg; copper (CuSO₄ · 5H₂O), 125 mg; selenium (Na₂SeO₃), 0.19 mg; cobalt (CoCl₂), 0.19 mg; iodine (Ca(IO₃)₂), 0.54 mg.

given an intraperitoneal (i.p.) injection of LPS (*E. coli* serotype O55: B5, Sigma-Aldrich, St Louis, MO) at 100 µg/kg BW. The LPS was dissolved in a sterile 9 g/L NaCl solution (500 mg LPS/L saline). All piglets were deprived of feed after the i.p. LPS challenge until slaughter but were allowed *ad libitum* access to water.

At day 21, blood samples were taken by venipuncture from the jugular vein of the selected challenged piglets before (0 h) and 4 h after the i.p. LPS injection to evaluate plasma indices. Blood samples were centrifuged (3000 × g for 10 min) to separate plasma that was stored at -20 °C until analysis. After the blood collection was completed, all piglets were euthanized by intravenous injection of pentobarbital sodium (6 mg/kg BW).

At necropsy, a length of approximately 3 cm of the jejunum (50 cm from the pylorus) and 15 cm of the ileum (10 cm from the caecum) of each piglet were collected. The jejunum and the first approximately 3 cm segment of the ileum were fixed in fresh 4 % paraformaldehyde for at least 24 h and then embedded in paraffin. The remaining segment of the ileum was cut longitudinally expose the mucosa and washed 3 times in PBS to remove the mucus and digesta. Then, the mucosa was gently scraped off with a glass microscope slide, placed in a sterile cryogenic vial (Corning Incorporated, NY, USA), rapidly frozen in liquid nitrogen and then stored at -80 °C until use for RNA extraction and secretory IgA determination.

2.4. Intestinal morphology

The jejunum and ileum specimens were dehydrated in a graded ethanol series, cleared with xylene and embedded in paraffin. Then, three pieces of 5 µm thick sections were prepared from the intestinal samples, stained with hematoxylin-eosin, and observed under an optical microscope. Ten fields were randomly selected to measure the villus height and the crypt depth, and the villus height-to-crypt depth ratio (V:C) was calculated. For the purpose of statistical analysis, the average of these values was used.

2.5. Assay of plasma antioxidant indices

Plasma antioxidant indices were measured spectrophotometrically with commercial assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, catalase (CAT) activity was measured with ammonium molybdate, and the change in absorbance was recorded at 405 nm. Glutathione peroxidase (GSH-Px) activity was measured with 5,5-dithiobis-p-nitrobenzoic acid, and the change in absorbance at 412 nm was recorded. Superoxide dismutase (SOD) activity was measured through a nonenzymatic NBT test, which measures the inhibition of the formation of superoxide anion free radicals that reduce the nitroblue tetrazolium of the sample. The change in absorbance at 450 nm was recorded. Malondialdehyde (MDA) concentration was analyzed with 2-thiobarbituric acid, and the change in absorbance was read at 532 nm.

2.6. Plasma immunoglobulin measurement

Immunoglobulin (Ig) A and M contents in plasma samples were measured using commercial ELISA kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Standard curves were generated using serial dilutions of the recombinant porcine immunoglobulin. The intra-assay coefficients of variation for IgA and IgM were 5.0 % and 7.3 %, respectively. The interassay coefficients of variation (average of category) for IgA and IgM were 5.0 % and 8.5 %, respectively. The results were expressed in mg/mL based on a standard curve.

2.7. Quantification of cytokines in plasma

Quantification of tumor necrosis factor (TNF)- α and interleukin (IL)-8 in plasma samples was assayed in duplicate using commercial porcine ELISA kits according to the protocols provided by the manufacturer (R&D Systems, Minneapolis, MN). Standard curves were generated using serial dilutions of the recombinant porcine cytokine. The intra-assay coefficients of variation for TNF- α and IL-8 were 4.6 % and 6.9 %, respectively. The interassay coefficients of variation for TNF- α and IL-8 were 4.5 % and 6.6 %, respectively. The results were expressed in pg/mL based on a standard curve.

2.8. Secretory IgA (sIgA) content in ileal mucosa

One milliliter of 4 °C PBS (including 10 microliters PMSF, a kind of protease inhibitor) was added to 50 mg of ileal mucosa and fully ground in a glass homogenizer. The homogenates were centrifuged ($4000 \times g$ for 15 min at 4 °C), and the supernatant was collected to measure the sIgA content using commercial ELISA kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The intra-assay and interassay coefficients of variation for sIgA were 6.5 % and 7.0 %, respectively. In addition, the protein in the supernatant of the ileal mucosa was quantified by the BCA method, and the final measurement of sIgA content in the ileal mucosa was normalized to total protein content ($\mu\text{g/g}$ protein).

2.9. Quantitative real-time PCR

Total RNA was extracted from ileal mucosa samples with Trizol reagent (Thermo Fisher Scientific, Inc., Boston, MA) according to the manufacturer's protocol. The concentration and purity of RNA were determined by a microplate reader, and the OD260/OD280 was between 1.8 and 2.0. Reverse transcription was performed with a Super Script III First-Strand Synthesis Super Mix reagent kit (Thermo Fisher Scientific, Inc., Boston, MA). The homology of each primer was evaluated by an NCBI BLAST search to ensure that it was specific for the target mRNA transcript. *GAPDH* was used as a control gene. The primers used for real-time PCR are presented in Table 2. Quantitative analysis of PCR was carried out on a QuantStudio Real-Time PCR System (Thermo Fisher Scientific, MA, USA) with a SYBR Green reagent. The levels of interleukin (*IL*)-1 β , *IL*-6, *IL*-8, tumor necrosis factor (*TNF*)- α , Cyclooxygenase-2 (*Cox*-2), *NF*- κ B1 (p50), *RelA* (p65) and *GAPDH* in the ileal mucosa samples were subsequently measured. The comparative CT method was used (Livak and Schmittgen, 2001) to determine the fold changes in gene expression and was calculated as $2^{-\Delta\Delta CT}$. The relative expression of each target gene was normalized to the mRNA levels of the *GAPDH* gene.

Table 2

List of primers used for real-time PCR.

Accession No.	Gene	Forward primers (5'-3')	Reverse primers (5'-3')	Product Length, bp
NM_001206359.1	<i>GAPDH</i>	GCTTGTCAATGGAAGG	CATACGTAGCACCAGCATCA	86
NM_214022.1	<i>TNF-α</i>	TGGCCCTTGAGCATCA	CGGGCTTATCTGAGGTTTGAGA	68
NM_214055.1	<i>IL-1β</i>	GTGGACTGCAAACTCCAGGACAAA	GCTCATGCAGAACACCACTTCTCT	119
NM_214399.1	<i>IL-6</i>	AATGTCGAGGCTGTGCAGATT	TGGTGGCTTTGTCTGGATTCT	82
NM_213867.1	<i>IL-8</i>	CCGTGTCAACATGACTTCCAA	GCCTCACAGAGAGCTGCAGAA	75
NM_214321.1	<i>COX-2</i>	AAGCGAGGACCAGCTTTCACAAA	GCGCAGTTTATGCTGTCTCTCCAA	93
NM_001048232.1	<i>NF-κB1</i>	GTAAGAATGGACAGGACAGC	CTGGATGTCATCTTCTGAA	87
NM_001114281.1	<i>RelA</i>	GCGATGAGATCTTCTGCTG	GCCACTTGTGCGTGACGCTC	121

Table 3Effect of dietary gallic acid (GA) supplementation on growth performance of weaned piglets.¹

Item	GA level (mg/kg feed)				P-value ²		
	0	100	200	400	SEM	Linear	Quadratic
Day 1–21							
ADG, g	200	224	207	190	16.0	0.455	0.337
ADFI, g	326	342	333	312	17.6	0.452	0.427
G:F, g/g	0.61	0.66	0.61	0.62	0.038	0.879	0.700

ADFI = average daily feed intake; ADG = average daily gain; G:F = gain-to-feed ratio;

¹ n = 24 (6 pens/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.² Orthogonal polynomial contrast was used to determine the effect of dietary GA levels.

2.10. Statistical analysis

The data related to growth performance, intestinal morphology and gene expression were analyzed by one-way ANOVA using the GLM procedure of SAS v. 9.2 (SAS Institute, 2009 Cary, NC). The model included the treatment effect, and the pen represented the experimental unit for growth performance, while individual piglets were the experimental unit for intestinal morphology and gene expression. The dose-related effect of supplemental GA was computed by GLM, using contrast command for the linear and quadratic effects. In addition, the results of plasma antioxidant capacity, immunoglobulin, and inflammatory cytokines were analyzed by repeated measures over time using the MIXED procedure, where the piglet was the experimental unit. Treatment comparisons were performed using Tukey's honest significant difference test for multiple testing. Moreover, the Chi-square test was used to analyze diarrhea incidence. Differences were considered statistically significant at $P \leq 0.05$, whereas a treatment effect trend was noted at $0.05 < P \leq 0.10$.

3. Results

3.1. Growth performance and diarrhea incidence

The effect of dietary GA on growth performance and diarrhea incidence of weaned piglets is shown in Table 3. No significant effect on ADG, ADFI, or G:F was observed ($P > 0.05$). Compared with piglets in the CTR group, piglets provided dietary GA at 400 mg/kg showed significantly reduced diarrhea incidence from day 1–10, from day 11–21 and from day 1–21 ($P < 0.01$; Table 4). Compared with the CTR group, the group supplemented with 100 mg/kg GA exhibited significantly reduced diarrhea incidence from day 11–21 ($P < 0.01$), while 100 mg/kg GA supplementation tended to reduce diarrhea incidence from day 1–21 ($P = 0.074$), and 200 mg/kg GA supplementation tended to reduce diarrhea incidence from day 1–21 ($P = 0.057$).

3.2. Intestinal morphology

The results of the effect of dietary GA on the jejunum and ileum morphology of weaned piglets are displayed in Table 5. In the jejunum, the ratio of villus height to crypt depth was increased ($P < 0.01$) in piglets administered dietary GA at 100–400 mg/kg compared with those fed the control diet. In addition, the jejunum crypt depth was reduced ($P < 0.01$) in piglets administered dietary GA at levels 200 and 400 mg/kg compared with those fed the control diet. Compared with the control diet, supplementation of GA at 100 mg/kg increased jejunum villus height (contrast, $P < 0.01$) and tended to decrease jejunum crypt depth (contrast, $P = 0.079$). Both linear ($P < 0.01$; $P < 0.01$) and quadratic ($P = 0.015$; $P < 0.01$) effects were observed on crypt depth and the ratio of villus height to crypt depth in the jejunum in response to dietary GA supplementation.

In the ileum, the ratio of villus height to crypt depth was increased ($P = 0.029$) in piglets fed dietary GA at 100 mg/kg compared

Table 4Effect of dietary gallic acid (GA) supplementation on diarrhea incidence of weaned piglets.¹

Item	GA level (mg/kg feed)				P-value
	0	100	200	400	
Diarrhea incidence, %					
Day 1–10	15.42 ^a	14.17 ^a	10.83 ^a	5.56 ^b	< 0.01
Day 11–21	9.17 ^a	3.33 ^b	6.25 ^{ab,x}	2.64 ^{b,y}	< 0.01
Day 1–21	12.29 ^{a,x}	8.75 ^{a,y}	8.54 ^{a,y}	4.12 ^b	< 0.01

^{a,b} Means listed in the same row with different superscripts are significantly different ($P \leq 0.05$).^{x,y} Means listed in the same row with different superscripts are tended to be different ($0.05 < P \leq 0.10$).¹ n = 24 (6 pens/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.

Table 5Effect of dietary gallic acid (GA) supplementation on intestinal morphology of weaned piglets.¹

Item	GA level (mg/kg feed)				P-value ²		
	0	100	200	400	SEM	Linear	Quadratic
Jejunum							
Villus height, μm	321 ^b	356 ^a	327 ^b	335 ^{ab}	6.0	0.713	0.137
Crypt depth, μm	222 ^{a,x}	204 ^{ab,y}	193 ^b	194 ^b	4.4	< 0.01	0.015
V:C, $\mu\text{m}/\mu\text{m}$	1.45 ^b	1.74 ^a	1.70 ^a	1.73 ^a	0.033	< 0.01	< 0.01
Ileum							
Villus height, μm	314	338	329	326	11.2	0.737	0.295
Crypt depth, μm	206	190	193	190	6.8	0.232	0.325
V:C, $\mu\text{m}/\mu\text{m}$	1.52 ^b	1.78 ^a	1.72 ^{ab}	1.71 ^{ab}	0.057	0.118	0.033

^{a,b} Means listed in the same row with different superscripts are significantly different ($P \leq 0.05$).^{x,y} Means listed in the same row with different superscripts are tended to be different ($0.05 < P \leq 0.10$).¹ n = 24 (6 piglets/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.² Orthogonal polynomial contrast was used to determine the effect of dietary GA levels.

with those fed the control diet, and a quadratic ($P = 0.033$) effect was observed on the ratio of villus height to crypt depth in response to dietary GA supplementation. However, villus height and crypt depth were not affected by the addition of GA to the diet of weaned piglets ($P > 0.05$).

3.3. Plasma antioxidant capacity in LPS-challenged piglets

The effect of dietary GA on the plasma antioxidant capacity of weaned piglets before and 4 h after LPS challenge is presented in Table 6. There was no interaction ($P > 0.05$) between dietary treatment and time on plasma antioxidant capacity. The injection of LPS significantly reduced the activities of CAT, GSH-Px and SOD ($P < 0.01$) and increased MDA production ($P = 0.054$). However, no significant difference among the dietary treatments was observed ($P > 0.05$).

3.4. Plasma immunoglobulin in LPS-challenged piglets

Table 7 shows the effect of dietary GA supplementation on plasma immunoglobulin before and 4 h post LPS challenge. There was no interaction ($P > 0.05$) between dietary treatment and time on IgA and IgM contents. LPS injection significantly increased the contents of IgA and IgM ($P < 0.01$). However, no significant difference among the dietary treatments was observed ($P > 0.05$).

3.5. Plasma concentrations of cytokines in LPS-challenged piglets

The concentrations of TNF α and IL-8 in the plasma of piglets before LPS challenge were almost undetectable (data not shown), while they were dramatically increased 4 h after the i.p. injection of LPS ($P < 0.01$). However, dietary GA supplementation had no effect on the plasma concentration of these cytokines in piglets after LPS challenge (Fig. 1).

Table 6Effect of dietary gallic acid (GA) supplementation on plasma antioxidant capacity of weaned piglets challenged with lipopolysaccharide.¹

Item	GA level (mg/kg feed)				P-value			
	0	100	200	400	Pooled SEM	GA	Time (T)	GA $\times$ T
CAT, U/mL								
0 h	24.24	23.41	24.42	25.26	1.784	0.830	< 0.01	0.992
4 hpc	15.00	14.97	16.09	16.21				
MDA, nmol/mL								
0 h	2.81	2.62	2.64	2.62	0.287	0.281	0.054	0.634
4 hpc	3.59	2.74	3.21	2.82				
GSH-Px, U/mL								
0 h	503	492	498	487	8.3	0.297	< 0.01	0.201
4 hpc	420	415	441	435				
SOD, U/mL								
0 h	40.02	41.94	43.98	43.43	2.252	0.613	< 0.01	0.909
4 hpc	34.48	35.02	36.55	34.62				

CAT = catalase; MDA = malondialdehyde; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase.

¹ n = 24 (6 piglets/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.

Table 7Effect of dietary gallic acid (GA) supplementation on plasma immunoglobulin (Ig) contents of weaned piglets challenged with lipopolysaccharide.¹

Item	GA level (mg/kg feed)				P-value			
	0	100	200	400	Pooled SEM	GA	Time (T)	GA × T
IgA, mg/mL								
0 h	0.41	1.22	0.86	1.06	0.631	0.881	< 0.01	0.885
4 hpc	2.37	2.28	2.74	2.57				
IgM, mg/mL								
0 h	1.93	3.96	3.11	2.10	1.165	0.747	< 0.01	0.605
4 hpc	8.38	7.28	8.78	7.40				

¹ n = 24 (6 piglets/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.

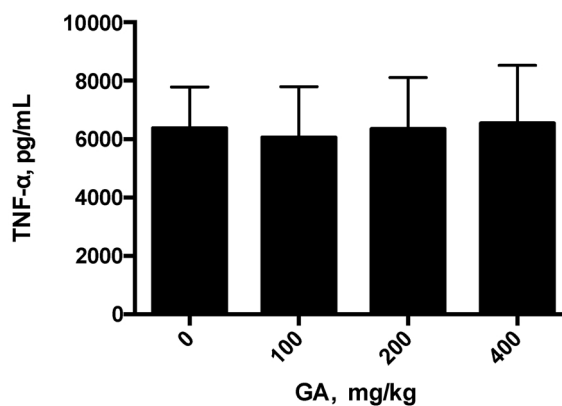
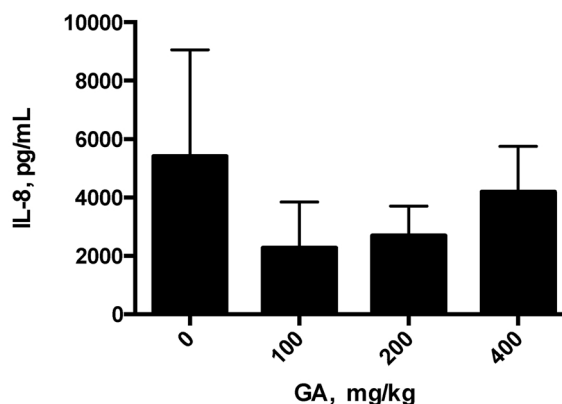
A**B**

Fig. 1. Effect of dietary gallic acid (GA) on plasma concentrations of (A) TNF-α and (B) IL8 in piglets 4 h after i. p. LPS challenge. Six piglets per treatment were used. Error bars correspond to the SE.

3.6. Ileal sIgA content

The effect of different doses of GA supplementation on sIgA content in the ileal mucosa of weaned piglets is reported in Table 8. Dietary GA had a tendency to linearly increase sIgA content ($P = 0.053$), but no significant difference among the treatments was observed ($P > 0.05$).

3.7. Ileal gene expression

The gene expression levels of inflammatory mediators in the ileal mucosa of weaned piglets are summarized in Table 9. Compared

Table 8Effect of dietary gallic acid (GA) supplementation on secretory IgA (sIgA) content in the ileal mucosa of weaned piglets.¹

Item	GA level (mg/kg feed)				P-value ²		
	0	100	200	400	SEM	Linear	Quadratic
sIgA, µg/g protein	1092	1334	1251	1668	171.8	0.053	0.790

¹ n = 24 (6 piglets/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen was intraperitoneally challenged with LPS on day 21 of the trial.

² Orthogonal polynomial contrast was used to determine the effect of dietary GA levels.

Table 9Effect of dietary gallic acid (GA) supplementation on gene expression of inflammatory mediators in the ileal mucosa of weaned piglets.^{1,2}

Item	GA level (mg/kg feed)				P-value ³		
	0	100	200	400	SEM	Linear	Quadratic
<i>TNF-α</i>	1.00 ^a	0.92 ^a	0.89 ^a	0.38 ^b	0.112	< 0.01	0.202
<i>IL-1β</i>	1.00	0.95	1.04	0.98	0.164	0.994	0.928
<i>IL-6</i>	1.00	1.02	0.94	0.60	0.160	0.063	0.437
<i>IL-8</i>	1.00	0.69	0.85	0.79	0.114	0.505	0.382
<i>COX-2</i>	1.00	0.78	0.86	0.83	0.147	0.665	0.587
<i>NF-κB1</i> (p50)	1.00	0.98	0.93	0.70	0.100	0.045	0.527
<i>RelA</i> (p65)	1.00	0.87	0.89	0.72	0.093	0.062	0.980

^{a,b} Means listed in the same row with different superscripts are significantly different ($P \leq 0.05$).

¹ n = 24 (6 piglets/treatment). Piglets were weaned at 25 ± 1 days of age, and one piglet in each pen were orally challenged with *E. coli* on day 21 of the trial.

² The relative expression of each target genes was normalized to mRNA levels of *GAPDH* gene. Data was expressed as fold change to control group of 0 mg/kg, which was set as 1.

³ Orthogonal polynomial contrast was used to determine the effect of dietary GA levels.

with the control diet, dietary GA supplementation at 400 mg/kg significantly downregulated the expression of *TNF-α* ($P < 0.01$), and GA supplementation also showed a linear effect on the expression *TNF-α* ($P < 0.01$). Dietary GA did not affect the expression of *IL-1β*, *IL-6*, *IL-8*, *COX-2*, *NF-κB1* or *RelA* ($P > 0.05$). However, the trend analysis showed a linear effect of dietary GA supplementation on the expression of *IL-6* ($P = 0.063$), *NF-κB1* ($P = 0.045$), and *RelA* ($P = 0.062$).

4. Discussion

The objectives of this study were to investigate whether the addition of GA to weaned piglets' diets would improve growth performance and reduce diarrhea incidence by enhancing systemic and/or intestinal defenses under an i.p. LPS challenge and whether there is a dose of GA with an optimum effect. It is quite common for piglets to present postweaning diarrhea in the early period after weaning. In the current study, we observed that the diarrhea incidence of piglets within 3 weeks postweaning was very high, which was attributed to the high incidence within the first 10 days; however, interestingly, animals fed 400 mg/kg GA showed significantly reduced diarrhea incidence. Both GA and grape seed procyanidins (GSPs) are active compounds of grape seed extracts and have similar physiological and biomedical functions due to their phenolic compounds (Kaur et al., 2009; Samuel et al., 2017). Hao et al. (2015) observed that supplementation with 50–150 mg/kg of GSPs decreased diarrhea incidence in weaned piglets. Song et al. (2011) demonstrated that dietary supplementation with 250 mg/kg GSPs had the potential to prevent weaning diarrhea by reducing intestinal permeability and improving antioxidant indices in rats. The current study indicated that GA could prevent diarrhea in weaned pigs, perhaps due to improvements in systemic defense and/or intestinal health. Therefore, further measurements were conducted to investigate our hypothesis and clarify the underlying mechanism.

In the present study, our results indicate that LPS challenge induced an acute systemic inflammatory response by increasing the production of proinflammatory cytokines (*TNF-α*) and chemokines (*IL-8*) and the contents of IgA and IgM in the plasma of piglets at 4 h after LPS inoculation. Similar to our study, it has been reported that intraperitoneal injection of LPS induces symptoms of acute bacterial infection (such as fever, lethargy, vomiting, etc.) in piglets, which leads to increased contents of inflammatory cytokines such as *TNF-α*, *IL-8* and *IL-6* in plasma and in some tissues (Leng et al., 2014; Bissonnette et al., 2016; Xiao et al., 2016). Cytokines play an important role in the regulation of intestinal function, while the overproduction of proinflammatory cytokines has a negative influence on intestinal homeostasis (Upadhyaya et al., 2015). Seeley et al. (2012) demonstrated that systemic inflammation develops secondary to local inflammation if proinflammatory substances such as proinflammatory cytokines do not successfully function in local inflammatory processes. Cytokines and immunoglobulin in the blood can reflect the immune system status of piglets (Xu et al., 2018). In addition, we observed that LPS challenge resulted in significant decreases in CAT, GSH-Px and SOD activities and an increase in MDA production in the plasma of piglets at 4 h after inoculation with LPS. The lower antioxidant enzymes and higher MDA content verified the imbalance in oxidative and antioxidant systems induced by LPS, which means that the redox balance of LPS-challenged piglets was destroyed. Cao et al. (2018) found that LPS injection reduced the activities of SOD and GSH-Px and

increased the content of MDA in the jejunum mucosa. Tanaka et al. (2018) demonstrated that GA exerted protective effects against LPS-induced inflammation and oxidative stress in RAW 264 macrophages by attenuating the expression of inflammatory mediators, the production of ROS, and the activation of mitogen-activated protein kinase and nuclear factor-kappa B and by increasing the expression of antioxidant enzymes. In contrast, our observations show that there was no dietary effect on the immune response and antioxidant indices in the blood before and after LPS challenge, which suggests that dietary GA may not affect the systemic defense in the blood of weaned piglets.

The intestine is an important digestive and immune organ in piglets, and the ratio of villus height to crypt depth represents the capacity of the small intestine for nutrient digestion and absorption (Pluske et al., 1996). A previous study demonstrated that the addition of GA or its derivatives to the diet can improve intestinal morphology and function in broiler chicks and then improve absorption function (Viveros et al., 2011; Yang et al., 2016; Samuel et al., 2017), but data on pigs in the literature are limited. In the present experiment, dietary supplementation of GA in piglets' diets from 100 to 400 mg/kg increased the V:C ratio in the ileum and jejunum of piglets by increasing villus height and decreasing crypt depth. Consistent with our study, Samuel et al. (2017) found that the addition of 50, 75, and 100 mg/kg GA to the diet could improve jejunum morphology by decreasing the villus depth and increasing the ratio of villus height to crypt depth in broilers, thereby improving digestion and absorption. Yang et al. (2016) found that feeding diets supplemented with 7.5 and 15 mg/kg grape proanthocyanidins could significantly reduce the crypt depth and increase the V:C ratio in the jejunum of broilers. Previous studies also reported increased V:C ratios in the ileum of weaned piglets fed essential oils (Jiang et al., 2015a,b). It has been well documented that a high villus and a short crypt can lead to improved nutrient absorption, decreased secretion in the gastrointestinal tract, increased disease resistance and improved gut health (Viveros et al., 2011). Accordingly, the increased villus height and V:C ratio observed in the current study may be partly the reason for the attenuated diarrhea incidence in weaned piglets supplemented with dietary GA.

The B cells in mucosa-associated lymphoid tissues secrete sIgA, the effector molecule of mucosal immunity and a major component of the local immune barrier of the intestine, which plays a crucial role in local immunity after exposure to antigens (Sun et al., 2013). In the current study, our results showed that sIgA content in the ileal mucosa of weaned piglets was linearly increased by dietary GA, which suggested that dietary GA might strengthen the local defense in the intestine against LPS infection. In the present study, dietary GA may have the potential to modulate the expression of proinflammatory mediators involved in the local immune defense against the LPS challenge. For instance, compared with the control group, the group administered dietary GA at 400 mg/kg significantly downregulated ileal *TNF- α* gene expression, and dietary GA linearly downregulated ileal *TNF- α* and *IL-6* gene expression. *TNF- α* is a multifunctional proinflammatory cytokine and has many actions, including the activation and induction of respiratory burst and degranulation, promotion oxygen free radical release, enhancement of neutrophil phagocytosis, and promotion of adhesion factor expression in neutrophils. Our results suggest that compared with a control diet, the administration of GA, particularly at 400 mg/kg, inhibits inflammation. In accordance with our observations, Jiang et al. (2015) reported that GA significantly inhibited *TNF- α* release by neutrophils in the blood of rats. Nuclear factor- κ B (NF- κ B) is a transcription factor that plays an important role in controlling the expression of proinflammatory genes, including *TNF- α* . In the current study, we observed that dietary GA linearly downregulated the expression of *NF- κ B1* (p50 subunit) and *RelA* (p65 subunit), suggesting that the suppression of *TNF- α* expression is partly attributed to the inhibition of *NF- κ B* activation (Zhao et al., 2004). Thus, the reduced diarrhea in piglets in the GA groups may be attributed to the modulated local defense in the intestine.

The obtained positive results on diarrhea incidence, gut morphology, and proinflammatory mediators in postweaning piglets were expected to lead to improve the growth performance of weaned piglets. However, the addition of dietary GA did not significantly affect growth performance within 21 days postweaning in our study. Although there is little published information about piglet postweaning diets supplemented with GA, previous studies reported that supplementation with GSPs did not significantly affect the growth performance of weaned piglets (Zhao et al., 2013; Hao et al., 2015). Therefore, we speculated that the lack of dietary GA-induced effect on growth might be due to the number of replicates, the experimental period and the administered dose of GA. Moreover, the lack of a challenge over experimental animals with pathogens can be another reason, as pathogen challenge can impair the health status of piglets and enhance the positive effect of additives on gut environments and eventually increase growth performance during a stressful challenge. In our study, a high dose of ZnO (3000 mg/kg) was supplemented in the basal diet. The pharmacological levels (up to 3000 mg/kg) have been well documented as an effective dietary tool to ameliorate and (or) prevent post-weaning diarrhea and improve the growth performance and gut health of weaned piglets (Heo et al., 2012; Li et al., 2018). Therefore, additional studies are needed to confirm whether GA has a beneficial role in the growth performance of weaned piglets under challenge situation or fed with low dose of ZnO.

5. Conclusions

The current study observed that dietary gallic acid at 400 mg/kg in the basal diet contained 3000 mg ZnO /kg decreased the diarrhea incidence of weaned piglets and that dietary GA positively modulated intestinal morphology. Dietary GA had no significant effect on plasma antioxidant and immune indices, but it increased sIgA content and downregulated proinflammatory cytokines in the ileal mucosa. Therefore, our study suggests that dietary GA attenuated diarrhea incidence and protected intestinal integrity, which may benefit the intestinal immune response through a mechanism other than systemic defense in weaned piglets.

CRedit authorship contribution statement

L. Cai: Investigation, Writing - original draft. Y.P. Li: Data curation, Validation. Z.X. Wei: Software. X.L. Li: Supervision,

Methodology, Project administration. **X.R. Jiang:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

We declare that there is no conflict of interest among contributors.

Acknowledgments

This study was financially supported by the National Key R&D Program of China (2018YFD0500600), the Beijing Natural Science Foundation (6174049), and the Elite Youth Program of Chinese Academy of Agricultural Sciences (to X.L. Li).

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