Original Paper

Clostridium Tyrobutyricum Protect Intestinal Barrier Function from LPS-Induced Apoptosis via P38/JNK Signaling Pathway in IPEC-J2 Cells

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Key Words

Clostridium tyrobutyricum • IPEC-J2 • Intestinal barrier function • Apoptosis • P38/JNK signaling pathway

Abstract

Background/Aims: The intestinal mucosa forms a physical and metabolic barrier against the diffusion of pathogens, toxins, and allergens from the lumen into the circulatory system. Early weaning, a critical phase in swine production, can compromise intestinal barrier function through mucosal damage and alteration of tight junction integrity. Maintenance of intestinal barrier function plays a pivotal role in optimum gastrointestinal health. In this study, we investigated the effects of *Clostridium tyrobutyricum* (C.t) on intestinal barrier dysfunction induced by lipopolysaccharide (LPS) and the underlying mechanisms involved in intestinal barrier protection. **Methods:** A Transwell model of IPEC-J2 cells was used to imitate the intestinal barrier. Fluorescence microscopy and flow cytometry were used to evaluate apoptosis. Real-time PCR was used to detect apoptosis-related genes and the downstream genes of the p38/c-Jun N-terminal kinase (JNK) signaling pathways. Western blotting was used to measure the expressions of tight junction proteins and mitogen-activated protein kinases. **Results:** C.t efficiently maintained trans-epithelium electrical resistance values and intestinal permeability after LPS-induced intestinal barrier disruption. The expressions of tight junction proteins (ZO-1, claudin-1, and occludin) were promoted when IPEC-J2 cells were treated with C.t. Fluorescence imaging and flow cytometry revealed that C.t qualitatively and quantitatively inhibited LPS-induced cell apoptosis. C.t also increased the relative expression of the anti-apoptotic gene Bcl-2 and decreased that of the apoptotic genes Bax and caspase-3/-8. Moreover, the protective effect of C.t on damaged intestinal cell models was associated with suppression of p38 and JNK phosphorylation, negative regulation of the relative expressions of downstream genes including AP-1, ATF-2, ELK-1, and p53, and activation of Stat3 expression. **Conclusions:** These findings indicate that C.t may promote intestinal integrity, suggesting a novel probiotic effect on intestinal barrier function.

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Introduction

The intestinal epithelial barrier, which is formed by intestinal mucosa, separates the internal milieu from the external environment and is the first line of defense against bacterial and toxin invasion of host tissues [1, 2]. At early weaning, the intestinal barrier function of pigs is vulnerable to damage. The epithelial layer impairment results in poor nutrient digestion and absorption, reduced transmucosal resistance, imbalanced secretion and absorption of water and electrolytes, and increased enteric pathogen infection. Disruption of the intestinal barrier disturbs immune homeostasis, increases inflammation in the intestine, and is associated with intestinal diseases such as diarrhea [3-5]. Therefore, maintenance of intestinal barrier function plays a pivotal role in optimum gastrointestinal health.

Probiotics, defined as live microorganisms, can maintain epithelial integrity and barrier function, stimulate the cellular repair mechanism, and reestablish well-balanced indigenous intestinal and respiratory microbial communities [6]. Probiotics such as *Lactobacillus* and *Enterococcus faecium* help to protect the intestinal epithelium after infection with *E. coli* or exposure to lipopolysaccharide (LPS) [7, 8]. LPS, produced by Gram-negative bacteria, plays important roles in the integrity of the outer membrane permeability barrier and participates extensively in host-pathogen interplay [9]. LPS interacts with and activates Toll-like receptors, which themselves activate mitogen-activated protein kinases (MAPK) [10]. The MAPK family can be classified into extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Signaling by these pathways govern fundamental cellular responses, such as cell proliferation, differentiation, inflammation, and apoptosis [11-12].

Clostridium tyrobutyricum (*C.t*), a Gram-positive anaerobic bacterium, can efficiently produce butyric acid and is a promising probiotic to protect intestinal epithelial function. Therefore, in this study, we investigated whether *C.t* could protect porcine intestinal epithelial barrier function, hypothesizing that its benefit might be associated with the MAPK signaling pathway in IPEC-J2 cells.

Materials and Methods

Cell culture and *C.t* preparation

Intestinal porcine epithelial cells (IPEC-J2) were obtained from the Institute of Subtropical Agriculture (Chinese Academy of Sciences) and cultured in Dulbecco's modified Eagle medium/F12 medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Gemini, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C with 5% CO₂ in a humidified atmosphere. No antibiotics were used during the treatment. *C.t* was obtained from Ohio State University and cultured anaerobically at 37 °C in clostridial growth medium (CGM) [13]. *C.t* was then collected after centrifugation at 12,000 rpm for 5 min, washed 3 times, and suspended in phosphate-buffered saline (PBS) at different concentrations (10⁶ to 10¹¹ CFU/mL) for cell treatments.

Cell viability and cytotoxicity assay

Cell viability following treatment with different concentrations of *C.t* (ranging from 10⁶ to 10¹¹ CFU/mL) for different hours (2, 4, 6, 8, and 10 h) was measured using the Cell Counting Kit 8 (MedChem Express, Monmouth Junction, NJ, USA). Cell cytotoxicity following the treatments with different concentrations of *C.t* (ranging from 10⁸ to 10¹¹ CFU/mL) for 8 h was measured using a lactate dehydrogenase (LDH) detection kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, IPEC-J2 cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well, cultured overnight to allow cell attachment, and then pretreated with *C.t* after reaching 70-80% confluence. To evaluate cell viability, 10 µL of the Cell Counting Kit 8 assay solution was added to each treated cell well and the cells were further incubated for 1 to 2 h. Subsequently, the optical density was measured using SpectraMax M5 (Molecular Devices, California, USA) at a 450 nm wavelength, and the percentage of living cells was calculated as described by Mosmann [14]. As for cell cytotoxicity, 150 µL LDH release solution was added to each cell well and incubated for 1 h. Next, 120 µL cell-free culture supernatants were collected into new 96-well plates after centrifugation at 1,200 rpm

for 5 min. Finally, 60 μ L LDH working assay solution was added to each cell well and the optical density was measured using the SpectraMax M5 at a 490 nm wavelength with a reference wavelength of 620 nm [15].

Trans-epithelium electrical resistance measurements

IPEC-J2 cells were seeded onto polycarbonate membrane filters (0.4- μ m pore size, 1.12 cm² growth area) inside Transwell® cell culture chambers (Corning Costar, Cambridge, MA, USA) at a density of 1×10^5 cells/cm². The culture medium was changed every day and trans-epithelium electrical resistance (TEER) values were measured every other day using the Millicell Electrical Resistance System (Millipore ERS-2, Massachusetts, USA). When a monolayer of cells was considered to be completely differentiated as previously described [16], cells were treated with PBS, LPS (LPS; 1 μ g/mL; LPS O55:B55, Sigma, Massachusetts, USA), *C.t*, LPS + *C.t* (cells were treated with 1 μ g/mL LPS, followed by *C.t*), or *C.t* + LPS (cells treated with *C.t*, followed by 1 μ g/mL LPS), and the TEER values were measured every 4 h.

Cell permeability to FD4

Immediately after the last TEER value measurement, 100 μ L of 1 mg/mL 4-kDa fluorescein isothiocyanate-dextran (FD4; Sigma-Aldrich, St. Louis, MO, USA) was added into the upper compartments of the Transwells. The Transwells were cultured at 37 °C for 30 min. Then, 100 μ L of medium of each well from the lower compartments of the Transwells was added into wells of a black 96-well plate and the fluorescence intensity was detected using a SpectraMax M5 at an excitation wavelength of 480 nm and emission wavelength of 520 nm [17].

Cell apoptosis detection

Apoptotic IPEC-J2 cells were detected using an Annexin V-FITC/PI kit (R&D Systems, Minnesota, USA) according to the manufacturer's instructions. Cells at a density of 1×10^6 cells/well were seeded in 6-well plates overnight. After the treatments were finished, 500 μ L of 1 $\times$ Binding Buffer, 5 μ L Annexin V-FITC, and 5 μ L propidium iodide (PI) were added into each well after two washes with PBS and the plates were incubated for 15 min at room temperature in the dark. Fluorescence images were taken by a TE2000 fluorescence microscope (Nikon, Tokyo, Japan). To determine apoptotic cells by flow cytometry, cells at a density of 1×10^6 cells/well were seeded in 6-well plates overnight. The medium and cells were digested by pancreatin without ethylenediaminetetraacetic acid (EDTA) (Beyotime), collected into 10-mL centrifugal tubes, and centrifuged for 5 min at 1,000 rpm. Cells were then suspended in 100 μ L 1 $\times$ Binding Buffer at a concentration of 1×10^5 cells/mL in 10-mL centrifugal tubes. Next, 5 μ L FITC Annexin V and 5 μ L PI were added into the tubes and they were incubated for 15 min at room temperature in the dark. Finally, 400 μ L 1 $\times$ Binding Buffer was added to each tube and cell apoptosis was analyzed by flow cytometry (FACSCalibur, B&D, Minnesota, USA) within 1 h.

Western blot analysis

Cells cultured in Transwells and 6-cell culture plates were first digested with 0.25% trypsin/EDTA (Biological Industries, Kibbutz Beit Haemek, Israel) into centrifuge tubes. Total cell protein was extracted using the Whole Cell Lysis Assay Kit (KeyGEN BioTech, Beijing, China) after two PBS washes. The protein concentration was determined using the BCA Assay Kit (KeyGEN BioTech). Approximately 30 μ g of protein was boiled in 5 $\times$ loading buffer (Sangon Biotech, Shanghai, China), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes (Millipore, Massachusetts, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated in the corresponding primary antibodies at 4 °C overnight. After being washed with TBST 3 times for 10 min each, the membranes were incubated with secondary antibodies for 1 h at room temperature. The membranes were then washed with TBST as before. The immunoreactive bands were visualized with ECL luminescence reagent (KeyGEN BioTech) using a ChemiScope Touch (Clinux, Shanghai, China). The band intensities were determined using ImageJ software. Primary antibodies ZO-1, Claudin-1, and Occluding were purchased from Proteintech (Wuhan, China). ERK1/2, phospho-ERK1/2, p-38, phospho-p38, JNK, phospho-JNK, and HRP-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Massachusetts, USA). β -actin was purchased from Abcam (Massachusetts, USA), and HRP-conjugated anti-mouse IgG was obtained from Beyotime.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA). RNA quantity and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). cDNA was synthesized with the PrimeScript RT reagent kit with gDNA Eraser (TAKARA, Dalian, China) according to the manufacturer's instructions. Briefly, 1 µg total RNA was used to erase gDNA at 42 °C for 2 min. The reverse transcription was conducted at 37 °C for 15 min and 85 °C for 5 s. Real-time PCR was performed on a CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA) in triplicate, in a total volume of 25 µL consisting of 12.5 µL SYBR Premix EX Taq (TAKARA), 0.5 µL of each primer (10 µM), 2 µL of cDNA template, and 9.5 µL double-distilled water. The PCR cycle conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analysis was used to confirm the specificity and reliability of the PCR products. β-actin was used as a house-keeping gene to normalize target gene levels. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [18]. Primers used in this study were designed with Primer 5.0 (Table 1) and synthesized in Tsingke (Beijing, China).

Statistical analysis

Statistical analysis was performed with one-way analysis of variance followed by a Duncan multiple range test with SPSS 17.0 (SPSS, Chicago, IL, USA). All data are expressed as the mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

Results

C. t boosted cell viability and reduced cytotoxicity in IPEC-J2 cells

IPEC-J2 cells were treated with *C. t* at different final concentrations (ranging from 10^6 to 10^{11} CFU/mL) for 2, 4, 6, 8, and 10 h. As shown in Fig. 1A, *C. t* treatment enhanced the growth of IPEC-J2 cells in a dose- and time-dependent manner. For instance, cell viability was increased significantly at 8 h ($P < 0.001$). When cells were treated with high concentrations (ranging from 10^8 to 10^{11} CFU/mL), cell viability was increased significantly compared with other concentrations ($P < 0.05$) (Fig. 1A). Cell cytotoxicity was measured after cells were treated with *C. t* (ranging from 10^8 to 10^{11} CFU/mL) for 8 h, with the results showing that LDH release was decreased when cells were treated with 10^8 and 10^9 CFU/mL *C. t* compared with the control ($P < 0.01$; Fig. 1B). Therefore, IPEC-J2 cells treated with 10^8 CFU/mL *C. t* for 8 h were used in the subsequent experiments.

C. t maintained permeability in IPEC-J2 cells

An intestinal barrier model was used to investigate whether *C. t* could inhibit the *in vitro* LPS-induced increase in intestinal permeability. When the TEER value reached $1524.43 \pm 15.02 \Omega \cdot \text{cm}^2$, the monolayer was considered to be completely differentiated (Fig. 2A). Cells were treated with PBS (the control), LPS, *C. t*, LPS + *C. t*, or *C. t* + LPS (Fig. 2B), and TEER measurements were conducted every 4 hours. The TEER value reduced to the lowest ($\sim 40 \Omega \cdot \text{cm}^2$) in 20 h with LPS treatment, indicating that the intestinal integrity was greatly disturbed. No significant differences were detected between the control and the *C. t* treatment, implying that *C. t* positively maintained the TEER values of IPEC-J2 cells with time. As for the LPS + *C. t* treatment, the TEER value decreased to $217 \Omega \cdot \text{cm}^2$ in the first 12 h when cells were pretreated with LPS. However, the TEER value was maintained at about $223 \Omega \cdot \text{cm}^2$ after the

Table 1. Primers for gene expression using real-time PCR

Gene	Accession No.	Primer sequence (5'-3')
β-actin	DQ845171.1	F:CCAGGTCATCACCATCGGCAAC R:CAGCACCGTGTGGCGTAGAG
Bcl-2	NM_214285.1	F:AGCAGGTATTGAACGAACCTCTCCG R:AGGCTCTAGGTGGTCAATTCAGTAAG
Bax	XM_003127290.5	F:CTACCAAGAAGTTGAGCGAGTGTCTC R:GTGTCCACGGCTGCGATCATC
Caspase-3	NM_214131.1	F:CGAGGCACAGAATTGGACTG R:CCAGGAATAGTAACCGGTGCTG
Caspase-8	NM_001031779.2	F:ACCAGGTCGGAATTGAAGGAC R:GCAAGGTCATGTCATCATCCAG
AP-1	NM_213880.1	F:AGGCGGAGAGGAAGCGTATGAG R:CTGAGCATGTTGGCGGTGGAC
ATF-2	XM_021075926	F:AGAAGACCTGTCAGTGCCGAGTAG R:GGAGGAAGGAGCCATAACAATCTGTG
ELK-1	XM_013990888.2	F:ACAGCGCGCAGCCTCTCAC R:GCCTCTCAGCCTCCAGACAG
P53	NM_213824	F:TCGGCTCTGACTGTACCAACATC R:TTCTCTGTGGCGCGGTCTC
Stat3	NM_001044580	F:GTGGAGAAGGACATCAGCGGTAAAG R:AGGTAGACAGCGGAGACACAAG

Fig. 1. Cell viability and cytotoxicity of *Clostridium tyrobutyricum* (*C. t*) in IPEC-J2 cells. (A) Cell viability of cells treated with different concentrations (10⁶, 10⁷, 10⁸, 10⁹, 10¹⁰, 10¹¹ CFU/mL) of *C. t* for different 0, 2, 4, 6, 8, 10 h. (B) Cell cytotoxicity of cells treated with different concentrations (10⁸, 10⁹, 10¹⁰, 10¹¹ CFU/mL) of *C. t* for different 8 h. Data were expressed as mean ± SEM (n=3). ^{ns}P>0.05, *P<0.05, **P<0.01 and ***P<0.001 compared with the control; ^{ns}P>0.05, #P<0.05, ##P<0.01, ###P<0.001 compared with LPS. Results represented one of the three independent experiments.

cells were treated with *C. t* in the next 8 h. For the cells treated with *C. t* + LPS, the TEER value was the same as the control when the cells were pretreated with *C. t* in the first 8 h, whereas the TEER value reached the minimum after LPS was added in the next 12 h (Fig. 2C).

After the TEER values were measured, FD4 was added into the upper compartments of the Transwells to ensure that *C. t* could suppress the LPS-induced increase in intestinal permeability. The concentration of FD4 was markedly increased by LPS treatment compared with the control and *C. t* treatment groups ($P < 0.01$) and no differences were observed between the control and *C. t*-treated cells. In addition, the concentrations of FD4 were decreased by the LPS + *C. t* and *C. t* + LPS treatments compared with LPS alone ($P < 0.01$; Fig. 2D).

The expressions of tight junction (TJ) proteins (ZO-1, Claudin-1, and Occludin) were also measured in this study. After treatment with LPS or *C. t* for 20 h, the expressions of TJ proteins were significantly decreased in cells treated with LPS ($P < 0.05$), whereas *C. t* increased the expressions of all three proteins ($P < 0.01$). In addition, we noted that *C. t* inhibited the LPS-induced decrease in TJ protein expressions (Fig. 2E–G).

C. t improved the expression of TJ proteins in IPEC-J2 cells

TJ protein expressions were measured to further investigate the protective effect of *C. t* on the intestinal barrier. Compared with the control, cells treated with LPS alone for 12 h showed decreased expressions of TJ proteins ($P < 0.01$; Fig. 3), which was consistent with the TEER, FD4, and TJ protein expression results obtained previously (Fig. 2). No differences could be observed between the control and 8-h *C. t* treatment groups in the expressions of ZO-1 and Claudin-1, but Occludin expression was much higher in the *C. t* group than in the control ($P < 0.01$). Cells treated with LPS + *C. t* showed increased expression of these three proteins compared with the LPS-alone group, whereas the expressions of TJ proteins were not different between the *C. t* + LPS and LPS treatments.

C. t suppressed LPS-induced cell apoptosis in IPEC-J2 cells

The bright-field images of cells pretreated with LPS showed nuclear and cytoplasmic condensation and some cellular fragments, which are typical characteristics of apoptosis. However, there was no characteristic difference between the control and *C. t* treatment groups (Fig. 4A). An annexin V-FITC/PI assay was conducted to further confirm whether *C. t* could attenuate LPS-induced apoptosis. Fluorescence images of FITC-/PI-stained apoptotic

Fig. 2. Effect of *Clostridium tyrobutyricum* (*C.t*) on IPEC-J2 cells permeability. Cells were treated with PBS (the control), LPS (1 $\mu\text{g}/\text{mL}$ for 20 h), *C.t* (10^8 CFU/mL for 20 h), LPS + *C.t* (1 $\mu\text{g}/\text{mL}$ LPS for 12 h followed by 10^8 CFU/mL *C.t* for 8 h), or *C.t* + LPS (10^8 CFU/mL *C.t* for 8 h followed by 1 $\mu\text{g}/\text{mL}$ LPS for 12 h) after IPEC-J2 cells were completely differentiated in the transwells. (A) Changes in the TEER values of IPEC-J2 cells over time for the completely differentiated monolayer; (B) Schematic illustration of the in vitro intestinal barrier model using transwells to evaluate the effects of *C.t* on intestinal permeability; (C) Effect of *C.t* on the LPS-induced TEER decrease in IPEC-J2 cells; (D) Effect of *C.t* on IPEC-J2 cells permeability to FD4; (E–G) Western blot and quantitative analysis of the expressions of ZO-1, Claudin-1, and Occludin. Data were expressed as mean $\pm$ SEM ($n=5$). $^{ns}P>0.05$, $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control; $^{ns}P>0.05$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with LPS.

IPEC-J2 cells showed that LPS considerably stimulated cell apoptosis. No differences were observed between the control and *C.t* treatments. Both LPS + *C.t* and *C.t* + LPS treatments partially inhibited the LPS-induced apoptosis (Fig. 4B).

The results of flow cytometry quantitatively verified the above results. LPS significantly increased ($P < 0.05$) the ratio of apoptotic cells, whereas *C.t* efficiently inhibited apoptosis induced by LPS (Fig. 4C). The flow cytometry results showed that 57.47% cells were alive when the cells were treated LPS alone, which was lower than the percentages in the control and *C.t* groups (94.4% live cells and 96.8% live cells, respectively; $P < 0.001$; Fig. 4D and E).

Fig. 3. Effect of *Clostridium tyrobutyricum* (*C.t*) on the expressions of tight junction proteins in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 μ g/mL for 12 h), *C.t* (10^8 CFU/mL for 8 h), LPS + *C.t* (1 μ g/mL LPS for 12 h followed by 10^8 CFU/mL *C.t* for 8 h), or *C.t* + LPS (10^8 CFU/mL *C.t* for 8 h followed by 1 μ g/mL LPS for 12 h). (A–C) Western blot and quantitative analysis of the expressions of ZO-1, Claudin-1, and Occludin. Data were expressed as mean $\pm$ SEM (n=3). ^{ns}P>0.05, *P<0.05, **P<0.01 and ***P<0.001 compared with the control; ^{ns}P>0.05, #P<0.05, ##P<0.01, ###P<0.001 compared with LPS. Results represented one of the three independent experiments.

Fig. 4. Effect of *Clostridium tyrobutyricum* (*C.t*) on LPS-induced apoptosis in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 μ g/mL for 12 h), *C.t* (10^8 CFU/mL for 8 h), LPS + *C.t* (1 μ g/mL LPS for 12 h followed by 10^8 CFU/mL *C.t* for 8 h), or *C.t* + LPS (10^8 CFU/mL *C.t* for 8 h followed by 1 μ g/mL LPS for 12 h). (A) Representative microscopy images of IPEC-J2 cells under the condition of bright field. The scale bar represents 100 μ m; (B) Fluorescence images of Annexin V-FITC/PI staining apoptotic cells. The scale bar represents 100 μ m; (C) Flow cytometry analysis of Annexin V-FITC/PI staining apoptotic cells; (D) Quantification of apoptotic cells (Q2+Q3); (E) Quantification of death cells (Q1+Q2+Q3). Data were expressed as mean $\pm$ SEM (n=3). ^{ns}P>0.05, *P<0.05, **P<0.01 and ***P<0.001 compared with the control; ^{ns}P>0.05, #P<0.05, ##P<0.01, ###P<0.001 compared with LPS. Results represented one of the three independent experiments.

Fig. 5. Effect of *Clostridium tyrobutyricum* (*C. t*) on mRNA relative expressions of cell apoptosis related genes in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 μ g/mL for 12 h), *C. t* (10^8 CFU/mL for 8 h), LPS + *C. t* (1 μ g/mL LPS for 12 h followed by 10^8 CFU/mL *C. t* for 8 h), or *C. t* + LPS (10^8 CFU/mL *C. t* for 8 h followed by 1 μ g/mL LPS for 12 h). (A) mRNA relative expression of the anti-apoptotic gene Bcl-2; (B) mRNA relative expression of the pro-apoptotic gene Bax; (C–D) mRNA relative expressions of apoptotic genes Caspase-3/8. Data were expressed as mean $\pm$ SEM (n=3). ns>P>0.05, *P<0.05, **P<0.01 and ***P<0.001 compared with the control; ns>P>0.05, #P<0.05, ##P<0.01, ###P<0.001 compared with LPS. Results represented one of the three independent experiments.

Fig. 6. *Clostridium tyrobutyricum* (*C. t*) protects intestinal barrier from LPS-induced apoptosis in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 μ g/mL for 12 h), *C. t* (10^8 CFU/mL for 8 h), Apoptosis inhibitor + LPS (50 μ M apoptosis inhibitor for 1 h followed by 1 μ g/mL for 12 h), or Apoptosis inhibitor (50 μ M apoptosis inhibitor for 1 h). (A) mRNA relative expressions of cell apoptosis related genes; (B–C) Western blot and quantitative analysis of the expressions of ZO-1, Claudin-1, and Occludin. Apoptosis inhibitor Z-VAD(OMe)-FMK was purchased from MedChem Express, Monmouth Junction, NJ, USA. Data were expressed as mean $\pm$ SEM (n=5). ns>P>0.05, *P<0.05, **P<0.01 and ***P<0.001 compared with the control; ns>P>0.05, #P<0.05, ##P<0.01, ###P<0.001 compared with LPS.

C.t affected the relative mRNA expressions of cell apoptosis-related genes in IPEC-J2 cells

We next analyzed the relative mRNA expression of several apoptotic markers, including caspase-3, caspase-8, Bax, and Bcl-2. As for the expression of the anti-apoptotic gene Bcl-2, the results showed that the relative Bcl-2 expression was significantly decreased with LPS compared with that in the control and *C.t* treatment groups ($P < 0.05$). In particular, *C.t* significantly increased the Bcl-2 expression compared with the control ($P < 0.05$), signifying its anti-apoptotic effects. Thus, for the LPS + *C.t* and *C.t* + LPS treatments, Bcl-2 expressions were higher than with LPS, although the differences were not significant (Fig. 5A). The relative expressions of the pro-apoptotic gene Bax and the apoptotic genes caspase-3/-8 were significantly increased in cells pretreated with LPS compared with the control and *C.t* treatment groups ($P < 0.01$). *C.t* reduced the expression of these apoptosis-related genes (Fig. 5B–D).

C.t protected the intestinal barrier from LPS-induced apoptosis

The apoptosis inhibitor Z-VAD(OMe)-FMK, a pan-caspase inhibitor, was used in this study to investigate whether *C.t* could protect intestinal barrier function from LPS-induced

Fig. 7. *Clostridium tyrobutyricum* (*C.t*) inhibits LPS-triggered phosphorylation of p38/JNK signaling pathway in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 μ g/mL for 12 h), *C.t* (10^8 CFU/mL for 8 h), LPS + *C.t* (1 μ g/mL LPS for 12 h followed by 10^8 CFU/mL *C.t* for 8 h), or *C.t* + LPS (10^8 CFU/mL *C.t* for 8 h followed by 1 μ g/mL LPS for 12 h). (A) Western blot analysis of ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK; (B–D) Quantitative analysis of the expression of phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK; (E–I) mRNA relative expressions of downstream genes AP-1, ATF-2, ELK-1, p53 and Stat3. Data were expressed as mean $\pm$ SEM (n=3). $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control; $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.001$ compared with LPS. Results represented one of the three independent experiments.

Fig. 8. *Clostridium tyrobutyricum* (*C.t*) protect intestinal barrier from LPS-induced apoptosis via p38/JNK signaling pathway in IPEC-J2 cells. Cells were cultured in 6-wells plates for 24 h and then treated with PBS (the control), LPS (1 $\mu\text{g/mL}$ for 12 h), *C.t* (10^8 CFU/mL for 8 h), JNK inhibitor + LPS (20 μM apoptosis inhibitor for 1 h followed by 1 $\mu\text{g/mL}$ LPS for 12 h), JNK inhibitor (20 μM apoptosis inhibitor for 1 h), p38 inhibitor + LPS (25 μM apoptosis inhibitor for 1 h followed by 1 $\mu\text{g/mL}$ LPS for 12 h), or p38 inhibitor (25 μM apoptosis inhibitor for 1 h). (A) Western blot bands of JNK, phospho-JNK, ZO-1, Claudin-1, and Occludin; (B, E) mRNA relative expressions of apoptosis related genes Bax, Caspase-3, Caspase-8, and Bcl-2; (C, F) Western blot analysis of ZO-1, Claudin-1, and Occludin. JNK inhibitor SP600125 and p38 inhibitor SB203580 were purchased from MedChem Express, Monmouth Junction, NJ, USA. Data were expressed as mean $\pm$ SEM ($n=5$). $^{ns}P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control; $^{ns}P>0.05$, $^{#}P<0.05$, $^{##}P<0.01$, $^{###}P<0.001$ compared with LPS.

apoptosis. The relative expressions of the apoptotic genes caspase-3/-8 were significantly decreased in LPS-treated cells after an apoptosis inhibitor was added ($P < 0.05$). However, no significant differences were observed in the relative expressions of Bax and Bcl-2 between the LPS and apoptosis inhibitor + LPS treatments. These results indicated that an apoptosis inhibitor could partly prevent LPS-induced apoptosis (Fig. 6A). The expressions of TJ proteins were increased dramatically after the addition of the apoptosis inhibitor to LPS-treated cells ($P < 0.05$), which were similar to the results of *C.t*-treated cells (Fig. 6B and C).

C.t suppressed LPS-induced apoptosis by inhibiting the p38 and JNK signaling pathways

To elucidate the mechanisms underlying the effects of *C.t* on LPS-induced apoptosis, we examined the effects of *C.t* on the MAPK signaling pathway. Phosphorylation of p38 (p-p38) and JNK (p-JNK) was observed after LPS treatment of cells. However, *C.t* inhibited the LPS-induced phosphorylation of p-p38 and p-JNK (Fig. 7A–D). We then measured the relative mRNA expressions of downstream genes in this pathway. The mRNA levels of AP-1, ATF-2, ELK-1, and p53 were increased ($P < 0.05$) and that of Stat3 was decreased ($P < 0.001$) in cells treated with LPS compared with the control, whereas *C.t* inhibited these changes (Fig. 7E–I).

Pharmacological inhibitors of p38 (SB203580) and JNK (SP600125) were used to investigate the underlying mechanisms of the protective effects of *C.t* on LPS-induced apoptosis in IPEC-J2 cells. The concentrations of p-p38 and p-JNK were suppressed when cells were treated with inhibitors or inhibitors + LPS (Fig. 8A and D). The mRNA levels of the anti-apoptotic gene Bcl-2 were increased in cells treated with inhibitors or with inhibitors + LPS compared with cells treated with LPS alone ($P < 0.05$). In addition, relative mRNA expressions of apoptosis-related genes, including Bax, caspase-3, and caspase-8, were significantly decreased when inhibitors were added to the cells ($P < 0.01$; Fig. 8B and

Fig. 9. Proposed model of *Clostridium tyrobutyricum* (*C.t*) protect intestinal barrier against LPS-induced apoptosis via inhibiting the phosphorylation of p38/JNK in IPEC-J2 cells. p38/JNK are activated in the presence of specific stress stimuli, LPS. The phosphorylated p38/JNK activate their downstream genes such as AP-1, ATF-2, ELK-1, p53 and Stat3, which induce apoptosis, eventually, result in intestinal permeability increasing and intestinal barrier damage (left panel and upper of the medium panel). The phosphorylation of p38/JNK and the expression of downstream genes are inhibited by *C.t*, which suppresses apoptosis and protects the normal intestinal barrier function (right panel and lower of the medium panel).

E). TJ proteins levels were also determined to verify whether *C.t* could protect intestinal barrier function from LPS-induced apoptosis via the p38/JNK signaling pathway. The results showed that expressions of TJ proteins were increased when p38/JNK signaling pathways were switched off ($P < 0.05$; Fig. 8C and F).

Discussion

C.t, a Gram-positive and strictly anaerobic bacterium, has received much attention from the dairy and chemical synthesis industries because of its efficiency in butyric acid production [19, 20]. However, the role of *C.t* in animal models or mammalian cells has not yet been reported. In this work, the biological function of *C.t* was investigated *in vitro*, with the results showing that *C.t* could protect the intestinal barrier from LPS-induced apoptosis in IPEC-J2 cells.

The intestinal epithelium, which represents the largest interface in the body, plays several important roles in the absorption of nutrients and prevention of water and electrolyte loss [21, 22]. In particular, the intestinal barrier can separate the internal milieu from the external environment via cell-cell adhesion and intercellular junctions that direct morphogenesis and maintain tissue integrity [2]. Intestinal permeability is controlled by epithelial adherens junctions and TJs, with the increased intestinal permeability contributing to the severity of some diseases, such as inflammatory bowel disease and irritable bowel syndrome [21]. In the present study, an *in vitro*-simulated intestinal barrier was established by culturing IPEC-J2 cells in Transwells. LPS has a negative effect on the TEER values and TJ protein contents of IPEC-J2 cells, ultimately resulting in increased permeability and intestinal barrier dysfunction [23, 24]. Many reports have also revealed that the TEER values and TJ protein expressions markedly decreased after cells were treated with LPS for 12 or 24 h [7, 25]. In our study, we successfully constructed a model of LPS-induced increased permeability. The TEER values decreased to their lowest level and the expression of these TJ proteins was completely reduced after the IPEC-J2 cells were treated with LPS for 20 h. In contrast, *C.t* stably maintained the TEER values and increased the expression of the TJ protein at 20 h. The decrease in the TEER values and TJ protein expression were also inhibited by *C.t*. Our further research on the expression of TJ proteins in cells treated with LPS for only 12 h or with *C.t* for 8 h also verified the protective function of *C.t*. These results indicate that *C.t* protected the intestinal barrier from the LPS-induced increase in intestinal permeability.

Apoptosis, which is a type of programmed cell death involving the activation of apoptotic caspases, can be initiated by a variety of stimuli arising from receptors on the cell surface or from the detection of events within the cell [26]. Tumor necrosis factor (TNF) or LPS stimulation increase apoptosis and consequent cell shedding which are associated with barrier loss [27]. We noticed in the present study that, after treatment with LPS, cells showed nuclear and cytoplasmic condensation, typical characteristics of apoptosis. LPS, which is a major component in the outer monolayer of most Gram-negative bacteria, can induce apoptosis and disturb intestinal integrity in IPEC-J2 cells [28]. Thus, in the present study, we hypothesized that *C.t* could inhibit LPS-induced apoptosis and ultimately protect the intestinal barrier. We found that LPS induced apoptosis at 12 h and that *C.t* efficiently inhibited this phenomenon. The relative mRNA expression levels of apoptosis-related genes, including Bcl-2, Bax, and caspase-3/-8, were also measured in this study; their levels were consistent with the results of the Annexin V-FITC/PI assay and flow cytometry. Moreover, intestinal barrier function was improved when LPS-induced apoptosis was prevented by a pharmacological apoptotic inhibitor. Our results qualitatively and quantitatively indicated that *C.t* may protect intestinal barrier function by markedly suppressing LPS-induced apoptosis in IPEC-J2 cells.

MAPKs play essential roles in some physiological cell processes, including inflammation, stress, growth, differentiation, and death. ERK1/2 are mostly activated by growth factors, such as epidermal growth factor and nerve growth factor, which play central roles in the control of cell proliferation [29, 30]. While p38 and JNK, known as stress-activated protein kinase, are strongly activated by various cellular stresses, such as heat shock and oxidative stress, TNF- α and LPS, which play roles in cell proliferation and survival, are associated with the induction of apoptosis by cellular stresses [30]. In the present study, p38 and JNK phosphorylation occurred after cells were treated with LPS for 12 h. The major functional role of phosphorylated p38 and JNK lies in mediating the downstream substrates involved in the cell cycle and apoptosis [29]. Our results also suggest that the relative mRNA expressions of downstream genes of the p38 and JNK signaling pathways, including AP-1, ATF-2, ELK-1, and p53, were increased and that Stat3 expression was dramatically decreased after cells were treated with LPS, which may activate caspases and induce apoptosis [31, 32]. Pharmacological inhibitors were used to switch off the activation of p38/JNK signaling pathways; we found that these inhibitors could restrain LPS-induced apoptosis and thus improve intestinal barrier function. Therefore, we speculated that *C.t* inhibited LPS-induced apoptosis by depressing the p38/JNK signaling pathway (Fig. 9). However, the molecular basis of how *C.t* suppresses the activity of the p38/JNK signaling pathway remains to be investigated.

Conclusion

Our findings suggest that *C.t* could protect against the LPS-induced increase in intestinal permeability and inhibit LPS-induced intestinal apoptosis via the p38/JNK signaling pathway in IPEC-J2 cells. These results suggest *C.t* is a potential probiotic additive for protecting the intestinal barrier and preventing diarrhea in early weaned piglets.

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Disclosure Statement

No conflict of interest exists.

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