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Gallic acid mitigates LPS-induced inflammatory response via suppressing NF-κB signalling pathway in IPEC-J2 cells

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Abstract

Gallic acid is a phenolic compound that exhibits antibacterial, antioxidative and anti-inflammatory functions. In a previous study, we found that dietary supplementation with gallic acid decreased incidence of diarrhoea and protected intestinal integrity in weaning piglets. However, the underlying mechanism remains unclear. Here, a pig intestinal epithelial cell line (IPEC-J2) was used as an in vitro model to explore the antioxidant and anti-inflammatory capacity of gallic acid. IPEC-J2 cells were stimulated with hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS) to establish oxidative and inflammatory models, respectively. Results showed that H_2O_2 significantly decreased catalase (CAT) secretion and CAT mRNA abundance in the cells ($p < 0.05$), while pretreatment with gallic acid did not prevent the decrease in CAT expression induced by H_2O_2. However, gallic acid pretreatment mitigated the increased expression of the tumour necrosis factor-α and interleukin-8 genes caused by LPS in IPEC-J2 cells ($p < 0.05$). In addition, pretreatment with gallic acid significantly suppressed phosphorylation of NF-κB and IκBα in LPS-stimulated IPEC-J2 cells. Moreover, LPS stimulation decreased the protein abundance of zona occludens 1 (ZO-1) and occludin, while pretreatment with gallic acid preserved expression level of tight junction proteins ZO-1 and occludin in LPS-stimulated IPEC-J2 cells ($p < 0.05$). In conclusion, gallic acid may mitigate LPS-induced inflammatory responses by inhibiting the NF-κB signalling pathway, exerting positive effects on the barrier function of IPEC-J2 cells.

KEYWORDS
anti-inflammation, barrier function, gallic acid, IPEC-J2 cells, LPS

1 | INTRODUCTION

The gut is an important first barrier to defend the body against diet-derived pathogens, mycotoxins and antigenic materials from external environment. The integrity of intestine is essential to barrier function. Intestinal integrity might be compromised by localized inflammatory responses in the small intestine of weaned pigs (Pitman & Blumberg, 2000; Suzuki, 2013; Wang et al., 2015). During the inflammatory reaction, intestinal epithelial cells (IECs) secrete a series of proinflammatory cytokines, chemokines, and various other inflammatory mediators (Abarikwu, 2014). These molecules help to battle the pathogens and harmful substances; however, if the immune system of the animals is overactivated, excessive expression of inflammatory cytokines will further exacerbate systemic inflammation (Kanda et al., 2006), and the structure and function of the intestine will be impaired (Odenwald & Turner, 2017). Impaired intestinal integrity leads to diarrhoea and stunted growth in weaning piglets, accounting for major losses for pig farms.

Long Cai and Zixi Wei contributed equally to this study

Previous studies have demonstrated that phenolic compounds exhibit gut protective functions in animals and humans (Bravo, 1998). Gallic acid (GA), a type of polyphenolic compound in pomegranate peel, has multiple beneficial effects, for instance antibacterial, antioxidant and anti-inflammatory (Shao et al., 2015; Yen et al., 2002; Kroes et al., 1992). Gallic acid has been applied as an antioxidant agent in animal feed (Samuel et al., 2017; Wei et al., 2016). Our previous study observed that dietary GA supplementation decreased the incidence of diarrhoea and protected intestinal integrity, which might be attributed to a moderate intestinal immune response in weaning piglets (Cai et al., 2020). However, there is limited data about the underlying mechanism of the protective effects of GA on pig intestinal epithelial cells.

Therefore, the aim of this study was to investigate the protective effect and mechanism of GA on pig intestinal epithelial cells under oxidative stress and inflammatory conditions. We hypothesized that GA suppresses oxidative stress and inflammatory response by modulating antioxidant and inflammatory signalling pathways. Pig intestinal epithelial cells (IPEC-J2) were used as a cell model to explore the antioxidant and anti-inflammatory effects of GA in this study. Results from this study provide the basis for the application of GA as a gut protective agent in the diet of weaning piglets.

2 | MATERIALS AND METHODS

2.1 | Materials

Gallic acid (GA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12), phosphate buffered saline (PBS), foetal bovine serum (FBS) and Penicillin-Streptomycin (pen-strep) were purchased from Gibco BRL Life Technologies (Thermo Fisher Scientific). Lipopolysaccharide (LPS) was procured from Beyotime Biotech. Cell Counting Kit (CCK-8) and bay 11–7082 were acquired from Medchem Express (MCE).

2.2 | Cell culture

Pig intestinal epithelial cells line (IPEC-J2) were cultured as in our previous study (Liu, Jiang, et al., 2019). Briefly, IPEC-J2 cells were cultured in DMEM/F12 medium supplied with 5% FBS, 1% pen-strep, 0.1% ITS solution (containing 5 μg/L insulin, 5 μg/L transferrin and 5 ng/L sodium selenite, Corning Inc.) and 0.01% EGF (5 μg/L, Corning Inc.), and grown in a humidified incubator at 37°C containing 5% carbon dioxide (CO_2) atmosphere. Culture medium was replaced daily. During 80–90% confluence, cells were passaged utilizing 0.25% trypsin-EDTA (Gibco, MA, USA) and subcultured for further investigations. We randomly designated a certain column (row) of plates as a treatment after we seeded cells into the well plates in this experiment.

2.3 | Cell viability assay

The cytotoxicity of GA was measured using the Cell Counting Kit 8 according to the manufacturer's instructions. Briefly, 5×10^3 cells per well were seeded into 96-well plates (Corning) with 6 replications (wells) per treatment, then cultured in medium until reaching 70–80% confluence. After 6 h or 24 h treated with various concentration of GA, cells were washed twice with preheated PBS. The IPEC-J2 cells were incubated with 100 μl of 10% CCK-8 reagent solution for 3 h at 37°C; then, optical density values (OD) of culture media at 450 nm were measured employing a microplate reader (BioTek Instruments, Inc). The cell viability was expressed as the ratio of the treatment group to the control group.

2.4 | H_2O_2 stimulation model

H_2O_2 was used to stimulate the IPEC-J2 cell line to build an in vitro injury model. Briefly, IPEC-J2 cells were seeded into 6 or 12-well plates (Corning) and cultured in medium until reaching 60–70% confluence. Then, the cells were incubated with 100 μg/L gallic acid for 24 h, followed by co-incubation for an another 1 h in the presence or absence of H_2O_2. The concentration of hydrogen peroxide was selected based on previous reports (Cao et al., 2020). After treatment, intracellular protein and RNA were prepared respectively and stored at −80 °C until analysis.

2.5 | LPS stimulation model

LPS at 40 μg/ml was used to stimulate the cells was to establish an in vitro inflammation model. Concisely, cells were seeded into 6 or 12-well plates and cultured in medium, after 80–90% confluence, cells were incubated with 100 μg/L gallic acid for 1 h, followed by incubation for an additional 4 h in the presence or absence of LPS. After treatment, intracellular protein and RNA were extracted respectively for further assays.

2.6 | Antioxidant index measurement

In accordance with the instructions supplied by manufacturer, the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in cells was determined with commercial assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) using the visible light method, nonenzymatic NBT test method and 5, 50-dithiobis-p-nitrobenzoic acid method, respectively.

2.7 | Real time quantitative -PCR (qPCR)

Total RNA in IPEC-J2 cells was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the protocol provided by the manufacturer. Then, the content and quality of total RNA were

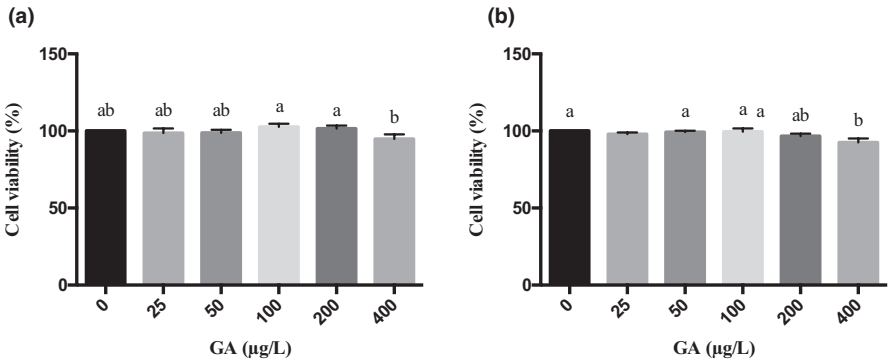

FIGURE 1 Effect of gallic acid on the viability of IPEC-J2 cells. Cells were incubated with various concentrations (0, 25, 50, 100, 200, or 400 μg/L) of gallic acid for 6 h (a) or 24 h (b), and cell viability was assayed using the CCK-8 reagent. The data are expressed as the mean ± SE, ($n = 6$). Different letters represent a significant difference ($p < 0.05$)

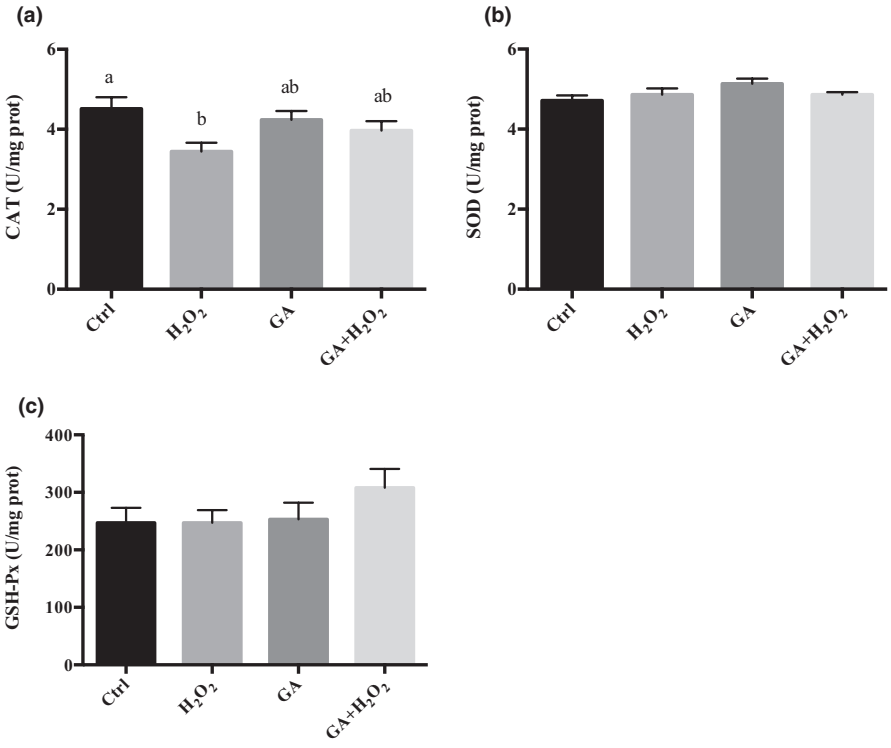

FIGURE 2 Effect of gallic acid on the activities of antioxidant enzyme in IPEC-J2 cells. IPEC-J2 cells were pretreated with gallic acid (100 μg/L) for 24 h before stimulation with H_2O_2 (0.6 mmol/L) for 1 h. Then, intracellular proteins were isolated, and the activities of CAT (a), SOD (b) and GSH-Px (c) were measured using commercial assay kit. The data are expressed as the mean ± SE, ($n = 6$). Different letters indicate a significant difference ($p < 0.05$)

detected using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.), and cDNA was synthesized using a TransScript First-Strand cDNA Synthesis Kit (TransGen Biotech) by reverse transcription. qPCR analysis was performed using SYBR Green reagent (Thermo Fisher Scientific) on an real-time fluorescence quantitative system (Thermo Fisher Scientific). Expression of target genes relative to GAPDH was analysed by the $2^{-\triangle\triangle Ct}$ method and using GAPDH as the housekeeping gene. GAPDH did not exhibit any difference among all groups (Liu, Li, et al., 2019). The relative expression of each target gene was normalized to the control group. The primers used for qPCR are listed in Table S1.

2.8 | Western blotting analysis

Cells were washed with chilled PBS three times and lysed in RIPA buffer freshly (Thermo Fisher Scientific) containing 1% protease inhibitors and phosphatase inhibitor cocktail (Thermo Fisher Scientific) for half hours min under ice bath conditions, protein content of the cell extracts was quantitated using a bicinchoninic acid (BCA) protein assay kit (Applygen) after centrifugation. 12% SDS-polyacrylamide gel electrophoresis was used to separate the cell samples, then were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc.) by the wet method. After blocking at room temperature for 3 h, membranes were incubated with diluted primary antibodies at 4°C overnight with gentle shaking. Tris buffer saline (TBST) added 0.1% Tween 20 was used to wash the membranes three times and then treated with corresponding HRP-conjugated secondary antibodies at 25°C for 1 h. At last, membranes were washed again with TBST and were visualized using the reagents in a Western Bright ECL Kit (Bio-Rad Laboratories Inc.) and then the images were detected using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc). All antibodies used in this study are listed in Table S2.

2.9 | Statistical analysis

The results are presented as the mean values ± SE. Data were analysed using one-way ANOVA in SAS v. 9.2 (SAS Institute Inc), and differences among treatment means were determined by the Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant, and $0.05 < p < 0.10$ was considered a tendency to be significant.

3 | RESULTS

3.1 | Effect of gallic acid on the viability of IPEC-J2 cells

The results of CCK-8 assay showed that concentrations of gallic acid lower than 400 μg/L had no obvious effect on the cell viability after incubation for 6 h (Figure 1a). However, the viability of IPEC-J2 cells was significantly decreased at a concentration of 400 μg/L compared to the control group when incubated with gallic acid for 24 h ($p < 0.05$) (Figure 1b). Therefore, we used 100 μg/L gallic acid in subsequent experiments.

3.2 | Effect of gallic acid on the activity of antioxidant enzymes in H_2O_2-stimulated IPEC-J2 cells

Levels of CAT were significantly reduced in response to H_2O_2 stimulation ($p < 0.05$) (Figure 2a), and pretreatment with 100 μg/L GA did not affect CAT levels compared to the H_2O_2-stimulated group. However, there was no difference in levels of SOD or GSH-Px among any groups (Figure 2b,2c).

3.3 | Effect of gallic acid on gene expression of antioxidant enzymes in H_2O_2-stimulated IPEC-J2 cells

Gene expression of CAT was significantly reduced in response to H_2O_2 stimulation ($p < 0.05$) (Figure 3a). Pretreatment with 100 μg/L GA did not prevent depressed CAT gene expression caused by H_2O_2 stimulation. There was no difference in gene expression of SOD1 or GPx1 among all groups (Figure 3b,3c).

3.4 | Effect of gallic acid on gene expression of inflammatory cytokine in LPS-stimulated IPEC-J2 cells

Based on the results in Figure S1, we used 40 μg/ml LPS to establish an LPS-stimulated model in this experiment. The result shown that expression of TNF-α (Figure 4a), IL-8 (Figure 4b) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Figure 4c) was significantly increased in response to LPS stimulation ($p < 0.05$). Gallic acid pretreatment suppressed the expression of TNF-α and IL-8 induced by LPS ($p < 0.05$), although suppression of GM-CSF expression was not significant. Adding gallic acid alone to the culture medium had no significant effect on expression of the above mentioned inflammatory factor genes. In addition, pretreatment with gallic acid had no restored effect to the expression of CAT induced by LPS (Figure S2).

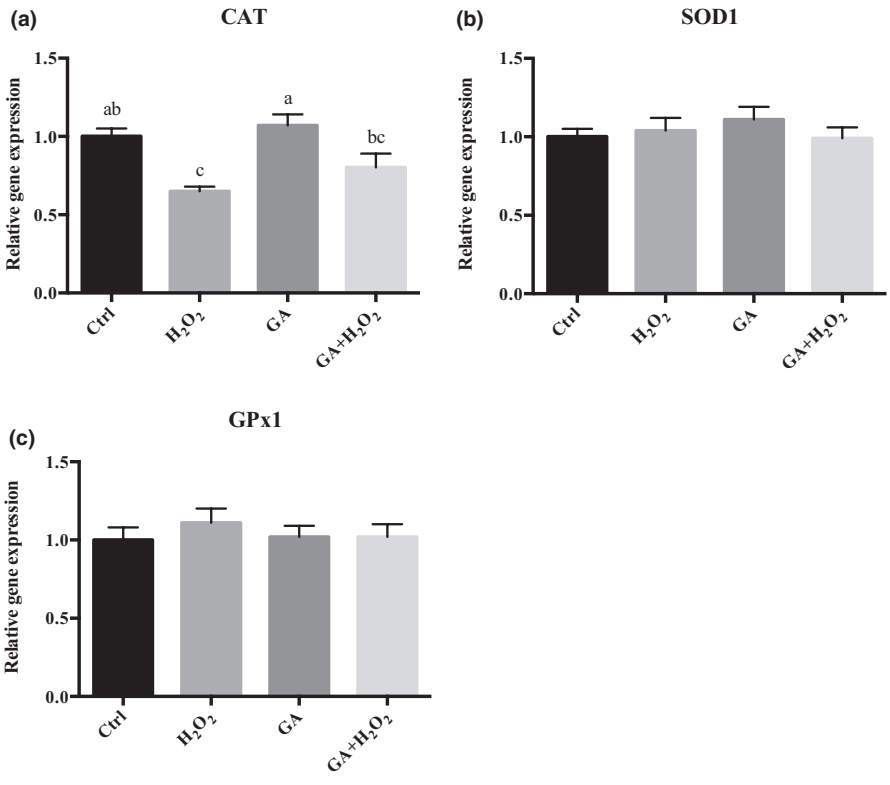

FIGURE 3 Effect of gallic acid on expression of antioxidant enzyme genes in H_2O_2-stimulated cells. IPEC-J2 cells were pretreated with gallic acid (100 μg/L) for 24 h before stimulation with H_2O_2 (0.6 mmol/L) for 1 h. Then, total RNA was collected using TRIzol reagents, and mRNA levels of CAT (a), SOD1 (b) and GPx1 (c) were quantified by qPCR. The data are expressed as the mean ± SE, ($n = 6$). Different letters indicate a significant difference ($p < 0.05$)

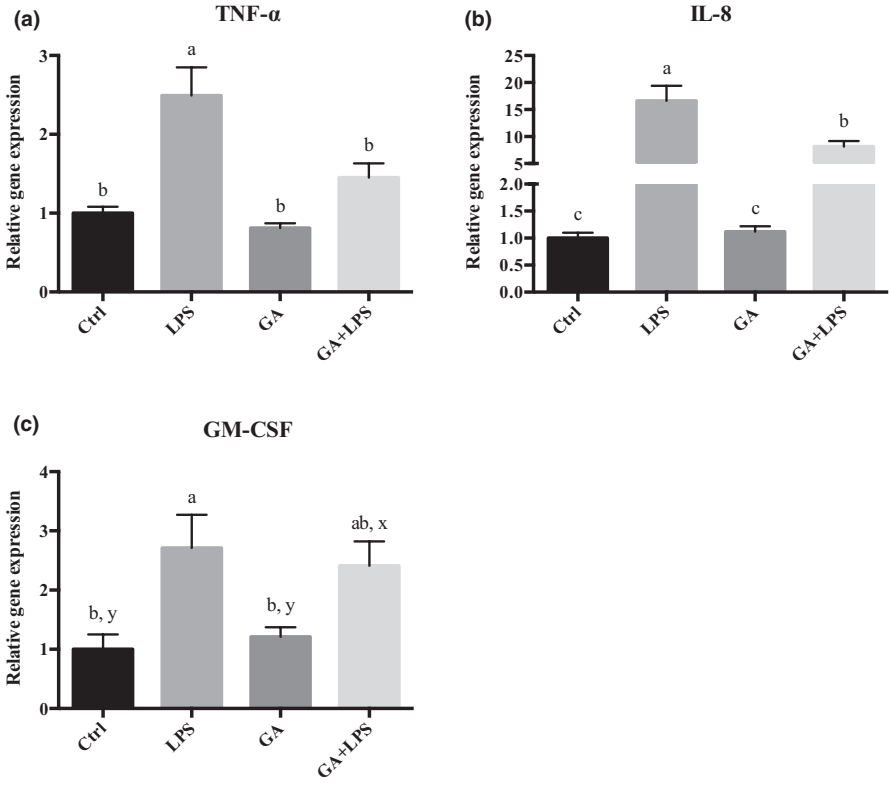

FIGURE 4 Effect of gallic acid on expression of inflammatory genes in LPS-stimulated cells. IPEC-J2 cells were pretreated with gallic acid (100 μg/L) for 1 h before being treated with LPS (40 μg/ml) for another 4 h. Then, total RNA was extracted using TRIzol reagent, and mRNA levels of TNF-α (a), IL-8 (b), GM-CSF (c) were quantified by qPCR. The data are expressed as the mean ± SE, ($n = 6$). Different letters a and b represent a significant difference ($p < 0.05$), while different letters x and y represent a tendency to be significant ($0.05 < p < 0.10$)

3.5 | Effect of gallic acid on activation of NF-κB and IκBα proteins in LPS-stimulated IPEC-J2 cells

As shown in Figure 5, LPS stimulation had no effect on total protein abundance of NF-κB or IκBα; however, LPS significantly increased IκBα phosphorylation and activation of NF-κB protein. Addition of GA to the media did not change total protein abundance or phosphorylation of NF-κB in IPEC-J2 cells in the absence of LPS challenge. In contrast, adding GA to the media enhanced total protein abundance and phosphorylation of IκBα but had no effect on the ratio of phosphorylated IκBα to total IκBα protein in IPEC-J2 cells in the absence of LPS challenge. Pretreatment with GA suppressed phosphorylated NF-κB and IκBα levels induced by LPS ($p < 0.05$).

3.6 | Effect of gallic acid on abundance of tight junction protein in LPS-stimulated IPEC-J2 cells

From Figure 6, the protein expression level of ZO-1 and occludin was significantly decreased in response to LPS stimulation ($p < 0.05$), whereas pretreatment of cells with GA prevented suppression of protein levels of ZO-1 and occludin by LPS stimulation ($p < 0.05$). Addition of bay 11–7082, an IκBα phosphorylation and NF-κB inhibitor, inhibited phosphorylation of NF-κB, which blocked the suppressive effect of LPS on ZO-1 and occludin expression (Figure S3).

4 | DISCUSSION

Gallic acid (GA) is a primary active compound of gallnuts (Liu, Li, et al., 2019), which has been shown its antioxidant activity in vitro (Phonsatta et al., 2017; Kroes et al., 1992). Moreover, it has been demonstrated that GA inhibited the production of reactive oxygen species in human peripheral blood mononuclear cells, due to its antioxidative stress effect (Jantan et al., 2014). However, there is seldom report concerning the effect of GA on intestine damage induced by oxidative stress in pig intestinal epithelial cells. Herein, we first established in vitro challenge models using IPEC-J2 cells stimulated with H_2O_2 to investigate the mechanism of GA in protecting IPEC-J2 cells as previous reports (Cao et al., 2020). In the present study, we found that pretreatment of cells with GA failed to alleviate oxidative stress caused by H_2O_2, which is reflected in the inability to effectively rescue the activity of catalase and its gene expression. Consistent with our results, it has been reported that pretreatment with different concentrations of GA was unable to significantly decrease intracellular reactive oxygen species (iROS) production in H2O2 stimulated porcine intestinal epithelial cells (Vergauwen et al., 2016). The present results are consistent with our previous in vivo study in which dietary GA supplementation did not affect plasma oxidative status in weaned piglets (Cai et al., 2020). Therefore, our results indicate that GA may have little antioxidant effect in IPEC-J2 cells in response to H2O2 stimulation.

Pro-inflammatory mediator are known to regulate the inflammatory response (Suzuki et al., 2011). GA serves to restrain the

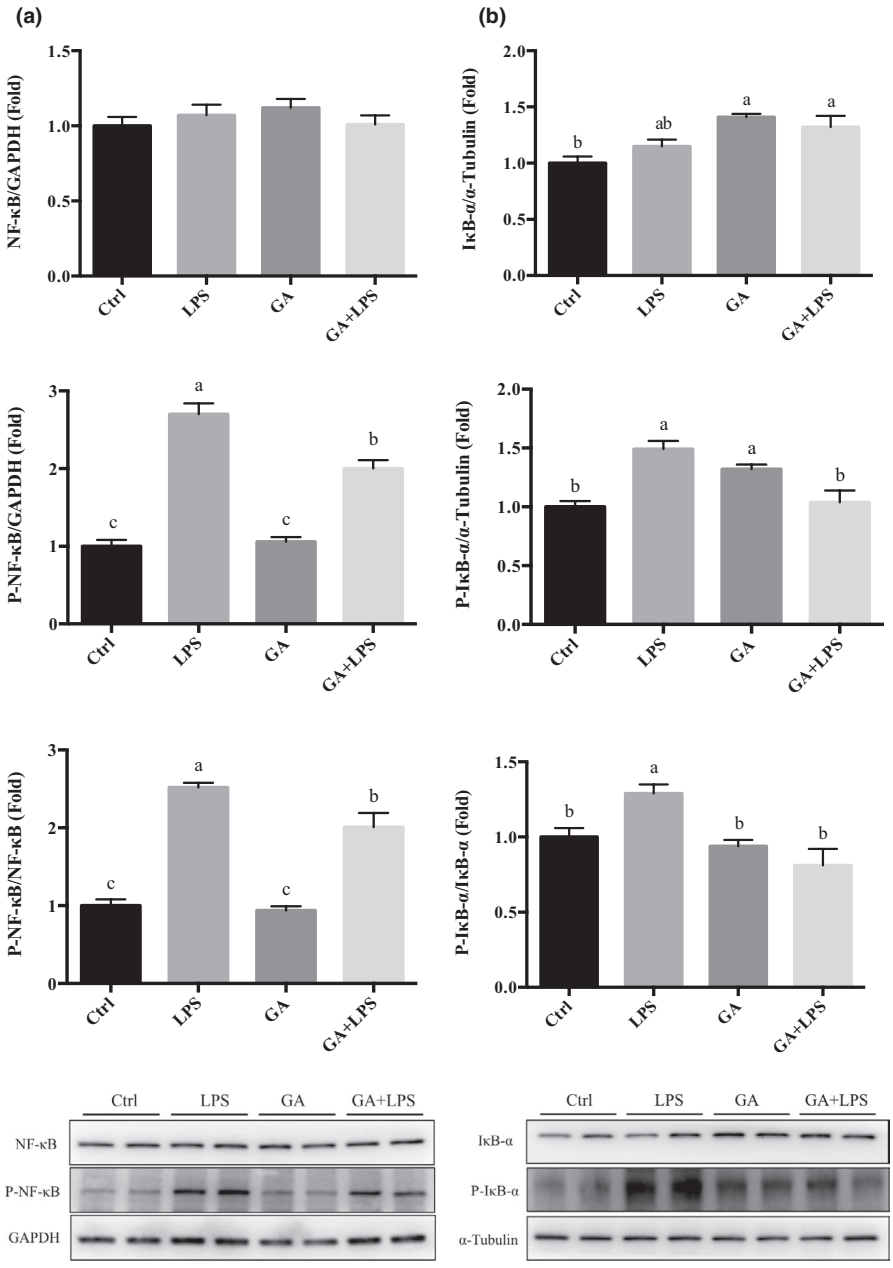

FIGURE 5 Effect of gallic acid on phosphorylation of NF-κB and IκB-α in IPEC-J2 cells. IPEC-J2 cells were pretreated with gallic acid (100 μg/L) for 1 h before stimulation with LPS (40 μg/ml) for 4 h. Then, intracellular proteins were isolated, and the abundance of NF-κB (a), P-NF-κB (a), IκB-α (b) and P-IκB-α (b) proteins were determined by western blotting. Protein levels were normalized to the housekeeping protein GAPDH (for NF-κB, and P-NF-κB) or α-tubulin (for IκB-α and P-IκB-α). The data are expressed as the mean ± SE, ($n = 6$). Different letters represent a significant difference ($p < 0.05$)

expression of inflammatory cytokines, and thus, the inflammatory response (Jantan et al., 2014). Consistent with the previous study, our results reveal that GA suppresses the inflammatory response as shown by a reduction in the expression of proinflammatory cytokines including TNF-α and IL-8 in porcine small intestinal cells. The anti-inflammatory function of GA has also been reported in other cell lines. It has been demonstrated that GA attenuated the expression of inflammatory cytokine genes in RAW 264 macrophages (Tanaka et al., 2018). Similarly, gallic acid also dose-dependently inhibited TNF-α and IL-6 gene expression in HMC-1 cells (Kim et al., 2006). Taken together, these data suggest that GA primarily inhibits the excessive expression of different inflammatory mediators in IPEC-J2 cells to alleviate the inflammatory response induced by LPS.

NF-κB signalling pathway plays pivotal roles in inflammatory responses and immunological reactions. NF-κB is the master regulator of inflammatory cytokine expression. In the canonical pathway, NF-κB proteins form an inactive cytoplasmic complex by binding to the inhibitory protein IκB. External stimuli, such as proinflammatory cytokines, LPS, and growth factors, activate IκKα/β by phosphorylation. Activated IκKα/β phosphorylates IκBα protein, resulting in its ubiquitination and proteasomal degradation. Then, NF-κB is released from binding IκB and translocates into the nucleus to induce target gene expression (Kundu et al., 2006; Uwe, 2008; Yoon et al., 2010). In the current study, our results showed that LPS significantly increased the phosphorylation of IκBα and NF-κB in IPEC-J2 cells. Pretreatment with gallic acid suppressed phosphorylation of IκBα and NF-κB, indicating that GA may mitigate the expression of proinflammatory cytokines by suppressing NF-κB signalling. Consistent with our results, a previous study found that the anti-inflammatory potential of GA

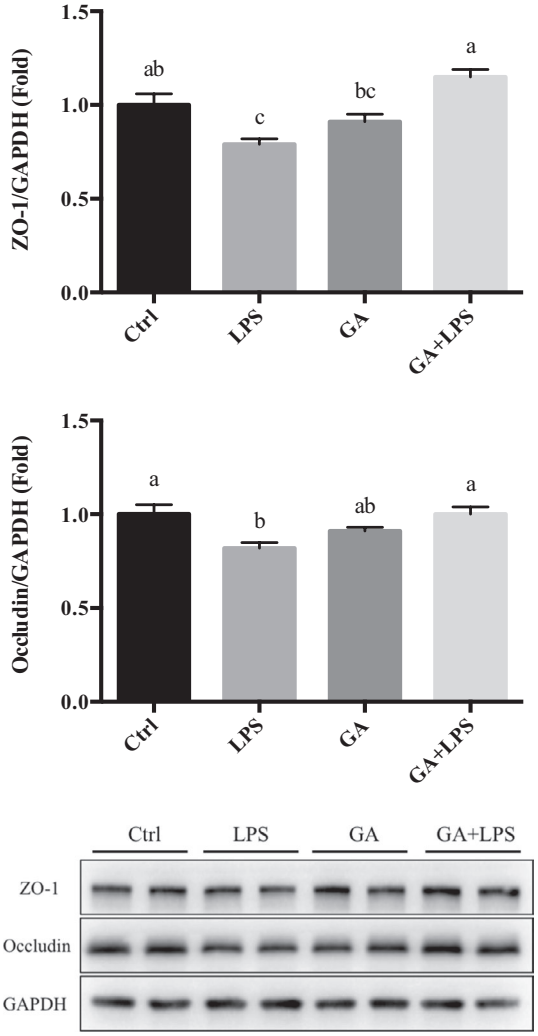

FIGURE 6 Effect of gallic acid on the abundance of barrier function proteins in LPS-stimulated cells. Intracellular proteins were isolated, and the abundance of ZO-1 and occludin proteins were determined by western blotting. Protein levels are normalized to the housekeeping protein GAPDH. The data are presented as the mean ± SE, ($n = 6$). Different letters represent a significant difference ($p < 0.05$)

was mediated by downregulation of the NF-κB pathway in mice (Hsiang et al., 2013). Other studies also found that *R. suavissimus* leaf extract, whose main biologically active ingredient includes GA, which inhibits the secretion of various proinflammatory factors by suppressing LPS-stimulated IκBα degradation and p65 nuclear translocation (Hu et al., 2014; Yang et al., 2001). Intriguingly, adding GA alone enhanced total protein abundance and phosphorylation of IκBα instead of the ratio of phosphorylated IκBα to its total protein, it has been well known that the activation of IκBα would not thoroughly reflect the expression of proinflammatory cytokines, but as a pivotal molecule, the phosphorylation of NF-κB coordinates the inflammatory response at numerous levels (Yoon et al., 2010). Taking together, these data indicate that GA exerts an anti-inflammatory function in IPEC-J2 cells, which might occur through suppressing NF-κB pathway-induced proinflammatory cytokine expression. To the best of our knowledge, this is the first study on the beneficial role of gallic acid on inhibiting porcine intestinal epithelial cell inflammatory responses in response to LPS stimulation in vitro.

The intestinal epithelial barrier plays a critical role in preventing the invasion of pathogens and other harmful chemicals, and tight junctions are the primary component of the intestinal barrier. Intestinal inflammation is closely associated with tight junction disruption and barrier dysfunction (Han et al., 2015; Lu et al., 2014; Mennigen et al., 2009; Schulzke et al., 2009). The current results demonstrated that excessive inflammation induced by LPS reduces protein levels of ZO-1 and occludin, two important tight junction proteins. This observation is consistent with previous studies (Chen et al., 2019; He et al., 2019). Importantly, pretreatment with GA recovered protein levels of ZO-1 and occludin induced by LPS stimulation, which may be closely related to the suppression of excessive inflammatory response. In the present study, we demonstrate that the protective effect of gallic acid on intestinal barrier function, this may explain the observation in our previous study that dietary supplementation with GA enhanced intestinal integrity (Cai et al., 2020). These findings contribute to understanding of the effects of gallic acid on maintaining intestinal barrier homeostasis, but the molecular basis for the effect of GA on barrier function needs further exploration, especially its molecular targets (e.g. protein kinases).

In conclusion, our study revealed that GA has few antioxidative effect in IPEC-J2 cells; however, it did alleviate the inflammatory response induced by LPS, which might occur through suppressing the NF-κB pathway. The reduced inflammatory response due to GA might benefit barrier function by enhancing the expression of tight junction proteins. Our study demonstrates the anti-inflammatory function of GA in pig intestinal epithelial cells, revealing that NF-κB signalling is involved in this process and implicating plant-derived phenolic compounds in the protection of intestinal health in weaning piglets.

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CONFLICTS OF INTEREST

The authors declare no competing financial interest.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as there is no animal trial conducted in this research article.

DATA AVAILABILITY STATEMENT

The data will be available on request from the corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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