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Original Research Article

Dietary emodin alleviates lipopolysaccharide-induced intestinal mucosal barrier injury by regulating gut microbiota in piglets

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ABSTRACT

This study was to determine the effects of dietary emodin (ED) on the intestinal mucosal barrier, nuclear factor kappa-B (NF-κB) pathways, and gut microbial flora in lipopolysaccharide (LPS)-induced piglets. Twenty-four weaned piglets were chosen and 4 treatments were created by randomly distributing piglets into CON, ED, LPS, and ED_LPS groups. Experiments were done in a 2 × 2 factorial arrangement and maintained for 21 d. Dietary treatment (a basal diet or 300 mg/kg ED) and immunological challenge (LPS or sterile saline) were 2 major factors. Intraperitoneal injections of LPS or sterilized saline were given to piglets on d 21. Six hours after the LPS challenge, all piglets were euthanized for sample collection and analysis. The results showed that piglets of the ED_LPS group had higher ($P < 0.05$) villus height to crypt depth ratio (VCR), and lower ($P < 0.05$) plasma D-lactate and diamine oxidase (DAO) than the LPS group. Furthermore, ED inhibited ($P < 0.05$) the decrease of glutathione peroxidase (GSH-Px) and catalase (CAT) activities and increase of malonaldehyde level ($P < 0.05$) in jejunal mucosa induced by LPS. The mRNA levels of pro-inflammatory cytokine genes (*IL-6*, *IL-1β*, and *TNF-α*) were significantly reduced ($P < 0.05$), and the mRNA levels of antioxidant enzyme genes (*GPX-1*, *SOD2* and *CAT*), as well as protein and mRNA levels of tight junction proteins (occludin, claudin-1, and *ZO-1*), were also significantly increased ($P < 0.05$) by ED addition in LPS-induced piglets. Meanwhile, ED supplementation significantly decreased the LPS-induced protein levels of cyclooxygenase-2 and phosphorylation levels of NF-κB p65 and IκBα in jejunal mucosa. Emodin had a significant effect on the composition of gut microbial flora at various taxonomic positions as indicated by 16S RNA sequencing. The acetic acid, isobutyric acid, valeric acid, and isovaleric acid concentrations in the cecum were also increased by ED addition in pigs ($P < 0.05$). Furthermore, the correlation analysis revealed that some intestinal microbiota had a potential relationship with jejunal VCR, plasma D-lactate and DAO, jejunal mucosa GSH-Px and CAT activity, and cecal short-chain fatty acid concentration. These data suggest that ED is effective in alleviating LPS-induced intestinal mucosal barrier injury by modulating gut microbiota in piglets.

1. Introduction

Weaned piglets are vulnerable to a variety of stressors (e.g., nutritional, environmental, and physiological stresses), all of which have negative impacts on piglet physiological health, resulting in considerable economic losses to the swine industry (Campbell et al., 2013). Damage to the intestinal barrier increases gut permeability, disturbs the tight junctions, and increases bacterial translocation and intestinal infection, subsequently leading to systemic inflammation and diarrhea in piglets (Jayaraman and Nyachoti, 2017). Previous studies suggested that intestinal barrier function requires critical

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pathways of phosphor-nuclear-factor kappa B (NF-κB). Inhibition of NF-κB ameliorates intestinal epithelial barrier dysfunction (Qi et al., 2014). Cyclooxygenase-2 (COX-2) and other key pro-inflammatory cytokines are transcriptional in a manner that is NF-κB dependent (van Uden et al., 2008; Fitzpatrick et al., 2011). Therefore, regulation of NF-κB pathways by nutritional supplements in the gastrointestinal tract, followed by down-regulation of pro-inflammatory factor genes, may have beneficial effects in alleviating intestinal injury. Moreover, the intestinal microbiome is also involved in maintaining the host's gut health. The presence of appropriate intestinal microbiota has been shown to resist pathogen invasion and thus boost mucosal immunity (Heo et al., 2013). Weaning reduces the diversity and alters the composition of intestinal microbes, which causes a decrease in obligate anaerobes and an increase in facultative anaerobes, thereby weakening the intestinal function (Winter et al., 2013). As a result, nutritional regulation of the intestinal microbiome is an effective method to improve intestinal health.

Nutritional supplements are known to be effective in preventing weaning stress (Zhuang et al., 2019; Zheng et al., 2020). Thus, supplementation with natural products has sparked interest in recent years owing to their potential roles in preserving the barrier function of the intestinal mucosa and alleviating the symptoms of intestinal diseases (Han et al., 2016; Zhao et al., 2020). Emodin (ED), a type of anthraquinone compound, is an effective component of the Chinese rhubarb *Rheum palmatum*. Studies suggest that ED possesses promising pharmaco-therapeutic effects, including intestinal microbiota regulation (Qu et al., 2013), anti-inflammatory, antioxidant, and anti-apoptotic activities (Qi et al., 2014; Wang et al., 2015; Tian et al., 2018). Emodin has been shown to improve the intestinal mucosal barrier affected by severe acute pancreatitis in rats (Tan et al., 2020). It has also been reported to provide tight junction structure protection and alleviate intestinal epithelial apoptosis (Fei et al., 2016; Wang et al., 2016). Moreover, ED could attenuate lipopolysaccharide (LPS) induced intestinal epithelial barrier dysfunction through NF-κB pathway modulation (Qi et al., 2014; Piao et al., 2020; Qian et al., 2020). Gao et al. (2021) reported that ED decreased the damage to the mucosal barrier function of the intestine in mice infected with *Escherichia coli* by altering the intestinal microbiota, thereby alleviating diarrhea.

However, the effect produced by ED on the intestine's mucosal barrier and microbiota in weaned piglets, as well as the correlation between barrier function and microbiota is not fully understood. We hypothesized that ED attenuated intestinal mucosal barrier injury probably through the inactivation of NF-κB pathways and regulation of gut microbiota. To this aim, an LPS-challenged piglet model was established to examine the effects of dietary ED supplementation on the intestinal mucosal barrier, NF-κB signaling, and gut microbiota. Our results suggested an important and conceptual basis for the potential administration of ED in regulating the intestinal health of weaned piglets.

2. Materials and methods

2.1. Animal ethics

All animal experiments comply with the ARRIVE guidelines. Animal handling protocols, experimental layouts, and approaches were approved by the Animal Care and Use Committee of Hainan University, Haikou, Hainan province, China (protocol code HNUAUCC-2022-000140).

2.2. Animals, diets and experiments

Twenty-four piglets (Duroc × Landrace × Yorkshire, weaned at 28 d) with an average initial body weight of 8.30 ± 0.25 kg were selected in a 2 × 2 factorial arrangement. Dietary treatment (i.e., a basal diet or 300 mg/kg ED) and immunological challenge (LPS or sterile saline) were considered as the main factors. Piglets were grouped by random selection into one of 4 treatments (CON, ED, LPS, and ED_LPS group) with 6 replicates per group ($n = 6$, 3 barrows and 3 gilts), and 1 piglet per replicate. Piglets in the LPS and CON groups were given a basal diet, and ED_LPS and ED group piglets were given a basal diet which was supplemented with 300 mg/kg ED (purity of 98%, Nanjing Jingzhu Bio-Technology Co., Ltd, Nanjing, China). The basal diet was formulated according to NRC (2012), and its ingredients and nutrient levels are shown in Table 1. The experiment lasted for 21 d. Feed and water were freely available during this period. On d 21 of the experiment, LPS and ED_LPS treated groups were injected with 100 μg/kg BW LPS (Sigma–Aldrich, St Louis, MO, USA) in accordance with previous study (Cao et al., 2018). The CON and ED groups were administered with an exact dose of sterile saline. The body weight of each piglet was documented on the morning of d 1 and 21. Feed intake by piglets was recorded every day for 21 d. Calculations for average daily gain (ADG), average daily feed intake (ADFI), and ratio of daily gain to daily feed intake (F:G) were calculated.

2.3. Sample collection

Four hours after LPS or saline administration, 5 mL of blood samples were collected in a heparin sodium-containing tube from the anterior vena cava of each piglet, and then centrifuged for 15 min at 4 °C and 3,000 × *g* for plasma separation. The processed samples were then stored at −80 °C for further analysis of the diamine oxidase (DAO) and D-lactate.

Table 1
Ingredients and nutrient levels of the basal diet (%, as-fed basis).

Item	Content
Ingredients	
Corn	62.50
Soybean meal	20.50
whey powder	5.50
Fish meal	4.50
Soybean oil	1.30
Sucrose	1.50
Limestone	0.60
Choline chloride	0.10
Calcium hydrophosphate	1.20
Complex acidifying agent	0.30
L-Lysine HCl	0.40
DL-Methionine	0.16
L-Tryptophan	0.04
L-Threonine	0.10
Salt	0.30
Vitamin–mineral premix [1]	1.00
Total	100.00
Nutrient levels [2]	
Digestible energy, MJ/kg	14.21
Crude protein	19.62
Calcium	0.75
Available phosphorus	0.40
Lysine	1.31
Methionine	0.42
Threonine	0.73
Tryptophan	0.20

[1] Provided the following per kilogram of diet: vitamin A 12,000 IU, vitamin D_3 3,000 IU, vitamin E 80 IU, vitamin K 2.5 mg, vitamin B_1 2.5 mg, vitamin B_2 6.0 mg, vitamin B_{12} 0.05 mg, folic acid 1.5 mg, nicotinic 40 mg, pantothenate 20 mg, biotin 0.20 mg, Cu 16.5 mg, Fe 100 mg, Mn 35 mg, Zn 100 mg, Se 0.3 mg, I 0.3 mg.
[2] Digestible energy was calculated from data provided by Feed Database in China (2012), while the other nutrient levels were measured values.

Piglets were euthanized with sodium pentobarbital at 80 mg/kg body weight at 6 h post-administration of LPS or saline. About 10 cm sections of jejunum were rinsed with phosphate buffer. The mucosa samples were gently scraped with sterilized glass slides and collected into cryo-tubes of 2 mL volume. Mucosa samples were snap-frozen with liquid nitrogen and then stored at −80 °C. The mid-jejunal samples (3 to 4 cm) were collected and then fixed with 4% paraformaldehyde for morphological analysis.

2.4. Hematoxylin and eosin (H&E) staining for morphological analysis

The protocol of Barea et al. (2011) was followed for the preparation of paraffin sections. After fixation with 4% paraformaldehyde for 24 h, the jejunum segments were dehydrated and then embedded in paraffin wax. The 5 μm sections were mounted on slides, de-paraffinized and re-hydrated. The slides for each sample were stained with H&E dye and then examined under a light microscope. The villus height (VH) and crypt depth (CD) were quantified on Image-pro Plus 6.0 (Media Cybernetics, Inc., US). The software assessed at least 20 well-oriented and intact crypt-villus units and finally calculated their average. The calculation of villus height to crypt depth ratio (VCR) was done as previously described (Zhu et al., 2013).

2.5. Plasma D-lactate and DAO analysis

Enzyme-linked immunosorbent assay (ELISA) kits were used for the measurement of plasma D-lactate levels and DAO activity. The manufacturer's protocols were followed (Mlbio, Shanghai, China).

2.6. Analysis of antioxidant parameters

The mucosa samples were added to 9 times the volume of normal saline and prepared into 10% tissue homogenate. All of this was performed on an ice water bath. After centrifugation at 2,500 × *g* for 10 min, the supernatant was collected in a new centrifuge tube and finally stored at 4 °C for antioxidant parameters analysis. Reagent kits were purchased from Nanjing Jiancheng Co., Ltd. (Nanjing, China) for the determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malonaldehyde (MDA), and catalase (CAT). The protein concentration of mucosa was measured using a BCA protein assay kit (Biosharp, Beijing, China). The methods were performed according to the manufacturer's protocols.

2.7. Quantitative real-time PCR

The collected jejunal mucosa samples were transferred into a sterile 1.5 mL centrifuge tube containing RNAiso plus (Vazyme, Nanjing, China). Extraction of total RNA was done as described by RNAiso plus kit instructions. The total RNA concentration was quantified using a Nano-Drop ND-1000 spectrophotometer. RNA quality was measured by 1% agarose gel electrophoresis. HiScript II One-step RT-PCR kit (Vazyme, Nanjing, China) was used for reverse transcription of total RNA into cDNA.

The relative expressions of mRNA for pro-inflammatory cytokine genes (*IL-6, IL-1β,* and *TNF-α*), antioxidant enzyme genes (*GPX-1*, *SOD2,* and *CAT*) as well as tight junction proteins (occludin, *ZO-1* and claudin-1) in the mucosa of the jejunum were quantitatively evaluated by real-time PCR by using a ChamQ Universal SYBR qPCR Master Mix kit (Vazyme, Nanjing, China). The reactions were performed in a total volume of 10.0 μL containing 5 μL of PCR premix, 0.2 μL of each primer, 1 μL of cDNA and 3.6 μL of DNase/RNase-free water. The cycling programme was 95 °C (30 s), followed by 40 cycles of denaturing at 95 °C (10 s), and annealing at 60 °C (30 s). The primers are listed in Table 2. The $2^{-\Delta\Delta CT}$ method was followed for calculating relative gene expression levels. GAPDH was set as the housekeeping gene, where ΔΔCT = ΔCT (treated group) − ΔCT (CON group) and ΔCT = CT (target gene) − CT (GAPDH).

2.8. Western blot analysis

Western blot analysis was performed to determine the jejunal mucosa protein expressions of occludin, claudin-1, ZO-1, p−NF−κB p65, NF-κB p65, p-IκBα and COX-2, as previously described (Xun et al., 2021). The primary antibodies were as follows: rabbit anti-β-actin (Boster, Wuhan, China) in the dilution of 1:2,000, rabbit anti-occludin (Proteintech, Wuhan, China) diluted at 1:1,000, rabbit anti-claudin1 (Bioss, Beijing, China) in 1:1,000 dilution, rabbit anti-ZO-1 (Proteintech, Wuhan, China) in 1:1,000 dilution, rabbit anti-COX-2 (Proteintech, Wuhan, China) in 1:1,000 dilution, rabbit anti−NF−κB p65 (Cell Signalling Technology, Danvers, MA, USA) at 1:1,000, rabbit anti-phospho−NF−κB p65 (Cell Signalling Technology, Danvers, MA, USA) in 1:1,000 dilution, and rabbit anti-phospho-IκBα (Cell Signalling Technology, Danvers, MA, USA) at 1:1,000 dilution. The secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cell Signalling Technology, Danvers, MA, USA) and it was diluted at 1:2,000. Protein abundance was normalized with housekeeping protein β-actin and stated as fold change. The results were communicated relative to the control piglet's levels.

2.9. DNA extraction and hiseq sequencing

DNA was extracted from samples using the E.Z.N.A. Stool DNA kit (D4015, Omega, Inc., USA). PCR amplification was performed according to Fu et al. (2021). The primers that were utilized are listed in Table S1. The 16S rDNA sequencing and bioinformatics analysis protocols are consistent with Fu et al. (2021).

2.10. Determination of cecal short-chain fatty acids (SCFA)

Cecal content (1 g) was weighed, and added to 1 mL of water, and then the combination was mixed ultrasonically for 20 min. The mixture was then centrifuged at 15,000 × *g* at 4 °C for 15 min. Then 3.6 mL of supernatant was removed and collected into an EP tube containing 0.4 mL of 25% metaphosphoric acid solution. This new solution was further centrifuged at 15,000 × *g* for 10 min. Filter membrane of 0.45 μm was used to filter the supernatant into a chromatographic injection vial for the determination of the SCFA. The concentrations of cecal SCFA were detected by using a gas chromatography (GC) system (Agilent Technologies 7890B GC System, Agilent, USA) and chromatographic column (30 m × 250 μm × 0.25 μm). Nitrogen was run as the carrier gas with a flow rate of 0.8 mL/min. The temperatures of the column, detector, and injector were 220, 280, and 250 °C, respectively.

2.11. Statistical analysis

ANOVA test was performed for statistical analysis, and general linear model procedures of SPSS 25.0 for a 2 × 2 factorial design were used (IBM SPSS Inc., Chicago, Illinois, USA). The statistical model involved dietary treatment (basal or 300 mg/kg ED) and immunological challenge (LPS or sterile saline) effects and their interactions. The differences were evaluated by one-way ANOVA with Duncan's multiple range tests. A value of $P < 0.05$ was deemed statistically significant. Results were reported as mean ± standard error of the mean (SEM). To analyze the complexity of species diversity for a sample using 4 indices, alpha diversity was used. These

Table 2
Primers used for real-time PCR.

Target	Accession number	Sense and antisense primer	Product size, bp
GAPDH	AF017079	F:5′GAAGGTCGGAGTGAACGGAT3′ R:5′CATGGGTAGAATCATACTGGAACA3′	149
Occludin	NM_001163647.1	F:5′ATGCTTTCTCAGCCAGCGTA3′ R:5′AAGGTTCCATAGCCTCGGTC3′	176
ZO-1	XM003353439.1	F:5′GAGGATGGTCACACCGTGGT3′ R:5′GGAGGATGCTGTTGTCTCGG3′	169
Claudin1	NM_001161635.1	F:5′GGACTAATAGCCATCTTTGT3′ R:5′CAGCCATCCGCATCTTCT3′	88
IL-1β	NM_214055.1	F:5′ACCTGGACCTTGGTTCTC3′ R:5′GGATTCTTCATCGGCTTC3′	124
TNF-α	NM_214022.1	F:5′ACGCTCTTCTGCCTACTGC3′ R:5′TCCCTCGGCTTTGACATT3′	162
IL-10	NM_214041.1	F:5′CACTGCTCTATTGCCTGATCTTCC3′ R:5′AAACTCTTCACTGGGCCGAAG3′	136
IL-6	NM_214399.1	F: 5′TTCAGTCCAGTCGCCTTCT3′ R:5′GTGGCATCACCTTTGGCATCTTCTT3′	91
SOD1	NM_001190422.1	F: 5′AAGGCCGTGTGTGTGCTGAA3′ R:5′GATCACCTTCAGCCAGTCCTTT3′	118
SOD2	NM_214127.2	F: 5′GGCCTACGTGAACAACCTGA3′ R: TGATTGATGTGGCCTCCACC3′	126
GPX-1	NM_214201.1	F: 5′CCTCAAGTACGTCCGACCAG3′ R: 5′GTGAGCATTTGCGCCATTCA3′	85
CAT	NM_214301.2	F: 5′AGCCTACGTCCTGAGTCTCTGC3′ R: 5′TCCATATCCGTTCATGTGCCTGTG3′	90

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; *ZO-1* = zonula occludens-1; *IL-1β* = interleukin 1β; *IL-6* = interleukin 6; *IL-10* = interleukin 10; *TNF-α* = tumor necrosis factor α; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2; *GPX-1* = glutathione peroxidase 1; *CAT* = catalase.

indices (Chao1, Goods coverage, Simpson, and Shannon) were all calculated using QIIME2. For each representative sequence, the feature sequences were annotated with the SILVA database using Blast. Other diagrams were formulated using R package (v3.5.2). Spearman's rank order correlation test in R was used to examine correlations between the top 20 cecum-associated microbiota and intestinal barrier function (v3.2.1).

3. Results

3.1. Growth performance

As shown in Table 3, piglet initial body weight (BW), final BW, ADG, ADFI, and F:G were not affected by dietary treatments during the 21-d feeding trial ($P > 0.05$). There were also no significant effects on ADG, ADFI, and F:G between LPS and ED_LPS groups ($P > 0.05$).

3.2. Small intestine morphology, plasma DAO and D-lactate

Compared with the CON group, the LPS challenged group showed a significant decrease in jejunal villus height ($P < 0.05$) and VCR ($P = 0.001$), whereas ED addition reversed the decrease of jejunal VCR ($P = 0.022$) induced by LPS. A significant LPS × diet relationship was discovered for jejunal VCR ($P = 0.048$). However, there were no distinct changes in crypt depth among treatments ($P = 0.098$) (Table 4).

When pigs that were challenged with LPS were compared with pigs injected with saline, higher plasma DAO activities and D-lactate levels were observed ($P < 0.05$), whereas ED supplementation reversed the increase of LPS-induced plasma DAO levels and D-lactate ($P < 0.05$). A LPS × diet interaction was noticed in plasma D-lactate ($P < 0.001$) (Table 4).

Table 3
Effects of dietary emodin (ED) on the growth performance in weaned piglets. [1]

Item	Saline		LPS		SEM	*P*-value		
	CON	ED	CON	ED		Diet	LPS	Interaction
Initial BW, kg	8.45	8.05	8.38	8.33	0.246	0.671	0.838	0.741
Final BW, kg	15.16	15.43	14.81	14.46	0.360	0.961	0.391	0.683
ADFI, kg/d	0.55	0.57	0.59	0.53	0.021	0.059	0.889	0.348
ADG, kg/d	0.34	0.37	0.32	0.31	0.013	0.456	0.211	0.616
F:G	1.68	1.58	1.85	1.75	0.066	0.977	0.219	0.436

LPS = lipopolysaccharide; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F:G = the ratio of average daily feed intake to average daily gain.
[1] Each value represents the mean ± SEM of 6 replicates ($n = 6$).

3.3. Antioxidant status in jejunal mucosa

In jejunal mucosa, no LPS × diet interaction was noted on antioxidant enzyme (SOD, T-AOC, and GSH-Px) activities and MDA levels ($P > 0.05$). LPS challenge reduced CAT and GSH-Px activities and increased the levels of MDA ($P < 0.05$) when compared with the CON group. Dietary ED supplementation improved ($P < 0.05$) LPS-induced CAT and GSH-Px activities and reduced ($P < 0.05$) the jejunal mucosal levels of MDA in comparison with the LPS group. However, the T-AOC and SOD activities remained unaffected by dietary treatments ($P > 0.05$) (Table 5).

Compared with piglets that were injected with saline, those which were LPS-challenged showed lower mRNA levels of *GPX-1*, *SOD2* and *CAT* ($P < 0.05$) in jejunal mucosa. Whereas the ED supplementation reversed the decrease of *GPX-1*, *SOD2* and *CAT* mRNA levels induced by LPS ($P < 0.05$). A significant LPS × diet relationship was discovered for *CAT* mRNA level ($P = 0.034$) (Table 6).

3.4. Tight junction mRNA and protein expression

When compared with the CON group, the LPS group showed decreased mRNA and protein expression of occludin, ZO-1 and claudin-1 in the jejunal mucosa ($P < 0.05$), whereas ED administration reversed the decrease of protein and mRNA expressions of occludin, claudin-1, and ZO-1 that was induced by LPS ($P < 0.05$) (Table 6 and Fig. 1). The protein expressions of ZO-1 and occludin

Table 4
Effects of emodin (ED) on jejunal morphology, plasma D-lactate and DAO after lipopolysaccharide (LPS) challenge.[1]

Item	Saline		LPS		SEM	*P*-value		
	CON	ED	CON	ED		Diet	LPS	Interaction
Villus height, μm	319.40^{b}	393.92^{a}	246.43^{c}	299.85^{bc}	14.20	0.003	0.001	0.589
Crypt depth, μm	188.84	232.37	204.74	199.77	7.32	0.183	0.557	0.098
VCR	1.70^{a}	1.72^{a}	1.21^{c}	1.50^{b}	0.05	0.022	0.001	0.048
DAO, U/L	7.22b^{c}	6.56^{c}	11.98^{a}	9.17^{b}	0.579	0.044	<0.001	0.197
D-Lactate, μmol/mL	1.16^{c}	1.14^{c}	2.10^{a}	1.57^{b}	0.082	<0.001	<0.001	<0.001

VCR = ratio of villus height to crypt depth; DAO = diamine oxidase.
^{a,b,c} Within a row, means values with unlike letters differ significantly ($P < 0.05$).
[1] Each value represents the mean ± SEM of 6 replicates ($n = 6$).

Table 5
Effect of emodin (ED) on antioxidant index in jejunal mucosa of piglets after lipopolysaccharide (LPS) challenge.[1]

Item	Saline		LPS		SEM	*P*-value		
	CON	ED	CON	ED		Diet	LPS	Interaction
T-AOC, U/mg prot	0.35	0.33	0.27	0.26	0.018	0.658	0.058	0.895
GSH-P_X, U/mg prot	77.82^{a}	76.73^{a}	27.30^{c}	43.64^{b}	4.93	0.084	<0.001	0.051
SOD, U/mg prot	301.00	313.73	255.12	267.67	13.36	0.640	0.099	0.997
CAT, U/mg prot	35.77^{a}	37.90^{a}	25.15^{c}	30.54^{b}	3.96	0.038	<0.001	0.346
MDA, nmol/mg prot	1.18^{b}	0.90^{c}	1.66^{a}	1.10^{bc}	0.069	<0.001	<0.001	0.099

T-AOC = total antioxidant capacity; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde.
^{a,b,c} Within a row, means values with unlike letters differ significantly ($P < 0.05$).
[1] Each value represents the mean ± SEM of 6 replicates ($n = 6$).

Table 6
Effect of emodin (ED) on mRNA levels of antioxidant enzymes, cytokines and tight junction proteins in jejunal mucosa of piglets.[1]

Item	Saline		LPS		SEM	*P*-value		
	CON	ED	CON	ED		Diet	LPS	Interaction
Antioxidant enzymes								
GPX-1	1.00^{b}	1.69^{a}	0.26^{c}	0.82^{b}	0.128	0.001	<0.001	0.664
SOD1	1.00	1.10	0.93	1.07	0.068	0.154	0.535	0.789
SOD2	1.00^{b}	1.83^{a}	0.41^{c}	0.98^{b}	0.121	<0.001	<0.001	0.310
CAT	1.00^{b}	1.23^{a}	0.60^{c}	1.13^{ab}	0.068	<0.001	0.002	0.034
Cytokines								
IL-1β	1.00^{c}	0.83^{c}	2.11^{a}	1.40^{b}	0.112	<0.001	<0.001	0.015
IL-6	1.00^{b}	0.87^{b}	1.87^{a}	0.93^{b}	0.110	<0.001	<0.001	<0.001
TNF-α	1.00^{b}	0.45^{c}	2.08^{a}	1.30^{b}	0.135	<0.001	<0.001	0.359
IL-10	1.00^{a}	1.09^{a}	0.53^{b}	0.88^{a}	0.085	0.043	0.007	0.155
Tight junction proteins								
ZO-1	1.00^{b}	1.46^{a}	0.39^{c}	1.12^{b}	0.086	<0.001	<0.001	0.042
Occludin	1.00^{b}	1.41^{a}	0.35^{c}	0.76^{b}	0.095	0.001	<0.001	0.995
Claudin	1.00^{b}	1.47^{a}	0.29^{d}	0.63^{c}	0.100	<0.001	<0.001	0.511

GPX-1 = glutathione peroxidase 1; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2; *CAT* = catalase; *IL-1β* = interleukin-1β; *IL-6* = interleukin-6; *TNF-α* = tumor necrosis factor-α; *IL-10* = interleukin-10; *ZO-1* = zonula occludens-1.
^{a,b,c,d} Within a row, means values with unlike letters are significantly different ($P < 0.05$).
[1] Each value represents the mean ± SEM of 6 replicates ($n = 6$).

were not significantly different among CON, ED, and ED_LPS groups ($P > 0.05$) (Fig. 1).

3.5. The mRNA expression of cytokines in the jejunal mucosa of piglets

LPS-challenged piglets showed significantly increased ($P < 0.05$) mRNA expression of *IL-1β*, *TNF-α*, and *IL-6* but reduced mRNA expression of *IL-10* ($P = 0.007$) when compared with piglets that were injected with saline. However, ED supplementation reversed the elevation of mRNA levels of *IL-1β*, *IL-6*, and *TNF-α* ($P < 0.05$) and decrease in mRNA expression of *IL-10* produced by LPS ($P = 0.043$) (Table 6).

3.6. Protein expression of NF-κB pathway-related genes

When compared with the CON group, LPS challenge group showed a significant increase in the protein expression of COX-2 ($P < 0.05$), as well as phosphorylation levels of NF-κB p65 and IκBα ($P < 0.05$) in jejunal mucosa, whereas ED supplementation reversed the increase in the phosphorylation levels of NF-κB p65 and IκBα, and COX-2 protein expressions caused by LPS ($P < 0.05$) (Fig. 2).

3.7. Bacterial composition in cecal digesta

The microbial community richness and diversity were evaluated by alpha diversity analysis. Simpson index, Shannon index, Chao1 index, and Good's coverage showed no significant differences among the 4 treatments (Fig. S1).

As shown in Fig. 3A, the most abundant phyla were Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, and Spirochaetes, in both groups and accounted for >98% of the total cecal bacteria. When compared with the CON group, ED supplementation decreased Firmicutes (79.1% vs. 72.05%) and increased the abundance of Bacteroidetes (14.97% vs. 21.2%). LPS injection increased the abundance of Proteobacteria (2.95% vs. 8.85%) in contrast to the CON group. However, it was reduced in ED_LPS compared to the LPS group (8.85% vs. 5.18%). Furthermore, the genus level is shown in Fig. 3B. In comparison with the CON group, the LPS challenged group showed an increased abundance of *Ruminococcaceae_UCG-005* (4.16% vs. 7.58%) and a reduced abundance of *Faecalibacterium* (5.43% vs. 1.02%). Conversely, when the LPS group was compared with the ED_LPS group, results showed a reduced relative abundance of *Ruminococcaceae_UCG-005* (7.58% vs. 6.22%) and increased abundance of *Faecalibacterium* (1.02% vs. 3.39%). Besides, the results revealed that ED supplementation increased the relative abundance of *Lachnospiraceae_unclassified* (2.89% vs. 4.39%) and *Alloprevotella* (2.79% vs. 3.75%) and decreased the relative abundance of *Subdoligranulum* (5.52% vs. 2.02%) in comparison with the CON.

To identify statistically significant biomarkers between the groups, linear discriminant analysis Effect Size (LEfSe) was performed. Those microorganisms which were statistically significant

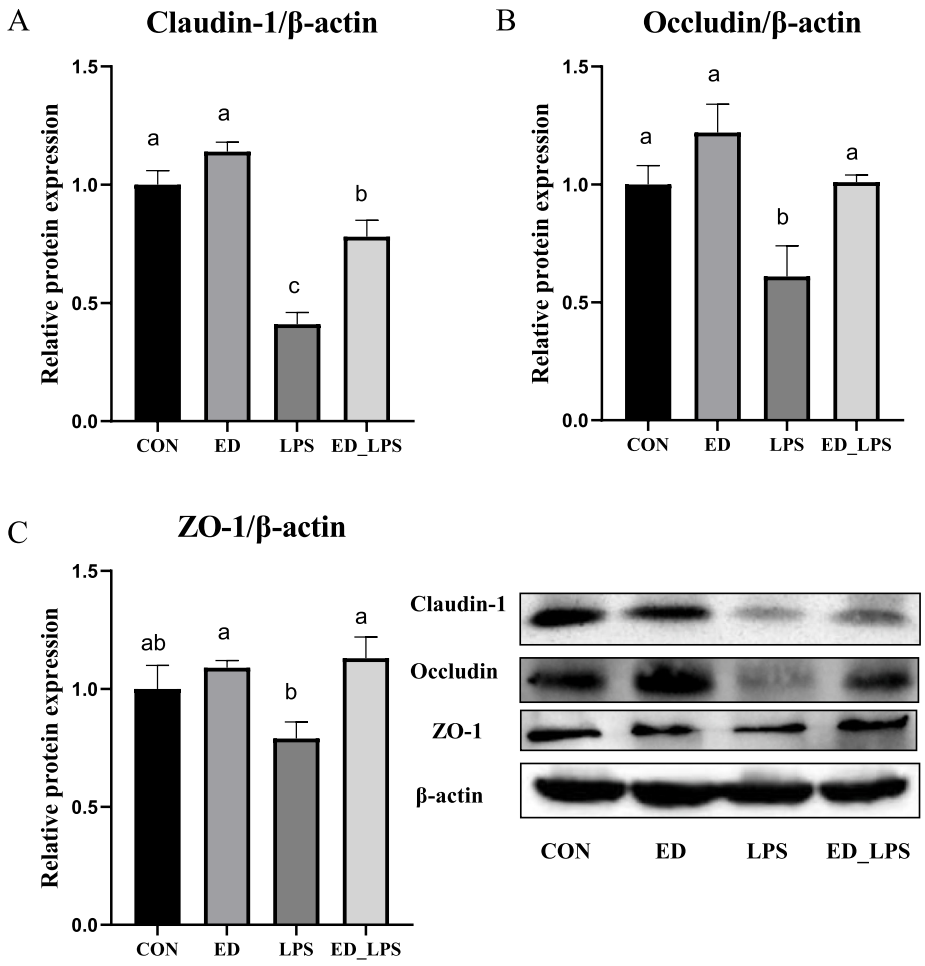

Fig. 1. Effect of dietary emodin (ED) supplementation on claudin 1 (A), occludin (B), and zonula occludens-1 (ZO-1) protein (C) expression in the jejunum mucosa of piglets. Values are means and standard deviations represented by vertical bars ($n = 6$). Bars with unlike letters indicate a significant difference ($P < 0.05$). LPS = lipopolysaccharide. CON group: piglets were fed the basal diet with sterile saline injection; ED group: piglets were fed the basal diet supplemented with 300 mg/kg ED and injected with sterile saline; LPS group: piglets were fed the basal diet with LPS injection; ED_LPS group: piglets were fed the basal diet supplemented with 300 mg/kg ED and injected with LPS.

were classified as those which belonged to phylum Proteobacteria in the LPS group, the family Lachnospiraceae in the ED group, and the order Aeromonadales in the ED_LPS group (Fig. 3C). ED supplementation up-regulated the abundance of *Oscillospira*, *Streptococcus*, *Lachnospiraceae_AC2044_group*, and *Ruminococcaceae_UCG-010* compared with the CON group (Fig. 3D). Moreover, the LPS challenge significantly up-regulated the abundance of *Lachnospiraceae_UCG-010*, *Coprococcus*, *Ursidibacter*, and *Pseudomonas* as compared to the ED_LPS group. Conversely, significant up-regulation was observed in *Lachnospiraceae_ND3007_group*, *Succinivibrio*, and *Mailhella* in the ED_LPS group as compared to the LPS group (Fig. 3E).

3.8. Determination of cecal SCFA concentration

As shown in Table 7, the ED group had higher concentrations of isobutyric acid, valeric acid, isovalerate, and acetic acid in cecal digesta compared with the LPS group ($P < 0.05$). There were no significant differences in acetic acid, butyric acid, isobutyric acid, valeric acid, isovalerate between the ED_LPS and CON groups ($P > 0.05$). A significant LPS × diet interaction was observed for acetic acid in cecal digesta ($P < 0.05$).

3.9. Correlation analysis

As shown in Fig. 4, the relative abundance observed in *Ruminococcus_1* and *Ruminococcaceae_UCG-014* was correlated positively with the concentration of cecal butyric acid, jejunal mucosa GSH-Px and CAT activities, and jejunal VCR. Conversely, *Ruminococcus_1* and *Ruminococcaceae_UCG-014* were negatively correlated with DAO activity and D-lactate level. A positive correlation was noted between the abundance of *Agathobacter*, *Lachnospiraceae_unclassified*, *Firmicutes_unclassified*, *Prevotellaceae_UCG-003*, and *Prevotella_9* with cecal acetic acid concentration.

4. Discussion

This study documented the effects of dietary ED addition on intestinal integrity and inflammation in piglets after a 6-h LPS challenge. Emodin, an active ingredient of Chinese rhubarb, is shown to exert numerous beneficial effects, including anti-oxidant anti-inflammatory, and anti-apoptotic properties (Qi et al., 2014). Studies showed that ED could promote the growth performance in aquatic animals, such as freshwater prawn (Tadese et al., 2020) and Wuchang bream (Song et al., 2019). However, ED failed to improve healthy piglets' growth performance prior to LPS challenge in our study. Straub et al. (2005) reported that when piglets were fed with 0.25% rhubarb for 31 d, the ADG and ADFI increased significantly, but when the rhubarb content increased to 1%, the ADG and ADFI decreased gradually. Therefore, the lack of ED effect on growth performance in the current study may be attributed to its supplemental dose.

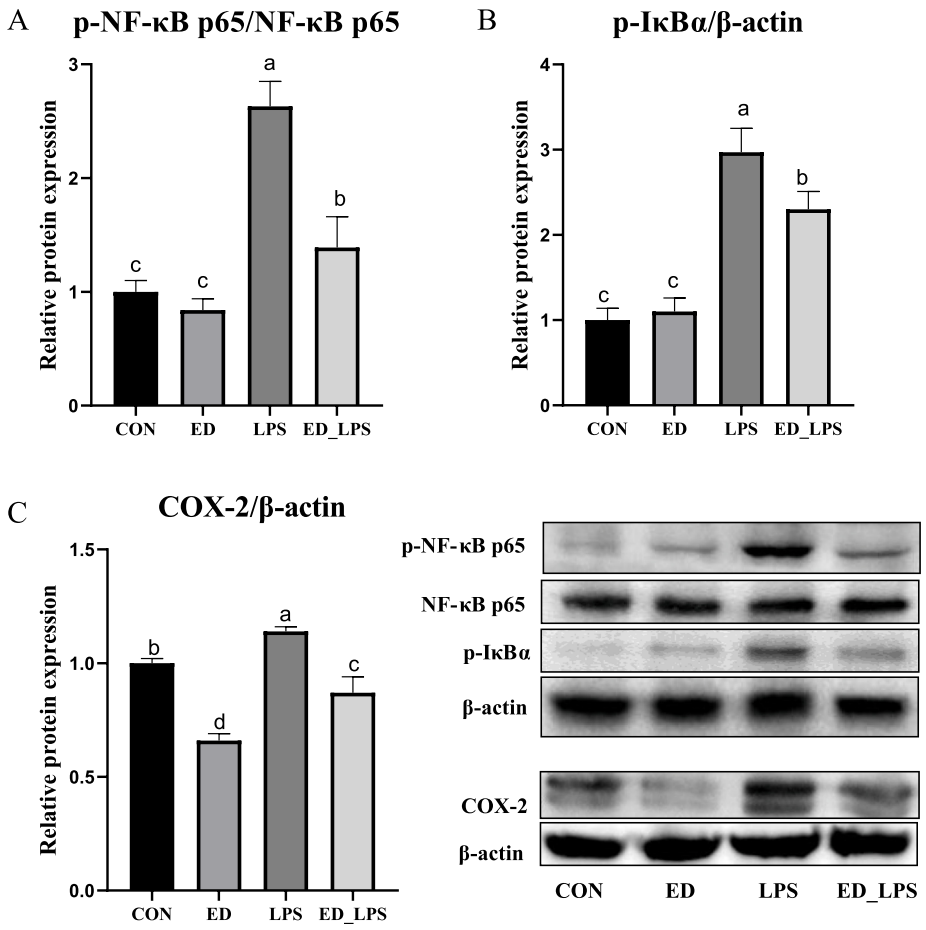

Fig. 2. Effect of emodin (ED) supplementation on nuclear factor kappa-B (NF-κB) pathways in the jejunal mucosa of piglets. Western blot analysis of p–NF–κB p65 (A), p-IκBα (B), and COX-2 (C) expression in jejunal mucosa. Beta-actin was used as housekeeping protein. Values are means and standard deviations represented by vertical bars ($n = 6$). Bars with unlike letters are significantly different ($P < 0.05$). COX-2 = cyclooxygenase-2. CON group: piglets were fed the basal diet with sterile saline injection; ED group: piglets were fed the basal diet supplemented with 300 mg/kg ED and injected with sterile saline; LPS group: piglets were fed the basal diet with LPS injection; ED_LPS group: piglets were fed the basal diet supplemented with 300 mg/kg ED and injected with LPS.

Intestinal integrity is critical to intestinal homeostasis. Intestinal morphology is an important indicator of nutrient absorption and gut health (Circu and Aw, 2012). The current study found that LPS reduced villus height and VCR in piglets, suggesting that LPS induced acute injury to the intestine. These new results are consistent with a previous study (Zheng et al., 2020). Supplemental ED relieved LPS-induced adverse effects on intestinal morphology of piglets. Similarly, ED was found to reduce pathological damage of intestinal mucosa in septic rats (Li et al., 2020) and *E. coli* challenged mice (Gao et al., 2021). Plasma D-lactate and DAO are sensitive circulating markers of intestinal permeability (Arrieta et al., 2006). In the present study, plasma D-lactate level and DAO activity showed a significant increase in animals after LPS challenge. Intriguingly, these adverse effects of LPS were prevented by ED supplementation. Intestinal epithelial tight junctions, including ZO-1, claudins, and occludin, are important components for maintenance of intestinal barrier integrity (Pan et al., 2013). To inhibit pathogens, toxins, or other exogenous substance invasion, a paracellular structure is formed by this complex between endothelial and epithelial cells (Förster, 2008). We further confirmed that ED reversed LPS-induced decline of mRNA and protein abundances of occludin, claudin-1 and ZO-1. Overall, these data demonstrated that ED had a protective effect on piglet intestinal morphology and barrier function under LPS challenge. Our findings are supported by the fact that ED reduces intestinal barrier permeability and maintains the barrier integrity by decreasing plasma DAO activity and increasing tight junction protein expression levels in septic rats (Li et al., 2020). Moreover, Wang et al. (2020) reported ED up-regulated mRNA levels of tight junction-related proteins, indicating a potential ED-induced mucosal regeneration on DSS-induced intestinal mucosal damage in rats.

Weaning stress can cause oxidative damage to the intestinal mucosa, resulting in intestinal barrier dysfunction, diarrhea, and systemic inflammatory responses (Heo et al., 2013). In the present study, LPS decreased CAT and GSH-Px activities and enhanced jejunal mucosa MDA levels, which was consistent with prior research (Cao et al., 2018). Furthermore, the mRNA levels of antioxidase-related genes *GPX-1*, *SOD2*, and *CAT* in jejunal mucosa were also decreased after injection with LPS, indicating that LPS induced oxidative stress to the intestinal mucosa. However, these adverse effects of LPS were prevented by ED supplementation, suggesting that ED may have a protective effect in alleviating oxidative stress caused by weaning in piglets. A similar phenomenon was also demonstrated by previous studies in mice (Li et al., 2016) and Wuchang bream (Song et al., 2019).

Apart from its antioxidant activity, ED may also alleviate LPS-induced jejunal damage through its anti-inflammatory effects. During weaning, LPS could activate TLR4-mediated NF-κB pathways, causing excessive pro-inflammatory cytokine production including *IL-6*, *TNF-α*, and *IL-1β*, which was thought to contribute to

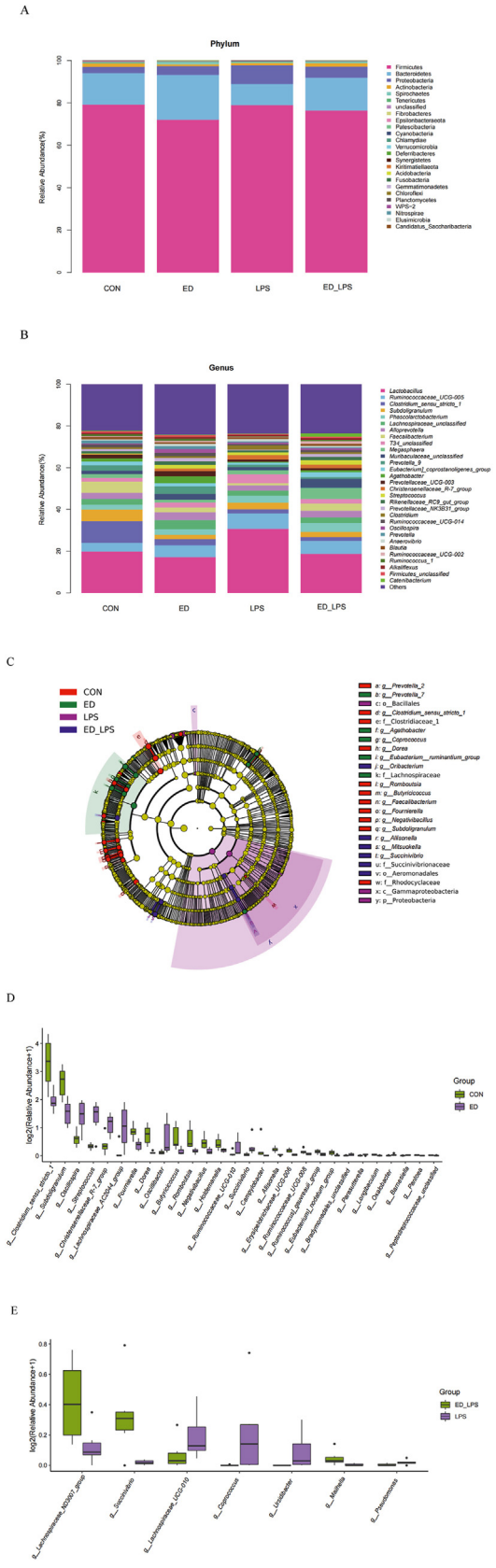

Fig. 3. Gut microbiome composition and community analysis. The relative abundance of cecal microbial phyla (A), cecal microbial genera (B), and the cladogram (C) by linear discriminant analysis Effect Size (LEfSe) analysis from CON, ED, LPS, and ED_LPS groups. The bar plot depicts microorganisms with significant differences at genus level between the CON and ED groups (D), as well as the LPS and ED_LPS group (E). ED = emodin; LPS = lipopolysaccharide.

increased intestinal tight junction permeability and promote inflammatory processes by increasing antigen penetration (Al-Sadi et al., 2014; Wu et al., 2017). In our study, ED supplementation reversed the increase in *IL-6*, *TNF-α*, and *IL-1β* mRNA levels and the decrease in *IL*-10 mRNA levels, which were induced by LPS. The overproduction of pro-inflammatory cytokines is associated with increased permeability of intestinal epithelium (Hu et al., 2013). This may partly explain why ED supplementation increased expression of occludin, claudin-1, and ZO-1. Consistent with our findings, Ning et al. (2017) and Shang et al. (2021) found that ED may be an effective anti-inflammatory agent that reduces intestinal mucosal damage by inhibiting excessive production of pro-inflammatory cytokines in a rat model.

Emodin's anti-inflammatory effects may be attributed to its capacity to prevent NF-κB pathway overactivation. The NF-κB pathways are regulated by inflammatory mediators, which have been confirmed to be deleterious to the intestinal barrier (Qi et al., 2014). COX-2 is critical in intestinal inflammatory responses and its transcriptional activation is regulated by the NF-κB pathway (Fitzpatrick et al., 2011). Paiotti et al. (2012a, b) reported that increased COX-2 expression resulted in intestinal epithelial damage, increased permeability, and disrupted tight junctions, whereas supplemental COX-2 inhibitors alleviated intestinal inflammation. In the present study, inhibition of protein expression of COX-2 and p−NF−κB p65 by ED in LPS-challenged piglet's jejunal mucosa was observed. Qi et al. (2014) observed that ED also decreased the dysfunction in the intestinal barrier by the inactivation of NF-κB signaling in LPS-exposed Caco-2 monolayers. Taken together, we demonstrated that ED attenuated intestinal barrier disorder by NF-κB signaling inactivation in LPS-challenged piglets.

Short-chain fatty acids are well known to decrease the production of proinflammatory cytokines and prevent many inflammatory disorders (Kobayashi et al., 2017). Butyrate improves the intestine barrier, and acetate alleviates metabolic syndrome by mediating the microbiome−brain−β-cell axis (Yan et al., 2020). The release of TNF-α and activation of the NF-κB pathway mediated by TNF-α were inhibited by acetate, propionate, and butyrate in response to LPS in a human colon adenocarcinoma cell line (Tedelind et al., 2007). In the present study, ED administration alleviated the LPS-induced decrease of SCFA (isobutyric acid, valeric acid, isovalerate, and acetic acid) concentration in piglets. This further demonstrated that ED improved LPS-induced intestinal integrity and reduced inflammatory responses possibly owing to increased SCFA production.

Intestinal microbiota is critical for gastrointestinal function and health of the host (Cresci et al., 2015). Studies have reported differences in gut microbiota composition between healthy and sick individuals, particularly Firmicutes and Bacteroidetes (Bervoets et al., 2013). Post-weaning diarrhea of piglets showed extensive intestinal microbiota imbalance, characterized by decreased abundance of Firmicutes and Bacteroidetes and increased abundance of Proteobacteria (Yang et al., 2016). In this investigation, LPS-challenge increased Proteobacteria abundance and decreased Bacteroidetes abundance, but ED reversed the adverse changes induced by LPS. Consistent with our findings, Gao et al. (2021) found that ED increased Bacteroidetes abundance while decreasing Proteobacteria abundance in a mice model of diarrhea. *Ruminococcaceae_UCG-005* in weaned piglets are known to be positively associated with metabolic diseases, chronic inflammation, and diarrheal incidence (Mateos et al., 2018; Hung et al., 2019). Whereas *Faecalibacterium prausnitzii,* belonging to the *Faecalibacterium* genus, is one of the symbiotic bacteria that blocks NF-κB activation and proinflammatory mediator secretion (Tang et al., 2018; Sokol et al., 2008). In the present study, LPS increased *Ruminococcaceae_UCG-005* and reduced *Faecalibacterium*, but ED

Table 7
Effect of emodin (ED) on short-chain fatty acids in the cecum of piglets after lipopolysaccharide (LPS) challenge.[1]

Item	Saline		LPS		SEM	*P*-value		
	CON	ED	CON	ED		Diet	LPS	Interaction
Acetic acid, mg/L	1,527.34^{b}	1,801.8^{a}	1,285.9^{c}	1,343.7^{bc}	42.7	0.003	<0.001	0.043
Propionic acid, mg/L	821.8	844.6	776.4	834.5	14.1	0.388	0.553	0.704
Isobutyric acid, mg/L	89.8^{ab}	95.1^{a}	74.2^{b}	87.8^{ab}	1.73	0.055	0.021	0.389
Butyric acid, mg/L	453.7	485.6	327.4	402.7	7.9	0.597	0.014	0.195
Isovaleric acid, mg/L	102.5^{a}	112.0^{a}	74.7^{b}	99.3^{ab}	2.25	0.020	0.007	0.289
Valeric acid, mg/L	149.9^{ab}	181.1^{a}	107.1^{b}	148.0^{ab}	3.66	0.031	0.023	0.765

^{a,b,c} Within a row, means values with unlike letters differ significantly ($P < 0.05$).
[1] Each value represents the mean ± SEM of 6 replicates ($n = 6$).

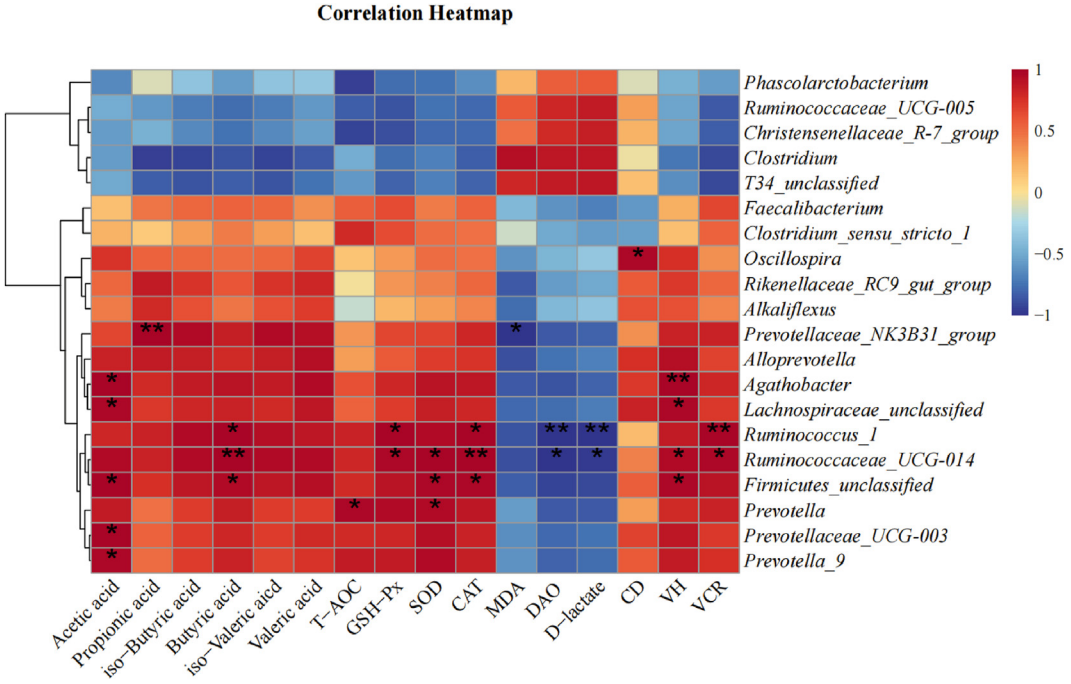

Fig. 4. The correlation coefficient between the microbial genus and short-chain fatty acid intestinal barrier parameters. Significance was set at *$P < 0.05$ and **$P < 0.01$, $n = 6$.

supplementation reversed these changes. In addition, we determined the inhibitory effect of ED on NF-κB signaling in jejunal mucosa, which may suggest that ED acted as an anti-inflammatory by positive regulation of *Faecalibacterium* in LPS-induced piglets.

Prevotella, a member of the phylum Bacteroidetes, is one of the most important dietary fiber fermenters in pigs' gut (Zhou et al., 2016). It is reported that *Prevotellaceae* are involved in polysaccharide degradation and SCFA formation (Wang et al., 2019b). The correlation analysis showed that *Prevotella_9* and *Prevotellaceae_UCG-003* were positively associated with acetic acid concentration. Besides, Firmicutes provide SCFA, which help to maintain intestinal health by reducing inflammation and supplying energy to the intestinal tract (Gophna et al., 2017), Our study found that *Firmicutes_unclassified* was positively associated with villus height, acetic acid and butyric acid concentrations. These findings further demonstrated that ED improves the barrier of the intestine by regulating SCFA concentrations and gut microbiota composition. Moreover, *Phascolarctobacterium* and *Ruminococcaceae_UCG-005*, as opportunistic pathogens, are significantly associated with systemic inflammatory cytokines, especially TNF-α (Wang et al., 2019a). This study demonstrated that the abundance of *Phascolarctobacterium* in the LPS group was increased compared to the CON group, and was negatively correlated with SCFAs and antioxidant enzyme activity and positively correlated with D-lactate levels, DAO activity, and MDA levels in intestinal mucosa. This could explain why LPS-induced piglets have lower antioxidant and anti-inflammatory activity. Consequently, the regulation by ED on intestinal microbiota may be a key factor in improving the barrier function of piglets' intestinal mucosa. The underlying mechanism needs to be further studied.

5. Conclusion

Our findings demonstrated that ED administration decreases LPS-induced injury in the intestinal mucosal barrier, probably by maintaining its integrity and alleviating oxidative damage and inflammatory responses by inactivation of NF-κB pathways. Moreover, ED attenuated LPS-induced intestinal barrier injury, potentially by modulating gut microbiota. Consequently, ED might act as an efficient additive to alleviate weaning-induced intestinal damage in piglets.

Author contributions

Wenjuan Xun: Conceptualization, Investigation, Data accumulation, Writing assessment and its editing, Project administration, Supervision and Funding acquisition. **Mengyao Ji:** Methodology, Data acquisition, Writing-original draft preparation. **Zhonghua**

Ma: Formal analysis, Data curation, Software. **Tanjie Deng:** Methodology, Software. **Wen Yang:** Software. **Liguang Shi:** Investigation, Supervision. **Guanyu Hou:** Methodology, Resources. **Ting Cao:** Methodology, Resources.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and 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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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.05.004.

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