In vitro protective effects of *Lactobacillus plantarum* Lac16 on *Clostridium perfringens* infection-associated intestinal injury in IPEC-J2 cells

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Research

Keywords: *Lactobacillus plantarum*, *Clostridium perfringens*, intestinal injury, intestinal barrier integrity, inflammation

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-463175/v1>

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Abstract

Background: *Clostridium perfringens* causes intestinal injury through overgrowth and secretion of multiple toxins, leading to diarrhea and necrotic enteritis in animals, such as pigs. *Lactobacillus plantarum* (L. *plantarum*) Lac16 has been reported to protect broilers against *C. perfringens* infection. This study aimed at investigating the protective effects of *Lactobacillus plantarum* Lac16 on *C. perfringens* infection-associated intestinal injury in intestinal porcine epithelial cell line (IPEC-J2).

Results: The results showed that *L. plantarum* Lac16 significantly inhibit the growth and biofilm formation of *C. perfringens* ($P < 0.001$). In the co-culture system, *L. plantarum* Lac16 significantly suppressed colony forming units (CFU) of *C. perfringens* ($P < 0.05$), which was accompanied by a decrease in pH levels ($P < 0.01$). Moreover, *L. plantarum* Lac16 significantly elevated the mRNA expression levels of host defense peptides (HDPs) in IPEC-J2 cells ($P < 0.05$), decreased *C. perfringens*-induced cellular cytotoxicity ($P < 0.01$) and adhesion to cells ($P < 0.05$). At the same time, *L. plantarum* Lac16 significantly attenuated *C. perfringens*-induced damage to intestinal barrier integrity and the decrease in claudin-1 ($P < 0.01$) as well as zona occludens 1 (ZO-1) expressions. Preincubation with *L. plantarum* Lac16 significantly suppressed mRNA expression levels of pattern recognition receptors (PRRs) (*Toll-like receptor (TLR) 1*, *TLR2*, *nucleotide-binding oligomerization domain (NOD) 1*) in *C. perfringens*-challenged IPEC-J2 cells ($P < 0.01$). *C. perfringens* significantly elevated the phosphorylation of p38 mitogen-activated protein kinase (MAPK), JNK, and p65 nuclear factor- κ B (NF- κ B) ($P < 0.05$) while *L. plantarum* Lac16 pre-incubation effectively inhibited phosphorylation of p65 ($P < 0.001$). Furthermore, *L. plantarum* Lac16 significantly suppressed *C. perfringens* induced gene expressions of proinflammatory cytokines (*interleukin (IL)-1 β* , *IL-6*, *IL-8*, *tumor necrosis factor- α* (*TNF- α*)) ($P < 0.05$).

Conclusions: Collectively, probiotic *L. plantarum* Lac16 exerts protective effects against *C. perfringens* infection-associated intestinal injury in IPEC-J2 cells.

Background

C. perfringens is a Gram-positive, spore-forming, anaerobic, rod-shaped bacterium [1, 2]. *C. perfringens* can be isolated from the natural environment (e.g., soil), and from human as well as animal intestines as a component of the normal microbial community[3]. However, under certain conditions, such as *C. perfringens* overgrowth or disruption of the intestinal microbiota, *C. perfringens* causes various intestinal diseases [4, 5]. *C. perfringens* strains have been reported to secrete more than twenty toxins or enzymes, which are principal virulence factors [4]. Based on the secretion of four major toxins (α , β , ϵ , and ι), *C. perfringens* can be grouped into five different toxin types (types A to E) [6]. Among them, *C. perfringens* type A is very common in the intestines of warm-blooded animals, and under the above mentioned conditions, it causes intestinal diseases in domestic animals, such as pigs, chicken, and sheep [3]. Symptoms of its infection in piglets include severe diarrhoea, accompanied by necrotic mucosa and intestinal villi atrophy [7, 8]. Currently, *C. perfringens* infections has become an important problem hindering the development of the pig industry [9, 10].

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [11]. In animal husbandry, beneficial effects of probiotics are mainly reflected in nutrition and immunity [12]. They promote animal health by regulating immune functions and maintaining dynamic

balance of intestinal microbiota [13, 14]. *Lactobacillus* spp. have been shown to exert protective effects against *C. perfringens* infection *in vivo* and *in vitro*. Guo et al. [15] reported that *L. acidophilus* and *L. fermentum* inhibited the growth and α -toxin production capacity of *C. perfringens* *in vitro*. At the same time, another study showed that *L. fermentum* also inhibited β 2-toxin secretion [16]. Furthermore, members of *Lactobacillus* spp., such as *L. fermentum*, *L. salivarius*, *L. plantarum*, and *L. acidophilus* inhibit *C. perfringens* infection associated necrotic enteritis by improving intestinal morphology and barrier integrity, reducing lesions, ameliorating inflammation, as well as by modulating intestinal microbiota [17-20].

In our previous study, we found that *L. plantarum* Lac16 effectively protected broilers from *C. perfringens* infection [21]. However, it has not been established whether *L. plantarum* Lac16 can protect pig intestines in cases of *C. perfringens* infection. The porcine jejunal epithelial cell line, IPEC-J2, has exhibited a high specificity in pig studies and is a suitable model for investigating the interactions between bacteria and intestinal epitheliums *in vitro* [22, 23]. Thus, this study aimed at investigating the protective effects of *L. plantarum* Lac16 on *C. perfringens* infection-associated intestinal injury in IPEC-J2 cells *in vitro*.

Methods

Bacterial strains and culture conditions.

L. plantarum Lac16 (CCTCC, No. M2016259) was isolated in our laboratory and preserved at the China Center for Type Culture Collection. *L. plantarum* Lac16 was cultured in Mann-Rogosa-Sharpe (MRS) medium and incubated at 37 °C for 18 h. *C. perfringens* type A (ATCC 13124) was cultured in Reinforced clostridium medium (RCM; Hopebio, Qingdao, China) and incubated at 37 °C in anaerobic conditions for 18 h.

To determine bacterial concentrations, we centrifuged the overnight-incubated bacterial cultures at 5000 rpm for 5 min. After being washed three times using sterile phosphate-buffered saline (PBS, pH = 7.2), bacteria were resuspended in PBS and their concentrations determined using a standard curve. Then, they were diluted to a certain concentration and stored at 4 °C.

Agar-diffusion method for detecting bacteriostasis of *L. plantarum* Lac 16 in a fermentation supernatant.

Agar-diffusion was performed as previously described by Wang et al. [24] with some modifications. Briefly, *L. plantarum* broth was centrifuged at 5000 rpm for 10 min to obtain the supernatant. The supernatant was filtered through a 0.22 μ m membrane to remove suspended bacteria and stored at 4 °C. About 0.2 % (v/v) of the overnight culture of *C. perfringens* was added to tryptose sulfite cycloserine (TSC; Hopebio, Qingdao, China) agar which cooled down to about 50 °C. Then, the medium was well mixed and poured into plastic plates which placed oxford cups in advance. The oxford cups were removed after the medium had solidified. Then, 100 μ L of *L. plantarum* Lac16 fermentation supernatants were injected into each well after which plates were placed in anaerobic gas generating packs (Hopebio, Qingdao, China) for 12 h. This bacteriostatic experiment was performed in triplicates.

Biofilm assays.

Biofilm assays were performed as previously described by Jiang et al. [25] with some modifications. *L. plantarum* and *C. perfringens* were cultured in modified RCM medium (glucose content was increased to 20 g/L

on the original basis) and incubated at 37 °C for 18 h, respectively. Then, the concentration of the *C. perfringens* culture was adjusted to 10⁷ CFU/mL using the modified RCM medium, after which the supernatant of *L. plantarum* was collected as described above.

Experimental groups were treated as: i. Sterile modified RCM medium (200 µL) were inoculated into 96-well culture plates and designed as the control group; ii. Resuspended *C. perfringens* (100 µL) or 100 µL of *L. plantarum* supernatant were added to 100 µL of sterile modified RCM medium, and designed as the CP or Lac16 fermentation supernatant group; iii. *L. plantarum* fermentation supernatant (100 µL) and 100 µL of resuspended *C. perfringens* were inoculated and designed as the experimental treatment group, which was labeled Lac16 fermentation supernatant + Cp group. The 96-well culture plate was incubated in an anaerobic environment at 37 °C for 12 h after which the bacterial proliferation index was read at OD₆₀₀ using SpectraMax M5 (Molecular Devices, USA). Then, bacterial cultures were removed with caution, wells were gently washed thrice using PBS and incubated with 100 µL of 1% crystal violet for 30 min. Crystal violet in the wells was removed and wells were gently washed thrice using PBS. Then, 100 µL of 95% alcohol was added into the wells to dissolve excess crystal violet and OD₅₉₀ in each well measured. The higher the optical density, the more biofilm formation. Experiments were done in triplicates.

Co-culture experiment and pH determination of cultures.

Bacterial co-culture experiments were done as previously described by Guo et al. [15] with some modifications. *L. plantarum* and *C. perfringens* were adjusted to 10⁷ CFU/mL using the modified RCM medium. Ten milliliter of the modified RCM medium were used as the blank control. At the same time, *L. plantarum* or *C. perfringens* suspensions (100 µL) were inoculated in 9.9 mL of modified RCM medium as single bacterial strain groups, respectively. Regarding the co-culture system, 100 µL of *L. plantarum* and *C. perfringens* were both inoculated in 9.8 mL of modified RCM medium. The above cultures were incubated at 37 °C for 12 h, their pH values were determined, after which they were serially diluted, cultured on TSC agar and incubated at 37 °C for 12 h to quantitate *C. perfringens* populations. These experiments were done in triplicates.

Cell cultures.

IPEC-J2 cells were cultured in Dulbecco's modified Eagle's F12 ham medium (DMEM/F12; Gibco, MA, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS; Gibco, MA, USA), 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, MO, USA). Incubation was done at 37 °C in an atmosphere of 90 % humidity and 5 % CO₂. When cell confluence reached 80 %, cells were digested using 0.25 % trypsin-EDTA solution (Gibco, MA, USA) and seeded in cell culture plates.

Determination of expression levels of HDPs by Real-Time PCR.

IPEC-J2 cells were seeded in 12-well cell culture plates (Corning Life Science, MA, USA) at a density of 5 × 10⁵ cells/well. *L. plantarum* cultures were centrifuged and resuspended in DMEM/F12 supplemented with 10% FBS and stored at 4 °C. When IPEC-J2 cells reached 80% confluence, they were co-incubated in cell culture media containing different concentrations of *L. plantarum* Lac16 (10⁶, 10⁷, and 10⁸ CFU/mL) for 6 h. After being washed three times using PBS, IPEC-J2 cells were lysed by RNAiso Plus (Takara, Dalian, China) to extract RNA.

Expression levels of porcine β -defensin (*pBD1*, *pBD2*, *pBD3*) and porcine epididymis protein 2 splicing variant C (*pEP2C*) were then determined by real-time PCR. All experiments were performed in triplicates.

Cytotoxicity Assay.

IPEC-J2 cells were seeded in 12-well culture plates at a density of 5×10^5 cells/well. When cells reached 80% confluence, they were incubated with or without *L. plantarum* (10^7 CFU/mL) for 6 h, respectively. After being washed three times using PBS, cells were infected with *C. perfringens* (10^6 CFU/well) under anaerobic conditions for 1 h or 3 h, respectively. Then, cell suspensions were collected and centrifuged at 10 000 rpm/min for 5 min to remove cell debris and bacteria. The release of lactate dehydrogenase (LDH) from damaged cells was measured using the LDH kit (Nanjing Jiancheng Biological Product, Nanjing, China), according to the manufacturer's instructions. Experiments were performed in triplicates.

***C. Perfringens* adhesion assay.**

Bacterial adhesion assay was performed as previously described by Jiang et al. [25] with some modifications. Briefly, IPEC-J2 cells were seeded in 12-well cell culture plates at a density of 5×10^5 cells/well. At 80% confluence, cells were pre-incubated with *L. plantarum* (10^7 CFU/mL) for 6 h, after which *C. perfringens* were added into the wells (10^6 CFU/mL) and incubated for 1 h under anaerobic conditions. Cells treated with *C. perfringens* only were used as the controls. Then, cells were washed three times using sterile PBS to remove non-adherent *C. perfringens*. Two hundred microliters of 0.25 % trypsin-EDTA solution was added to the wells and digested for 15 min, then, 800 μ L sterile PBS was added to each well and completely mixed. Liquids containing bacteria were serially diluted and incubated in TSC agar for 12 h to quantitate *C. perfringens* populations. Each assay was performed in triplicate.

At the same time, we used fluorescein isothiocyanate (FITC; Solarbio, Beijing, China) labeling method to observe the adhesion effect of *C. perfringens*. Briefly, the concentration of *C. perfringens* culture, after centrifuged, was adjusted to 10^7 CFU/mL with diluted FITC-solution (200 μ g/mL). Avoid light and incubate for 2 h at 37 °C. Then the bacteria were washed with sterile PBS for three times and stored at 4 °C. After incubation with *L. plantarum* Lac16 for 6 h, the cells were washed three times with sterile PBS and co-incubated with *C. perfringens* (10^6 CFU/mL), which was labeled with FITC, for 1 h under anaerobic conditions and away from light. Then the cells were washed three times with sterile PBS and examined under a fluorescence microscope (Nikon, Japan). All experiments were performed in triplicate.

Cell permeability to fluorescein sodium.

Cell permeability to fluorescein sodium was assessed as previously described by Nie et al. [26] with some modifications. Briefly, IPEC-J2 cells were seeded in 12-well transwell inserts (Corning Life Science, MA, USA), with pore sizes of 0.4 mm and membrane areas of 1.12 cm², at a concentration of 1×10^5 cells/mL. Since IPEC-J2 cells can develop tight junctions and differentiate into tight monolayers after 9 days of culture on transwell filters [22], we renewed the culture medium in both apical and basolateral sides of the filters every 24 h for 9 days. On the 10th day, the culture medium on the apical side of the filters was removed and IPEC-J2 cells were treated as described in adhesion assay section. Then, 100 μ g/mL fluorescein sodium (Sigma-Aldrich, MO, USA), dissolved in PBS, was added to the apical inserts for 1 h, after which 200 μ L of medium from each

basolateral side was collected. Fluorescence intensity was determined using a SpectraMax M5 (Molecular Devices, USA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Then, we calculated apical to basolateral flux of fluorescein sodium using the standard curve. Apparent permeability coefficient (P_{app}) was calculated using the formula: $P_{app} = \Delta Q / \Delta t \times (1 / AC_0)$ [26]. Whereby, $\Delta Q / \Delta t$ is the permeability rate ($\mu\text{g/s}$), A is the diffusion area of the monolayer (cm^2), while C_0 is the initial concentration ($\mu\text{g/mL}$) of fluorescein sodium in the transwell apical inserts. All experiments were performed in triplicates.

Immunofluorescence analysis.

IPEC-J2 cells (5×10^5 cell/mL) were seeded on glass coverslips in a 12-well flat-bottom culture plate for at least 9 days to form tight junctions. On the 10th day, the monolayer reaching polarization was treated with bacteria as described in adhesion assay section. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature and blocked with 2.5% bovine serum albumin (BSA; Solarbio, Beijing, China) for 1 h at room temperature. Cells were incubated with rabbit polyclonal anti-ZO-1 primary antibody (Invitrogen, MA, USA) for 12 h at 4 °C, after which they were incubated with secondary antibody Alexa fluor 488 goat anti-rabbit (Abcam, Cambridge, UK) for 1 h at room temperature and away from light. Nuclei were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Beyotime, Shanghai, China). Fluorescence images were obtained through laser scanning confocal microscopy (LSM 880 with AiryScan) (Zeiss, Germany). All experiments were performed in triplicates.

Periodic acid-Schiff (PAS) staining.

The IPEC-J2 cells (5×10^5 cell/mL) were grown in 24-well plates (Corning Life Science, MA, USA) for 9 days and pretreated with bacteria as described in adhesion assay section. Then, cells were washed using PBS and fixed in 70% ethanol for 10 min at room temperature. Periodic acid-Schiff staining was performed according to the manufacturer's instructions (Beyotime, Shanghai, China). Images were obtained using a light microscope (Leica, Germany). Experiments were performed in triplicate.

Quantitative Real-Time PCR.

IPEC-J2 cells were pretreated with bacteria as described in adhesion assay section. Then, cells were lysed using RNAiso Plus (Takara, Dalian, China) to extract RNA. Total RNA was reverse transcribed to cDNA using the HiScript II Q Select RT SuperMix (Vazyme, Nanjing, China). The qRT-PCR analysis was performed using StepOne Plus Real-Time PCR system (Applied Biosystems, USA) and the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Primer sequences used in this study are shown in Table 1. All samples were run in triplicates. β -actin was selected as an endogenous control and relative gene expressions were analyzed using the $2^{-\Delta\Delta C_t}$ method [27]. Each assay was performed in triplicate.

Table 1. Primers used for quantitative real-time PCR

Gene Name	Forward sequence (5'→3')	Reverse sequence (5'→3')	Accession Number
β -actin	CCAGGTCATCACCATCGGCAAC	CAGCACCGTGTGGCGTAGAG	DQ845171.1
pBD1	TTCCTCCTCATGGTCCTGTT	AGGTGCCGATCTGTTTCATC	NM_213838.1
pBD2	TGTCTGCCTCCTCTCTTCC	AACAGGTCCCTTCAATCCTG	AY506573.1
pBD3	CCTTCTCTTTGCCTTGCTCTT	GCCACTCACAGAACAGCTACC	XM_021074698.1
pEP2C	ACTGCTTGTTCTCCAGAGCC	TGGCACAGATGACAAAGCCT	BK005522.1
IL-1 β	AGAGGGACATGGAGAAGCGA	GCCCTCTGGGTATGGCTTT	NM_001302388.2
IL-6	ATCAGGAGACCTGCTTGATG	TGGTGGCTTTGTCTGGATTC	NM_001252429.1
IL-8	TCCTGCTTTCTGCAGCTCTC	GGGTGGAAAGGTGTGGAATG	NM_213867.1
TNF- α	CTGTAGGTTGCTCCACCTG	CCAGTAGGGCGGTACAGAC	NM_214022.1
IL-10	GGTTGCCAAGCCTTGTCAG	AGGCACTCTTCACCTCCTC	NM_214041.1
TGF- β	GAAGCGCATCGAGGCCATTC	GGCTCCGGTTCGACACTTTC	XM_021093503.1
TLR-1	GTCAGTCAGCACCGCAGTAA	CAGACAAACTGGAGGGTGGT	NM_001031775
TLR-2	TCACTTGTCTAACTTATCATCCTCT	TCAGCGAAGGTGTCATTATTGC	NM_213761.1
TLR-4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCG	NM_001113039.2
NOD-1	CTGTCGTCAACACCGATCCA	CCAGTTGGTGACGCAGCTT	AB187219.1
NOD-2	CCTTTTGAAGATGCTGCCTG	GATTCTCTGCCCCATCGTAG	NM_001105295.1

Western blot analysis.

After pretreatment with bacteria as described in adhesion assay section, IPEC-J2 cells were lysed using the RIPA Lysis Buffer (Beyotime, Shanghai, China) involving protease inhibitors. Protein concentrations were measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Briefly, protein samples were separated by SDS-PAGE and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Membranes with migrated proteins were blocked with 5 % dried skimmed milk for 2 h at room temperature and incubated overnight at 4 °C with the following primary antibodies: claudin-1 (Abcam, Cambridge, UK), occluding (Abcam, Cambridge, UK), ERK1/2 (Cell Signaling Technology, MA, USA), phospho-ERK1/2 (Cell Signaling Technology, MA, USA), p38 (Cell Signaling Technology, MA, USA), phospho-p38 (Cell Signaling Technology, MA, USA), JNK (Cell Signaling Technology, MA, USA), phospho-JNK (Cell Signaling Technology, MA, USA), p65 (Cell Signaling Technology, MA, USA), phospho-p65 (Cell Signaling Technology, MA, USA), and β -actin (Abcam, Cambridge, UK). After being washed three times for five minutes using Tris-Buffered-Saline with Tween (TBST), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Then, protein bands were detected on an image system (Tanon, China)

using the chemiluminescent HRP substrate kit (Millipore, MA, USA). Intensities of protein bands were determined using the ImageJ software. All experiments were performed in triplicate.

Statistical Analysis.

Experimental data were analyzed by IBM SPSS Statistics 20 and presented as mean $\pm$ SD. Statistical significance between two groups was determined by two-tailed Student's *t* test, while multiple comparisons were performed by one-way ANOVA. $P \leq 0.05$ was considered significant. Graphs were drawn using the OriginPro 2018 software.

Results

***L. plantarum* Lac16 and its fermentation supernatant significantly inhibited the growth of *C. perfringens*.**

As shown in Fig. 1A, the positive group (100 μ g/mL of ampicillin) exhibited the best bacteriostatic effect on *C. perfringens*, with *L. plantarum* Lac16 fermentation supernatant also exhibiting good bacteriostatic effects, forming a clear boundary of bacteriostatic zone. In the biofilm formation experiment (Fig. 1B), after 12 h of culture, optical density (OD₆₀₀) of the CP group increased to 0.837 ± 0.031 , while the OD₆₀₀ of the control group was 0.090 ± 0.002 , indicating that *C. perfringens* proliferated rapidly. However, in the group in which *C. perfringens* were co-incubated with the *L. plantarum* Lac16 fermentation supernatant, the OD₆₀₀ only increased to 0.189 ± 0.008 . These results indicate that *L. plantarum* Lac16 fermentation supernatant could significantly inhibit the growth of *C. perfringens* ($P < 0.001$). Similar results were obtained from the biofilm assays (Fig. 1C). The *L. plantarum* Lac16 fermentation supernatant significantly inhibited the formation of *C. perfringens* biofilms when compared to the CP group ($P < 0.001$).

After that, we co-cultured *L. plantarum* Lac16 with *C. perfringens* and determined the corresponding pH. It was found that CFUs of *C. perfringens* in the co-culture group were significantly decreased when compared to the CP group ($P < 0.05$) (Fig. 1D). Although *L. plantarum* Lac16 and *C. perfringens* could reduce the pH of the medium, the pH of Lac16 and co-culture groups were lower relative to those of the CP group ($P < 0.01$) (Fig. 1F).

***L. plantarum* Lac16 enhanced HDP mRNA expression in IPEC-J2 cells.**

Expression levels of HDP genes, including *pBD1*, *pBD2*, *pBD3* and *pEP2C*, were all found to be elevated after co-incubation with *L. plantarum* Lac16 (Fig. 2). Specifically, expression levels of *pBD1* were significantly elevated after co-incubation with *L. plantarum* Lac16 at concentrations of 10^7 CFU/mL and 10^8 CFU/mL ($P < 0.001$) (Fig. 2A). The best effect was obtained at the concentration of 10^7 CFU/mL. At the same time, the trend in *pBD2* gene expression level was similar (Fig. 2B), except that the group with a concentration of 10^7 CFU/mL was the only one that exhibited a significant increase in gene expression ($P < 0.05$). Gene expression levels of *pBD3* and *pEP2C* were significantly elevated in a concentration dependent manner after co-incubation with different concentrations of *L. plantarum* Lac16 ($P < 0.05$; Fig. 2C and D). To some extent, we found that *L. plantarum* Lac16 at elevated concentrations (10^8 CFU/mL) competed with cells for nutrients, therefore, we selected the medium concentration (10^7 CFU/mL) for the follow-up experiments.

***L. plantarum* Lac16 alleviated *C. perfringens* infection-associated LDH leakage.**

Pretreatment with *L. plantarum* Lac16 did not increase LDH release in both groups ($P > 0.05$; Fig. 3A). However, when IPEC-J2 cells were infected with *C. perfringens*, LDH release was significantly elevated ($P < 0.01$), which was alleviated by pre-incubation with *L. plantarum* Lac16 ($P < 0.01$). However, the increased LDH release, which was associated with *C. perfringens* infection for 3 h, was not alleviated to normal levels after *L. plantarum* Lac16 pre-incubation ($P < 0.001$), indicating cytotoxic induction due to the infection. Therefore, for subsequent experiments, we selected IPEC-J2 cells infected with *C. perfringens* for 1 h as the time point for sampling.

***L. plantarum* Lac16 suppressed the adhesion of *C. perfringens* to IPEC-J2 cells.**

For the bacterial adhesion assay, we defined adhesion rate as 100 % in the CP group, in which cells were only co-incubated with *C. perfringens* (Fig. 3B). However, when cells were pre-incubated with *L. plantarum* Lac16, the adhesion rate of *C. perfringens* to IPEC-J2 cells decreased significantly ($P < 0.05$). Similar results were obtained in the images of fluorescence labeling method (Fig. 3C). When IPEC-J2 cells were only infected with *C. perfringens*, the quantity of fluorescent labeled pathogens that adhered to cells was significantly larger than that of the group pretreated with *L. plantarum* Lac16.

***L. plantarum* Lac16 attenuated *C. perfringens*-induced damage to intestinal barrier function.**

When IPEC-J2 cells developed tight junctions and were completely differentiated into tight monolayers, they were infected with *C. perfringens*. At the same time, the flux of fluorescein sodium significantly increased ($P < 0.001$; Fig. 4A). However, *L. plantarum* Lac16 pretreatment significantly alleviated *C. perfringens*-induced increase in Papp of the IPEC-J2 monolayers ($P < 0.001$). Then, we evaluated the effects of bacterial pretreatment on the production of mucins (Fig. 4B). After PAS staining, it was found that *C. perfringens* infection inhibited mucin production. Moreover, *L. plantarum* Lac16 pretreatment effectively inhibited *C. perfringens*-mediated decrease in mucin production.

To evaluate *C. perfringens*-induced damage to intestinal epithelial barrier functions and the corresponding protective effects of *L. plantarum* Lac16, we determined the expression levels of tight junction proteins in IPEC-J2 cells (Fig. 4C). Compared to the control group, there were no significant differences in expression levels of occludin in each group after incubation with bacteria ($P > 0.05$). However, *C. perfringens* significantly suppressed the expression levels of claudin-1 in IPEC-J2 cells ($P < 0.01$). *L. plantarum* Lac16 treatment significantly elevated the expressions of claudin-1 ($P < 0.05$) and alleviated the decrease in protein expressions caused by *C. perfringens* infection ($P < 0.01$). Regarding immunofluorescence images of ZO-1, we found that *L. plantarum* Lac16 pretreatment had no effect on protein expression levels, whereas *C. perfringens* infection significantly suppressed the expression of ZO-1 (Fig. 4D). At the same time, compared to the CP group, IPEC-J2 cells that had been co-incubated with *L. plantarum* Lac16 and *C. perfringens* exhibited increased expressions of ZO-1.

***L. plantarum* Lac16 alleviated the increase in mRNA expression levels of PRRs after *C. perfringens* infection.**

mRNA expression levels of several PRRs in IPEC-J2 cells after co-incubation with *L. plantarum* Lac16 and *C. perfringens* were investigated (Fig. 5). Specifically, when cells had been co-incubated with bacteria, mRNA expression levels of TLRs were significantly up-regulated ($P < 0.05$) (Fig. 5A-C). The most significant increase in mRNA expression levels of TLRs was in the group infected with *C. perfringens* only. However, the increased mRNA expression (*TLR1* and *TLR2*) caused by *C. perfringens* infection was effectively inhibited ($P < 0.01$) by

co-incubation with *L. plantarum* Lac16. The trend in mRNA expression levels of NODs was similar to that of TLRs (Fig. 5D and E). Although both bacteria increased *NOD1* gene expression ($P < 0.001$), preincubation with *L. plantarum* Lac16 significantly reduced the increased gene expression associated with *C. perfringens* infection ($P < 0.01$) (Fig. 5D). However, this effect was not significant in *NOD2* gene expression ($P > 0.05$) (Fig. 5E).

***C. perfringens* induced inflammatory effects through MAPK and NF- κ B signaling pathways in IPEC-J2 cells.**

To investigate the potential signaling pathway that led to the release of inflammatory cytokines, phosphorylation levels of certain proteins of the MAPK and NF- κ B were determined (Fig. 6). Specifically, *C. perfringens* infection enhanced the phosphorylation of p38, JNK, as well as p65 when compared to the control group ($P < 0.05$). Furthermore, ERK phosphorylation in each group did not change significantly ($P > 0.05$). Moreover, preincubation with *L. plantarum* Lac16 significantly inhibited *C. perfringens* infection-associated p65 phosphorylation ($P < 0.001$).

***L. plantarum* Lac16 alleviated *C. perfringens* infection-associated increase in pro-inflammatory cytokine gene expression levels.**

We determined mRNA expression levels of inflammatory cytokines in IPEC-J2 cells after co-culture with bacteria (Fig. 7). After co-incubation with *L. plantarum* Lac16, mRNA expression levels of pro-inflammatory cytokines, such as *interleukin (IL)-6* and *IL-8*, were significantly elevated ($P < 0.05$; Fig. 7B and C). At the same time, gene expression levels of pro-inflammatory cytokines, such as *IL-1 β* , *IL-6*, *IL-8* and tumor necrosis factor (*TNF*)- α , were sharply elevated after *C. perfringens* infection ($P < 0.001$), which was significantly alleviated by pre-incubation with *L. plantarum* Lac16 ($P < 0.05$). *L. plantarum* Lac16 was very effective in alleviating the increase in *C. perfringens* infection-associated gene expressions of *IL-8* and *TNF*- α ($P < 0.001$). Furthermore, when cells were respectively incubated with *L. plantarum* Lac16 or *C. perfringens*, gene expression levels of anti-inflammatory cytokines, such as *IL-10* and *transforming growth factor (TGF)*- β , did not change significantly ($P > 0.05$) (Fig. 7E and F). However, mRNA expression levels of anti-inflammatory cytokines in the Lac16+CP group decreased significantly when compared to the CP group ($P < 0.05$).

Discussion

Probiotics provide benefits to the host through various mechanisms, including producing anti-bacterial substances, competing with pathogenic microorganisms for enterocyte binding, regulating the secretion of pro- and anti-inflammatory cytokines, and maintaining intestinal barrier integrity [28]. There were several studies indicated that *Lactobacillus* spp. could resist the infection of pathogenic bacteria, including *Escherichia coli*, *C. perfringens*, and *Salmonella Enteritidis* [29, 30, 28, 15]. Previously, we found that *L. plantarum* Lac16 exhibits protective effects against *C. perfringens* infection in broilers [21]. In this study, we have shown that *L. plantarum* Lac16 inhibits the growth of *C. perfringens* and attenuates *C. perfringens* infection-associated intestinal injury in IPEC-J2 cells.

Probiotics exert antimicrobial activities by secreting antimicrobial substances, such as bacteriocins, organic acids, and hydrogen peroxide [31]. Cell-free supernatants containing antimicrobial substances secreted by probiotics effectively inhibit the growth of various pathogens [32]. In addition, *C. perfringens* strains form

biofilms to enhance their persistence and increase resistance to various stressors, such as oxidative and antibiotic stress [33], and this phenomenon has been proved to be involved in a large proportion of bacterial infections [34]. In the current study, we found that the fermentation supernatant of *L. plantarum* Lac16 inhibited the growth of *C. perfringens*. Furthermore, the fermentation supernatant of *L. plantarum* Lac16 significantly suppressed *C. perfringens* biofilm formation. The antimicrobial activity of *L. plantarum* is associated with the production of organic acids, which decreases environmental pH [35]. *C. perfringens* is a pH sensitive bacterium [36]. Acidic environments downregulate the expression of virulence factors of *C. perfringens* while inhibiting its growth [36, 37]. These findings indicate that *L. plantarum* Lac16 and its metabolites decreased environmental pH, and inhibited biofilm formation as well as the growth of *C. perfringens*.

HDPs, as important components of the innate immune system, play critical roles in infection resistance [38]. They are mainly secreted by intestinal epithelial cells and phagocytes in the gastrointestinal tract [24]. HDPs are involved in the maintenance of intestinal homeostasis and innate immune defenses during infection through multiple mechanisms. Specifically, HDPs secreted by intestinal epithelial cells exert direct antimicrobial effects on invading bacterial pathogens and intestinal microbiota [39]. There were several studies indicated that *Lactobacillus* spp. enhance the expression of HDPs [40–42]. In this study, we found that different concentrations of *L. plantarum* Lac16 promoted the expression of endogenous HDPs in IPEC-J2 cells. Increased secretion of endogenous HDPs improves early immune system responses to pathogenic infections and inflammation [43]. Our findings are consistent with those of Wang et al. who concluded that *L. plantarum* ZLP001 upregulates the expression of HDPs, and they also concluded that *L. plantarum* enhances intestinal defense responses by promoting the secretion of HDPs [24].

LDH is a stable cytoplasmic enzyme that possesses oxidation-reduction activities. When cells are subjected to cell membrane damage that is caused by intracellular or extracellular stress, LDH will rapidly release into the extracellular environment [44]. Alpha toxin, which is produced by *C. perfringens* type A, can result in extensive degradation of the plasma membrane, leading to LDH release [45]. Elevated LDH release is a key feature of apoptosis, necrosis, and other forms of cellular damage [46, 47]. We found that *C. perfringens* infection elevated LDH release from IPEC-J2 cells, implying that *C. perfringens* damaged the intestinal epithelial cells, resulting in intracellular enzyme leakage. However, when cells were pre-incubated with *L. plantarum* Lac16, the *C. perfringens* infection-associated increase in LDH release was effectively alleviated. Similar protective results have been reported [48–51].

Pathogenic adherence to host epithelial cells is an indispensable step in the occurrence of infection [52]. In the meantime, *Lactobacillus* could effectively prevent pathogenic adhesion to intestinal epithelial cells and play an important role in maintaining intestinal homeostasis [53]. Thus, one way for *Lactobacillus* exerts its antibacterial activity is by occupying the adhesion site of pathogens to intestinal epithelial cells [54]. Probiotic adhesion to intestinal epithelial cells can optimize the balance and activities of intestinal microbiota [55]. It is worth mentioning that adherence of *C. perfringens* strains increases toxin production [56]. In this study, we found that *L. plantarum* Lac16 significantly suppressed *C. perfringens* adhesion to IPEC-J2 cells, thereby resisting *C. perfringens* infection and protecting intestinal epithelial cells. This finding is similar to that of another study, which showed that *L. rhamnosus* effectively inhibited the adhesion of *C. perfringens* to pig intestinal mucosa [57].

Epithelial cells form a layer that acts as a physical barrier connected by tight junctions between each cell [58]. The main components of tight junction proteins are claudins, zona occludens, and occludin [59]. Tight junctions regulate paracellular transport of various substances, such as ions, solutes, molecules, and water across the intestinal epithelium [60], thereby maintaining physiological functions of epithelial cells [61]. Probiotics and pathogens can alter the expression of tight junctions [61, 62]. Moreover, dysregulation of tight junction protein integrity enhances intestinal barrier permeability [63]. It is worth noting that virulence factors produced by *C. perfringens*, such as *C. perfringens* enterotoxins, can effectively impair tight junctions [9, 64]. In this study, *C. perfringens* infection suppressed the expression levels of claudin-1 and ZO-1, thereby increasing intestinal barrier permeability. However, these effects were effectively alleviated by *L. plantarum* Lac16 preincubation. In addition, the mucus layer covering the surface of intestinal epithelium plays a crucial role in protecting intestinal epithelial barrier integrity [65]. We found that *L. plantarum* Lac16 preincubation attenuated *C. perfringens*-induced disruption of mucus production. Our results are consistent with those of studies reporting on the effects of probiotics in the alleviation of intestinal barrier dysfunction caused by pathogens [65, 66, 28].

PRRs, including TLRs and NOD-like receptors (NLRs), are important receptor molecules in host immune system [67]. PRRs play a crucial protective role in the immune system via identifying pathogen-associated molecular patterns (PAMPs), such as bacterial nucleic acids and flagellin, to resist pathogenic infections [68]. Furthermore, PRRs initiate the activation of intracellular signaling pathways, thereby increasing the expression and release of chemokines, cytokines and antibacterial peptides [69]. It is worth mentioning that TLRs play a crucial role in regulation of mucosal immune responses and maintenance of intestinal homeostasis [70]. In the current study, we found that *L. plantarum* Lac16 elevated the expression levels of TLRs. Changes in expression levels of TLRs in intestinal epithelial cells regulate β -defensin expression [71], corresponding to our previous conclusion that *L. plantarum* Lac16 enhances the expression of endogenous HDPs. The *C. perfringens* challenge sharply elevated the mRNA expression levels of TLRs, including TLR1, TLR2, and TLR4, whereas preincubation with *L. plantarum* Lac16 attenuated this dramatic increase. When pathogens gain entry into the cytoplasm, NLRs have been shown initiate innate immune responses [72]. We found that *L. plantarum* Lac16 alleviated *C. perfringens*-induced elevations in NOD1 expression, in tandem with other studies that reported that probiotics attenuate pathogen associated elevations in PRR expression [73, 28]. In our opinion, *L. plantarum* Lac16 activates PRR-dependent signaling pathways and strengthens the immune system to resist *C. perfringens* infection.

MAPK signaling pathways are signal transduction modules that transform extracellular signals into intracellular responses that regulate the processes of cell growth, differentiation, and migration [74, 75]. In addition, MAPK play a crucial role in modulating the synthesis and release of inflammatory mediators during inflammatory responses [76, 77]. Elevated expressions of PRRs enhance the phosphorylation of MAPK [78, 79]. In this study, *C. perfringens* infection significantly elevated p38 and JNK phosphorylation in IPEC-J2 cells. However, preincubation with *L. plantarum* Lac16 did not significantly attenuate these phenomena. We indicated that *C. perfringens*-induced inflammatory responses are partly mediated by MAPK signaling pathways.

NF- κ B, an important transcription factor, is a key factor for modulating the expression of genes and proteins involved in inflammatory responses [80]. For example, production of proinflammatory cytokines, such as TNF- α , are closely associated with activation of NF- κ B [81]. Probiotics have also been shown to exert their protective

mechanisms against pathogenic infections by modulating the NF- κ B signaling pathways [82, 83, 28]. In this study, preincubation with *L. plantarum* Lac16 significantly attenuated *C. perfringens*-induced increase in p65 phosphorylation, implying that *L. plantarum* Lac16 prevents *C. perfringens* infection-associated excess immune responses by attenuating p65 phosphorylation.

Infections with pathogenic microbes, such as *C. perfringens*, often leads to significant inflammatory responses [84, 85]. Pro-inflammatory cytokines mediate inflammatory responses to invading pathogens through multiple modulatory mechanisms, such as lymphocyte activation, neutrophil migration, and cell proliferation [86]. However, excess secretion of pro-inflammatory cytokines has deleterious effects on the host [87]. In this study, although *L. plantarum* Lac16 elevated the expression levels of pro-inflammatory cytokines, such as *IL-6* and *IL-8*, preincubation with *L. plantarum* Lac16 significantly inhibited *C. perfringens* associated inflammatory responses. In our previous study involving broilers, we found similar protective effects, whereby *L. plantarum* Lac16 alleviated *C. perfringens* infection-associated inflammatory responses in the ileum mucosa [21]. In general, we postulate that one of the mechanisms through which *L. plantarum* Lac16 protects intestinal epithelial cells from *C. perfringens* injury is by relieving inflammation. Interestingly, when intestinal epithelial cells were incubated with *L. plantarum* Lac16 or *C. perfringens*, mRNA expression levels of anti-inflammatory cytokines were not significantly altered, while expression levels in the Lac16 + Cp group were significantly suppressed. These findings should be confirmed in more studies.

Conclusions

In summary, our study used a model system of IPEC-J2 cells to indicate the protective effects of *L. plantarum* Lac16 via several mechanisms against *C. perfringens* infection, including suppressing the growth and biofilm formation abilities of *C. perfringens*, reducing pH levels in the environment, preventing pathogenic adhesion to epithelial cells, promoting the expressions of endogenous host defense peptides, protecting intestinal epithelial barrier integrity, and alleviating inflammatory responses by attenuating p65 phosphorylation. These findings highlight the significance of *L. plantarum* Lac16 as a potential therapeutic strategy against *C. perfringens* infection and provide a theoretical basis for the application of *L. plantarum* Lac16 in animal husbandry to resist pathogen infections.

Abbreviations

BSA: Bovine serum albumin; CFU: Colony forming units; *C. perfringens*: *Clostridium perfringens*; DAPI: 4',6-Diamidino-2-phenylindole dihydrochloride; DMEM/F12: Dulbecco's modified Eagle's F12 ham medium; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; HDPs: Host defense peptides; HRP: Horseradish peroxidase; LDH: Lactate dehydrogenase; IL: Interleukin; IPEC-J2: Intestinal porcine epithelial cell line; *L. plantarum*: *Lactobacillus plantarum*; MAPK: Mitogen-activated protein kinase; MRS: Mann-Rogosa-Sharpe; NF- κ B: Nuclear factor- κ B; NLRs: NOD-like receptors; NOD: Nucleotide-binding oligomerization domain; OD: Optical density; PAMPs: Pathogen-associated molecular patterns; Papp: Apparent permeability coefficient; PAS: Periodic acid-Schiff; pBD: Porcine β -defensin; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; pEP2C: Porcine epididymis protein 2 splicing variant C; PRRs: Pattern recognition receptors; PVDF: Polyvinylidene difluoride; RCM: Reinforced clostridium medium; TBST: Tris-Buffered-Saline with Tween; TGF:

transforming growth factor; TLR: Toll-like receptor; TNF- α : Tumor necrosis factor- α ; TSC: Tryptose sulfite cycloserine; ZO-1: Zona occludens 1

Declarations

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author by request.

Authors' contributions

WL, YZ, and LG conceptualized the experiments; YZ performed the experiments, analyzed the data and wrote the original draft; BK and the rest of the authors revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This study is supported by the Natural Science Foundation of Zhejiang province, China (No. LZ20C170002), the National Natural Science Foundation of China (No. 32072766, 31672460, and 31472128), National High-Tech R&D Program (863) of China (No. 2013AA102803D), the Major Science and Technology Project of Zhejiang Province (No. 2006C12086), PRC.

Acknowledgements

The authors would like to thank members of their laboratory for helpful and constructive advice.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

Figure 1

Antimicrobial activity of *L. plantarum* Lac16 on *C. perfringens*. (A) Agar well diffusion assay. 1: MRS broth; 2: 100 μ g/mL of ampicillin; 3 and 4: *L. plantarum* Lac16 fermentation supernatant. (B) The growth of *C. perfringens* in different groups was measured by OD600 after 12 h of incubation. (C) Biofilm formation was measured by OD590. (D) *C. perfringens* levels in the co-culture experiment. (E) pH values of cultures in different groups. Data are presented as the means $\pm$ SD for n = 3; * P < 0.05, ** P < 0.01, *** P < 0.001 (t test).

Figure 2

Gene expression levels of HDPs in *L. plantarum* Lac16 treated IPEC-J2 cells. (A) pBD1, (B) pBD2, (C) pBD3, (D) pEP2C. mRNA expression was standardized to β -actin expression. Data are presented as the means $\pm$ SD for $n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA).

Figure 3

Cytotoxicity and C. Perfringens adhesion assays. (A) Concentrations of LDH in the supernatants of IPEC-J2 cells. (B) Adhesions of C. perfringens to IPEC-J2 cells were detected by on the TSC agar. Adherence ratio of the C. perfringens group was normalized to 100%. (C) Adhesions of C. perfringens (green) to IPEC-J2 cells were detected by fluorescence labeling method. Data are presented as the means $\pm$ SD for n = 3; * P < 0.05, ** P < 0.01, *** P < 0.001 (t test).

Figure 4

Intestinal barrier functions and tight junction proteins of IPEC-J2 cell monolayers. (A) Papp of IPEC-J2 monolayers based on apical-to-basolateral flux of fluorescein sodium. (B) Mucin production were detected by PAS staining. (C) Western blot detection of occludin and claudin-1 in IPEC-J2 cells. β -actin was used as an indicator of protein loading. (D) Immunofluorescence staining of ZO-1 (green) in IPEC-J2 cells. Nuclei were counterstained using DAPI (blue). All images were obtained at 63 $\times$ magnification in oil. Data are presented as the means $\pm$ SD for n = 3; * P < 0.05, ** P < 0.01 (t test).

Figure 5

Relative gene expression levels of PRRs during *C. Perfringens* infection in IPEC-J2 cells preincubated with *L. plantarum* Lac16. (A) TLR1, (B) TLR2, (C) TLR4, (D) NOD1, (E) NOD2. mRNA expression was standardized to β -actin expression. Data are presented as the means $\pm$ SD for $n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t test).

Figure 6

Western blot detection of MAPK and NF- κ B pathways in IPEC-J2 cells. Quantitative analysis of the expression levels of phospho-p38/p38, phospho-JNK/JNK, phospho-ERK/ERK, and phosphor-p65/p65. Data are presented as the means $\pm$ SD for $n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t test).

Figure 7

Relative gene expression levels of inflammatory cytokines during *C. Perfringens* infection in IPEC-J2 cells preincubated with *L. plantarum* Lac16. (A) IL-1 β , (B) IL-6, (C) IL-8, (D) TNF- α , (E) IL-10, (F) TGF- β . mRNA expression was standardized to β -actin expression. Data are presented as the means $\pm$ SD for $n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t test).