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Research Article

Synergistic Effect of B-Type Lotus Seedpod Oligomeric Procyanidin and Probiotics against Adhesion of Enterotoxigenic *Escherichia coli In Vitro*

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Enterotoxigenic *Escherichia coli* (ETEC) adhesion to gut epithelial cells is a prerequisite for diarrhea. Here, we studied the synergistic effect and potential mechanism of B-type lotus seedpod oligomeric procyanidin (LSPC) combined with probiotics against adhesion of ETEC. The results indicated that LSPC exhibited an effective anti-ETEC adhesion effect. LSPC showed signally synergistic effects with probiotics, e.g., *Streptococcus thermophilus* (ST) and *Lactobacillus rhamnosus* (LGG) in response to ETEC adhesion, with the combination of LSPC and LGG being the most efficacious. This may be attributed to the restoration of transmembrane resistance of cells, the increased expression of anti-inflammatory factors (IL-10, 1.89-fold), and the reduction of cellular inflammatory factor levels (TNF-α and IL-8), regulated by LGG-LSPC, resulting in a better enhancement of cellular immune defense and barrier function. In addition, the results of gas chromatography showed that LSPC, as a prebiotic, could significantly increase the total amount of short-chain fatty acids (especially, butyric acid) produced by probiotics (e.g., LGG), thus better maintaining intestinal health against ETEC infection. In conclusion, the synergistic effect of LSPC and probiotics (represented by LGG) against ETEC adhesion in epithelial cells may be achieved through the enhancement of cellular immune defense, cellular barrier function, and maintenance of homeostasis in the gut.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a primary contributor to bacterial diarrhea in humans, especially children younger than 5 years of age in developing countries, and farm animals, causing substantial mortality and morbidity [1]. ETEC could attach to intestinal epithelial cells by specific fimbriae and secrete two enterotoxins, via glycoprotein receptors in host cells [2, 3], causing intestinal damage, electrolyte imbalance, and dehydration [4]. The adhesin-mediated bacterial attachment to host small intestinal epithelial cells is the first step of pathogenic bacteria infection, involving interactions between bacterial ligands and cell receptors [5]. It is therefore reasonable to assume that acting on bacterial ligands or host cell receptors through suitable inhibitors, and subsequently effectively inhibiting the adhesion of pathogenic bacteria to intestinal epithelial cells, may be an effective way to combat ETEC diarrhea.

Currently, antibiotics are commonly used to treat diarrhea [6]. However, with the emergence of antibiotic-resistant pathogens, novel alternative treatment and prevention strategies for ETEC infections are being investigated. Probiotics, in particular, were considered to be one of the most promising countermeasures [7, 8]. Existing studies have indicated that probiotics exert antidiarrheal effects through complex anti-infective mechanisms, such as regulation of intestinal microbiota and modulation of the host immune system [9]. For instance, *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* (LGG) may protect enterocytes from ETEC K88-induced inflammatory-

related responses *in vitro* by modulating chemokine and cytokine expression (IL-1β, TNF-α, and IL-8), in part by reducing pathogen adhesion and counteracting neutrophil migration [10]. Ren et al. reported that the probiotic *Lactobacillus paracasei* ameliorated diarrhea by inhibiting the activation of the nuclear factor kappa B/MLCK pathway and increasing the abundance of short-chain fatty acids (SCFAs)-producing gut microbiota in mice [11]. In addition, Baillo et al. demonstrated that *Lactiplantibacillus plantarum* strains can modulate intestinal innate immune response, thereby increasing the ETEC clearance in mice [12]. Nevertheless, available evidence also suggests that the anti-invasive effect of probiotics on ETEC is closely related to the type and action mode of probiotics [13, 14], which needs further comparative study.

Plant polyphenols are widely available natural compounds that, in addition to probiotics, are thought to inhibit bacterial growth and protect gut health [15]. Xiao et al. reported that protocatechuic acid and quercetin attenuated endotoxin secretion and bacterial adhesion after ETEC K88 infection, thereby preventing diarrhea caused by ETEC K88 [16]. Proanthocyanidins (PA), a typical polyphenol, have been reported to regulate host health by increasing microbial diversity and their metabolites, such as SCFAs [17]. Moreover, the large number of hydroxyl groups in PA inhibits bacterial adhesion and coaggregation, reducing biofilm formation and decreasing inflammation [18]. That is, PA may possess the potential for anti-ETEC adhesion effects in intestinal epithelial cells.

In fact, polyphenols (including PAs) and probiotics have been reported to have a joint effect. Polewski et al. found that cranberry proanthocyanidins in combination with probiotics were able to inhibit the ability of extraintestinal pathogenic ETEC to invade intestinal epithelial cells *in vitro* [19]. Reciprocal benefits of wine polyphenols and lactic acid bacteria probiotics were reported, and more importantly the antiadhesive capacity of probiotics against *E. coli* CIAL-153 on intestinal cells was also strengthened by wine polyphenols [20]. In addition, Zhu et al. reported that the addition of anthocyanins significantly increased the number of *Bifidobacteria*, *Lactobacilli*, and probiotics simultaneously promoting the metabolism of anthocyanins [21]. Our previous study found that B-type oligomeric procyanidin from lotus probiotics combination had significant antidiarrhea and anti-ETEC K88 effects in mice, but the mechanisms and their synergistic effect were not clear [22]. ETEC K88 is one specific porcine pathogen, and the intestinal porcine epithelial cell (IPEC) J2 is commonly used as a porcine intestine in *in vitro* model [23]. Therefore, the aim of this study is to investigate the antiadhesive effects and mechanisms of LSPC in combination with probiotics on ETEC using IPEC-J2 cells. First, the concentration of ETEC and the concentration and action mode of LSPC or probiotics were screened by IPEC-J2 cells. Then, the antiadhesion effects of LSPC, probiotics, and their combination were further investigated. In the meantime, the regulatory effects of LSPC and probiotics on ETEC-induced inflammatory response and barrier function disruption, as well as the underlying mechanisms of antiadhesion, were explored.

2. Materials and Methods

2.1. Materials. Fetal calf serum, 0.25% Trypsin-EDTA, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), and penicillin-streptomycin solution (100×) were purchased from GIBCO (Life Technologies Corporation, Grand Island, NY, USA GIBCO, USA). The lactate dehydrogenase (LDH) kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Luria Bertani (LB) broth, LB Agar, MacConkey Agar, de Man Rogosa and Sharpe (MRS) broth, and MRS Agar medium were purchased from Hope Bio-Technology (Qingdao, China). All other reagents used in this study were of analytical grade, and the water was of Milli-Q quality.

2.2. Bacterial Strains and Culture Conditions. The ETEC strain K88ac (C83715) was obtained from the China Institute of Veterinary Drug Control. The strain was stored in 15% (v/v) glycerol stocks at −80°C. *Lactobacillus rhamnosus* HN001 (ATCC SD5675), *Lactobacillus plantarum* LP-115 (SD5209), *Lactobacillus acidophilus* NCFM (ATCC SD5221), *Lactobacillus paracasei* LPC-37 (ATCC SD5275), and *Streptococcus thermophilus* ST-21 (ATCC SD5207) were purchased from DuPont™ Danisco® (Shanghai, China). All the probiotics were prepared from freeze-dried powder stocks.

The bacteria's culture conditions were slightly modified according to our previous study [24]. In brief, K88ac was cultured in LB broth at 37°C for 4–6 h with shaking at 200 rpm routinely to obtain cells in the exponential growth phase. All the *Lactobacillus* powders were incubated in MRS agar plates at 37°C for 24 h and then recultured overnight in the fresh MRS broth (37°C). These bacteria were harvested by centrifugation (4500 × g, 5 min) and then washed three times in 0.9% NaCl, and the optical density of the suspension at 600 nm was measured by 1800 UV-VIS spectrophotometer (Shimadzu, Japan), and the number was calculated from the growth curve.

2.3. Cell and Cell Culture. The IPEC-J2 cell was obtained from the Stem Cell Bank of the Chinese Academy of Sciences. The cell recovery and passaging were performed according to a previous study with slight modifications [25]. IPEC-J2 cells were grown in DMEM/F12 complete medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2. Then, the appropriate volume of cell suspension was inoculated into 96-well plates, 6-well plates, or Transwell chambers, and the cells were cultured until they had fully spread on the bottom surface and were ready for experiments.

2.4. LDH Release Rate of IPEC-J2 Cells with ETEC Treatment. The cytotoxicity of ETEC with different treatment times and concentrations on IPEC-J2 was measured using an LDH kit. In brief, the ETEC with different concentrations was added to IPEC-J2 cells and treated for 1.5 h, or the ETEC with 10^9 CFU/mL was added to cells and treated for 10, 30, 60, 90, and 120 min. The cell treatment solution was centrifuged at

21000 × g for 5 min, and 20 mL of the supernatant was taken. Then, the reaction reagents were added following the instructions provided in the assay kit, using the standard solution as the positive control.

After the reaction was complete, the absorbance values of the positive control group (Ap) and the treated group cells (At) at 490 nm were measured. Simultaneously, we measured the absorbance values of the corresponding blanks group (Ac and Ab), and the LDH release rate was calculated by using the following equation:

$$\text{LDH release rate \%} = \frac{(\text{At-Ab})}{(\text{Ap-Ac})} \times 100\%. \quad (1)$$

2.5. Effect of LSPC and Probiotics on IPEC-J2 Cells. The effect of LSPC on cell viability was determined by the methyl thiazolyl tetrazolium (MTT) method [26]. Temporarily, IPEC-J2 cells were seeded in 96-well plates at $1.0–1.5 \times 10^4$ cells/well and then incubated with LSPC (0, 100, 200, 300, and 400 μg/mL) after the bottom wall was covered. After incubating for 24 h, using new media (160 μL) without fetal calf serum or antibiotic, 40 μL of the MTT (1.25 mg/mL) solution was added to each well. Then, the supernatant was removed after incubating for 4 h, and 150 μL of dimethyl sulfoxide was added. The crystals were shaken at low speed to fully dissolve, and the absorbance values (A) of the cells in the administration group (A_a) and the control group (A_c) were measured at 490 nm. The cell viability was calculated by using the following equation:

$$\text{cell viability} = \left(1 - \frac{\text{Aa}}{\text{Ac}}\right) \times 100\%. \quad (2)$$

The effect of probiotics on cell viability was then determined using the LDH kit method according to Section 2.4.

2.6. Observations of ETEC and Probiotic Adhesion on IPEC-J2 Cells. The effect of ETEC and ETEC-probiotic (1×10^9 CFU/mL) on the cell morphology was also observed [26]. The coverslips (2.5 cm × 2.5 cm) were soaked in acid, washed and sterilized, and then placed in medical alcohol, dried over an alcohol lamp, and placed in a 6-well plate. The cells were inoculated in the 6-well plate and ETEC/ETEC-probiotic was added after the cells had been fully coated in the bottom wall by a competitive antiadhesion method. After 1 h, the cells were washed 4-5 times by PBS buffer carefully and 1 mL of glutaraldehyde fixative (2.5%) was added to each well. The solution was placed at 4°C for 2 h, and then the coverslips were removed. Cell staining was performed according to the instructions of the Gram staining kit, and morphology was observed under a microscope.

2.7. Effect of LSPC on ETEC Antiadhesion. The LSPC, with 99.56 ± 1.2% purity, (+)-catechin (33.07%), (−)-epicatechin (12.95%), dimer (30.4%), trimer (12.14%), and tetramer (1.83%), was extracted from fresh lotus seedpod, according to previously described laboratory methods [22, 24]. IPEC-J2 cells were seeded into 6-well plates and grown until 90% fusion, according to previously published methodology [27]. The DMEM/F12 basal medium solution containing LSPC (200 μg/mL) was passed through a 0.2 μm sterile filter membrane. 0.1 mL of ETEC suspension (1.5×10^9 CFU/mL) was added to 0.9 mL of LSPC/blank DMEM/F12, and the mixture was cocultured in a 37°C, 5% CO_2 cell incubator for 2 hours. After this period, nonadhered bacteria were washed off with PBS. Then, 0.1 mL of Triton X-100 (1%) was added in each well, mixed, and allowed to stand for 10 min. It was then mixed well to release the adhesive bacteria. After that, a 0.9 mL PBS buffer was added to terminate the reaction. Sterile normal saline was used for multiple dilutions, and the live bacteria were counted by the MacConkey plate method [6].

The effects of LSPC treatment ways, concentration, and treatment time on the ETEC's adhesion rate were explored according to Table 1. The adhesion rate is calculated as follows:

$$\text{adhesion rate \%} = \frac{N_n}{N_0} \times 100\%, \quad (3)$$

where N_0 and N_n are the number of live bacteria in the LSPC-ETEC and nontreated group.

2.8. Effect of Probiotics and LSPC-Probiotic on Antiadhesion of ETEC. The antiadhesion effects of *Lactobacillus rhamnosus* (LGG), *Streptococcus thermophilus* (ST), and *Lactobacillus plantarum* (LP) on ETEC by different action modes (competition and exclusion) were measured as described in Section 2.7.

The effect of different LSPC-probiotics on the adhesion rate of ETEC on cells was explored. Specifically, *Lactobacillus rhamnosus* (LGG, 1×10^8 CFU/mL), *Lactobacillus paracasei* (LPC, 1×10^8 CFU/mL), *Lactobacillus plantarum* (LP, 1×10^8 CFU/mL), *Lactobacillus acidophilus* (LA, 1×10^8 CFU/mL), or *Streptococcus thermophilus* (ST) was cocultured with LSPC (200 μg/mL) for 2 h, respectively, and then the adhesion rate of ETEC was measured.

2.9. Synergistic Antiadhesion Effect of LSPC and Probiotics. Based on the results of the abovementioned experiments, LSPC concentration, treatment time, and antiadhesion exclusion were selected to investigate the synergistic effect of LSPC and probiotics in inhibiting ETEC adhesion by a mixed-level full factorial design (Table 2). The experiment was designed as per the previous reports [28].

2.10. Effect of LSPC on Cellular Zeta Potential. The cells with good logarithmic growth were digested by trypsin, and the cell suspension with a concentration of 4×10^4 cells/mL was prepared using pH 7.4 HBS buffer. The same volume of HBS-dissolved LSPC was then added, with LSPC concentrations of 0, 50, 100, and 200 μg/mL, respectively. After coincubation for 1 h, 4 h, and 20 h, the Zeta potential of the system was measured [29].

Table 1: Different processing types and cultivation methods.

Type	Treatment
Competition	LSPC + ETEC coculture 30 min
Exclusion	LSPC preculture 1 h + ETEC cocultured 30 min
Displacement	ETEC preculture 1 h + LSPC cocultured 30 min

Table 2: Full factorial design.

Factor	Number	Level		
LGG (CFU/mL)	A	0	10^8	10^9
LSPC (mg/mL)	B	0	200	

2.11. Measurement of Transepithelial Electrical Resistance (TEER). The measurement of TEER was evaluated as previously reported with a slight modification of IPEC-J2 cells seeded on Corning Transwell inserts (Ø12 mm, 0.4 μm, 1.12 cm^2), at a density of 1×10^5 cells per well [30]. Cells were then allowed to grow for 11 d at 37°C and 5% CO_2. The medium was changed every 2-3 d (500 µL in the apical compartment and 1500 µL in the basolateral compartment). After 12 days of cell differentiation, the TEER value ($t = 0$) was measured in every well. A LSPC solution with different concentrations was premixed with the same volume of ETEC bacteria, incubated in an incubator for 10–15 min, and then added to the cells. After incubation for 2 h and 4 h, the TEER was measured using a cell resistivity meter and the resistivity was calculated (relative to blank cells).

2.12. The Effect of LSPC and Probiotics on Cellular Immune Regulation. The effect of LSPC and probiotics on cellular immune regulation was investigated using real-time fluorescence quantitative PCR analysis [31]. The samples of TNF-α, IL-8, and IL-10 were prepared as follows: 200 μg/mL of LSPC and 10^8 CFU/mL of LGG were added to each cell wall and precultured for 2 h. After incubation for 3 h with the addition of ETEC (10^8 CFU/mL), the samples were washed with PBS for 4-5 times.

1 mL of TRIzol was added to the previously treated samples for homogenization and transferred to RNase-free EP tubes for lysis for 10 minutes. Then, 200 μL of chloroform was added and thoroughly mixed. After allowing it to stand at room temperature for 5 minutes, the supernatant was removed through centrifugation. Subsequently, 400 μL of isopropanol was added and mixed well, and the supernatant was discarded after standing for 10 minutes and centrifuged. Then, 1 mL of 75% ethanol (RNase-free) was added to the precipitate for purification, and this step was repeated twice. The extracted RNA was finally precipitated in 20 μL of DEPC water. The purity and concentration of RNA were estimated by measuring the OD260 and OD280 values with a microspectrophotometer. Then, oligo, dNTP, and other reverse transcription solvents were added in RNA (5 μg). Reverse transcription was performed according to the following procedures: 25°C, 5 min; 50°C, 15 min; 85°C, 5 min; and 4°C, 10 min. After reverse transcription, 4 μL of 1/10 cDNA was taken, and the reaction reagent such as SYBR Green Master Mix was added and treated at 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, and 60°C for 30 s. After 40 cycles of amplification, the qPCR analysis was performed. The mRNA level relative to β-actin content was calculated using $2^{-\Delta\Delta Ct}$ by drawing a dissolution curve.

2.13. Determination of Contents of SCFAs. The effect of LSPC on the secretion of SCFAs (acetic, propionic, *n*-butyric, i-butyric, *n*-valeric, and i-valeric acids) by different probiotic bacteria was analyzed by gas chromatography (GC). MRS solution with LSPC (500 μg/mL) was passed through a 0.2 μm sterile filter membrane, and an equal volume of probiotic suspension (2×10^7 CFU/mL) was added and incubated at 37°C for 24 h. After centrifugation at 4°C, 7000 × g for 5 min, the supernatant was passed through a 0.45 μm filtration membrane. Then, put 5 mL of supernatant into a 10 mL centrifuge tube, and add 0.5 mL sulfuric acid (50%) and 2 mL diethyl ether into the centrifuge tube. The mixture was vortexed for 1 min and then centrifuged at 10,000 × g for 5 min. After being placed at 4°C for 2 h, the upper clear night was taken for GC analysis [31].

The GC analysis was performed as follows: the mixture was filtered and analyzed by using an Agilent 6890N gas chromatography (Agilent Technologies, Santa Clara, CA, USA) equipped with a FFAP capillary column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector. The initial temperature was 100°C which was maintained for 1 min, then raised to 150°C at a rate of 5°C/min, and held at this temperature for 5 min. The flow rate of high-purity nitrogen (≥99.99%) was 2 mL/min. The inlet temperature was 270°C; the injection method was nonshunt injection with a volume of 2.0 μL. The temperature of the FID detector was 280°C.

2.14. Statistical Analysis. All experiments were repeated three times, and the results were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used for mean comparisons, and univariate linear models were employed for variance analysis of mixed-level design experiment data. Differences were considered significant when $P < 0.05$. Statistical analysis was conducted using IBM SPSS Statistics 20.0.

3. Results and Discussion

3.1. The Cytotoxicity of ETEC on IPEC-J2 Cells. The cytotoxicity of the drug to cells can be characterized by measuring the lactate dehydrogenase (LDH) released after the cell damage. In this study, the effects of different treatment concentrations and treatment duration of both ETEC and probiotics on IPEC-J2 cells were explored. As shown in Figure 1(a), the cytotoxicity of IPEC-J2 enhanced with ETEC concentration increase (1×10^7 CFU/$m^{-1} \times 10^9$ CFU/mL) at the same treatment time (1.5 h). When the ETEC concentration increased to 1×10^9 CFU/mL, the LDH release rate increased to 38.82 ± 3.92% accordingly. In addition, under the same concentration of ETEC (1×10^9 CFU/mL), the cell damage of IPEC-J2 increased with the extension of treatment time, and even the LDH release rate increased to 64.12 ± 1.29% after 2 h of ETEC treatment (Figure 1(b)). In

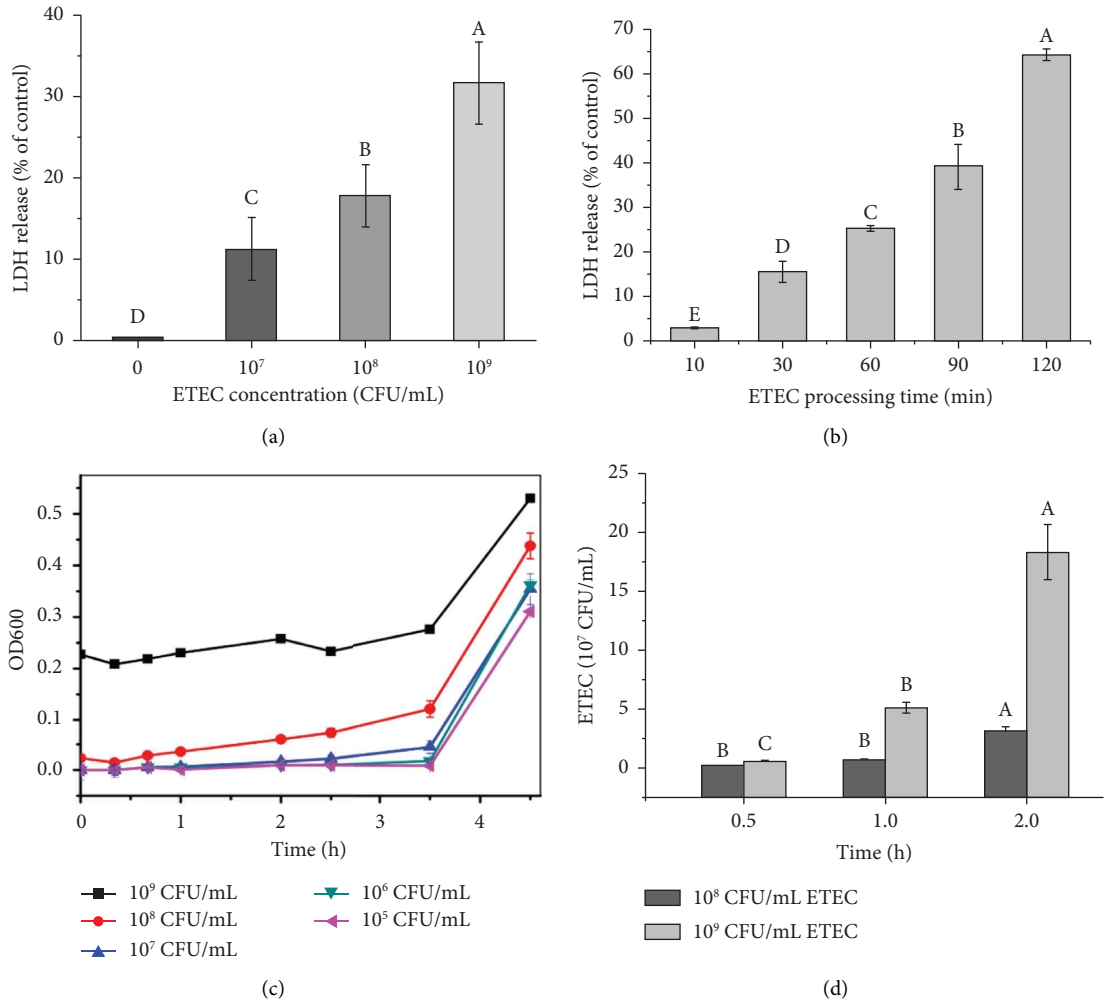

Figure 1: The LDH release of different concentrations (a) and treatment times (b) of ETEC on IPEC-J2 cells, and the growth curves of ETEC in DMEM/F12 medium (c), and the adhesion amount of ETEC to IPEC-J2 at different culture times (d). Columns with different letters indicate significant differences between groups ($P < 0.05$), while those with the same letters show no significant difference.

order to reduce the effect of ETEC on cells, the concentration of ETEC was controlled below 1×10^8 CFU/mL, and the treatment time was less than 1 h.

The concentration and the incubation time of pathogenic bacteria (e.g., ETEC) may be important factors affecting their cell adhesion. First, the growth curves and adhesion quantities of the ETEC in the DMEM/F12 system are measured. As shown in Figure 1(c), for ETEC at concentrations of 1×10^5–1×10^7 CFU/mL, a moderate increase did not occur until 3.5 h of incubation whereas for ETEC concentrations up to 1×10^8 CFU/mL, a linear increase in OD600 values was observed at only 45 min of incubation. This trend was also verified in the adhesion of ETEC (Figure 1(d)). With the extension of culture time, the adhesion amount of ETEC at 1×10^9 CFU/mL on IPEC-J2 increased significantly ($P < 0.05$), while for ETEC at a concentration of 1×10^8 CFU/mL, a slow increase in adhesion was observed after 1 h of incubation. In this work, ETEC concentration of 1×10^8 CFU/mL and incubation for 40 min were selected for antiadhesion assay. Furthermore, the effect of ETEC on cell morphology has been observed microscopically. Obviously, when IPEC-J2 was incubated with ETEC (1×10^9 CFU/mL) for 1 h, the cellular compact structure was disrupted and the cellular chromatin was concentrated (Figure 2(B)). This phenomenon further confirmed that ETEC could damage IPEC-J2 cells.

3.2. Effect of LSPC and Probiotics on the Cell Viability of IPEC-J2. As shown in Figure 3(a), a trend of increasing and then decreasing cell viability of IPEC-J2 was observed with increasing LSPC treatment concentration. When the concentrations of LSPC were increased to 200 μg/mL, the cell survival rate of IPEC-J2 was $127.29 \pm 5.82\%$, which was 1.27 times higher than that of the control group. These phenomena indicated that LSPC has a more pronounced proliferative effect on IPEC-J2. However, cytotoxicity appeared when the concentrations of LSPC were increased to 300 μg/mL, and the cell survival was reduced to $29.11 \pm 4.12\%$. Consequently, LSPC concentrations below 200 were selected in subsequent cell therapy experiments.

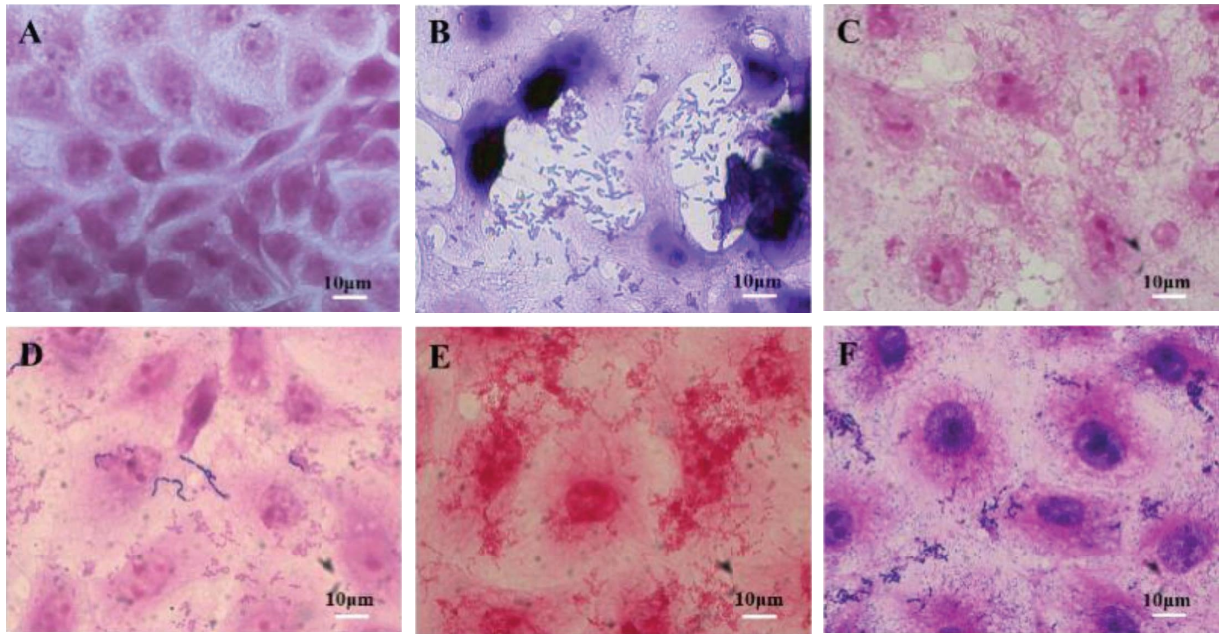

Figure 2: Adhesion morphology of control IPEC-J2 cell (A) and the cells cocultured with ETEC (B), LGG (C), LGG + ETEC (D), ST (E), and ST + ETEC (F).

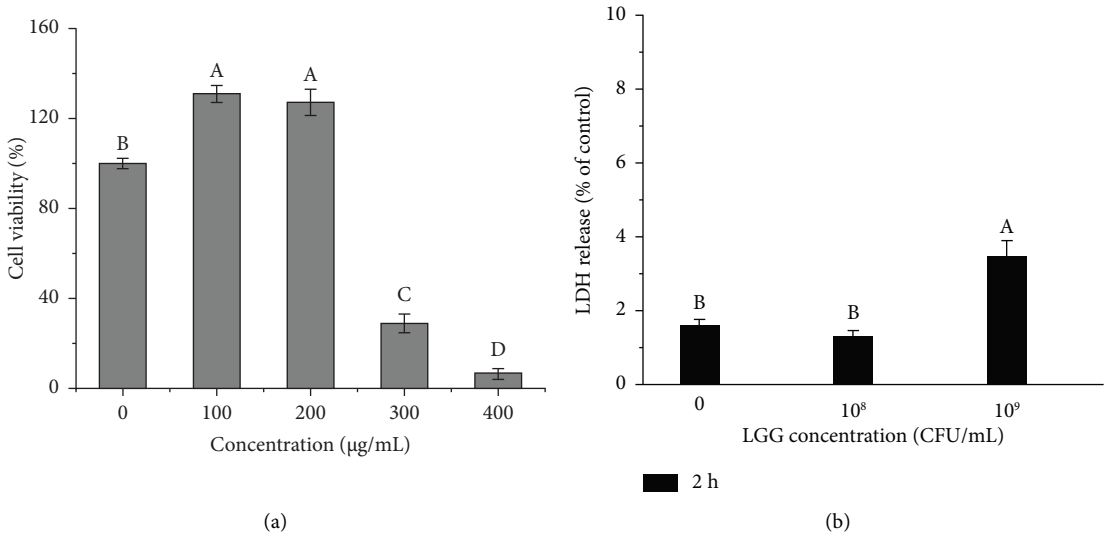

Figure 3: Effect of different concentrations of LSPC (a) and LGG (b) on cell viability of IPEC-J2 cells. Columns with different letters are significantly different between groups ($P < 0.05$), while no significant difference was observed with the same letters.

Simultaneously, the effect of probiotics (e.g., LGG) on IPEC-J2 was also analyzed (Figure 3(b)). The LDH release rate of IPEC-J2 was only $3.47 \pm 0.44\%$ after 2 h incubation with a LGG concentration of 1×10^9 CFU/mL. It became clear that the probiotic had little cytotoxicity against IPEC-J2 cells. After LGG/ST (1×10^9 CFU/mL) and IPEC-J2 were cultured for 1 h (Figures 2(C) and 2(E)), there was no significant difference in cell morphology compared with the control group (Figure 2(A)), and the results showed that probiotics (LGG and ST) did not have toxic effects on cells under these cultivation conditions. Notably, the morphology of IPEC-J2 cells cocultured with ETEC-LGG/ST was not different from that of normal cells, suggesting that LGG and ST may have a protective effect on IPEC-J2 cells against ETEC damage.

3.3. Effect of LSPC and Probiotics on ETEC Adhesion in IPEC-J2 Cells. Competition, exclusion, and displacement assays were performed to elucidate the manner in which LSPC inhibited ETEC adhesion to cells. As shown in Figure 4(a), at low concentrations of LSPC (50 μg/mL), none of the three action modes exhibited antiadhesive effects of ETEC. When the concentration of LSPC increased to 200 μg/

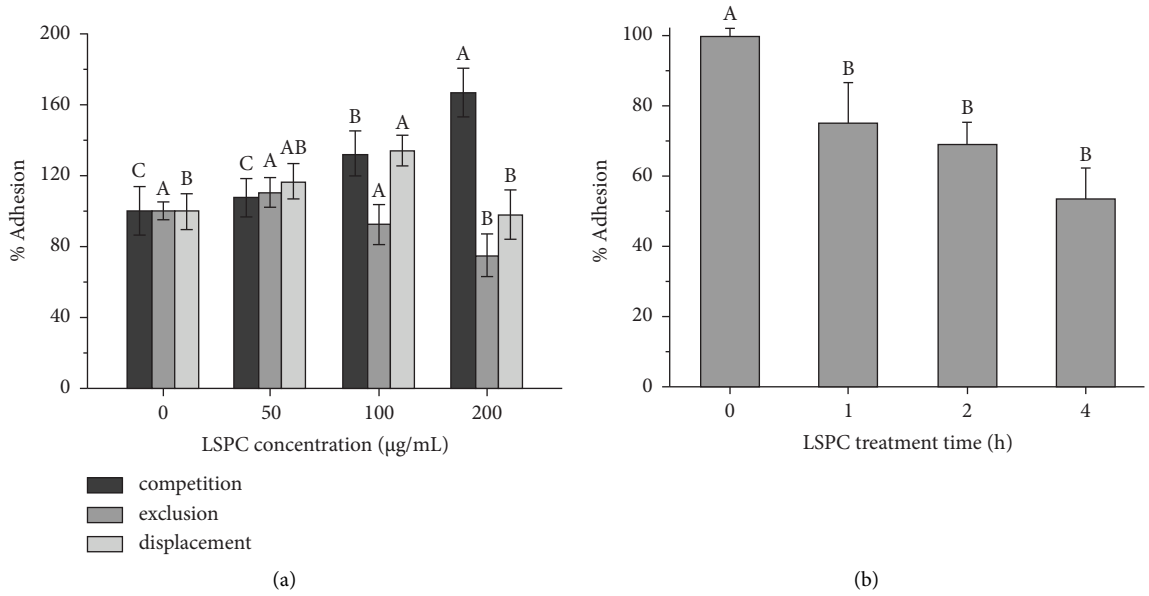

Figure 4: The inhibition of ETEC adhesion by LSPC and probiotic treatments. (a) The effects of different treatment methods (competition, exclusion, and displacement) (b) and pretreatment time of LSPC on the adhesion rate of ETEC to IPEC-J2. Columns with different letters are significantly different between groups ($P < 0.05$), while no significant difference was observed with the same letters.

mL, the adhesion rate of ETEC on cells was significantly decreased ($74.87 \pm 11.76\%$) only under the exclusion mode (preincubation for 1 h). At high concentrations of LSPC (200 μg/mL), preincubation time against the effect of adhesion was probed (Figure 4(b)). The adhesion rate of ETEC decreased as the preincubation time increased, from $69.00 \pm 6.20\%$ to $53.30 \pm 9.04\%$ when the incubation time was increased from 2 h to 4 h. The results suggested that as the duration of LSPC action increases, it may cause cell structure or stimulate cells to produce active factors against bacterial adhesion, thus decreasing the adhesion rate of pathogenic bacteria.

It was believed that the antiadhesive effect of probiotics on pathogenic bacteria is also closely related to their action mode [27]. Hence, the effect of probiotics (1×10^8 CFU/mL) on ETEC's (1×10^8 CFU/mL) adhesion rate under different action modes (competition and exclusion) was investigated. As shown in Figure 5(a), ST pretreatment, in a repulsive manner, had no inhibitory effect on ETEC adhesion, and ST coincubation with ETEC (competitive effect) significantly reduced ETEC adhesion ($P < 0.05$). In contrast, LP was able to significantly reduce the adhesion rate of ETEC only in repulsion ($P < 0.05$). Notably, LGG can obviously reduce the adhesion rate of ETEC in both competition and exclusion modes, with the adhesion rate of ETEC being slightly lower in competition ($75.60 \pm 6.52\%$) than in exclusion ($81.43 \pm 6.52\%$). The abovementioned results showed that the probiotics were more effective in inhibiting bacterial adhesion in the competitive mode.

Furthermore, the effect of probiotics and LSPC on ETEC adhesion was explored under competing action patterns. As shown in Figure 5(b), in addition to *Lactobacillus plantarum* (LP), treatment with ST, *Lactobacillus acidophilus* (LA), *Lactobacillus paracasei* (LPC), and LGG alone was able to reduce the adhesion rate of ETEC on IPEC-J2, and the magnitude of inhibition of ETEC adhesion was in the following order: LGG > ST > LPC > LA. Notably, the addition of LSPC altered the antiadhesive effect of probiotics on ETEC. Of these, LSPC-LGG, LSPC-ST, and LSPC-LP reduced ETEC adhesion rates by 25.23%, 18.69%, and 14.48%, respectively, compared to probiotics. In contrast, LSPC-LA/LPC did not show an inhibitory enhancement of cell adhesion to ETEC. It has been suggested that LSPC in combination with some probiotics, especially LGG, has a reinforcing effect on ETEC antiadhesion. The results suggested that LSPC selectively binds to probiotics, which may be related to their different interaction.

3.4. Synergistic Antiadhesive Effect of LSPC and Probiotics and Its Mechanism on ETEC

3.4.1. Full Factorial Experiment of Interaction between LGG and LSPC. Based on the experimental results, the ETEC treatment concentration was selected to be 1×10^8 CFU/mL, and the treatment was carried out under the exclusion mode for 2 h. LSPC (A) concentration of 200 μg/mL and LGG (B) concentrations of 1×10^8 CFU/mL (level 1) and 1×10^9 CFU/mL (level 2) were selected for a mixed-level full factorial experimental design. It can be seen in Table 3 that the treatment group with the lowest adhesion rate of ETEC on IPEC-J2 was A_2B_1, i.e., 1×10^9 CFU/mL of LGG combined with 200 μg/mL of LSPC. Of these, LGG has a greater impact than LSPC on the adhesion rate of ETEC on cells. The results of ANOVA in Table 4 showed that LGG, LSPC, and LSPC + LGG were able to significantly ($P < 0.05$) affect the adhesion rate of ETEC under the selected conditions and levels. These results suggested that there was a synergistic effect between LSPC and LGG on inhibiting ETEC adhesion on intestinal epithelial cells.

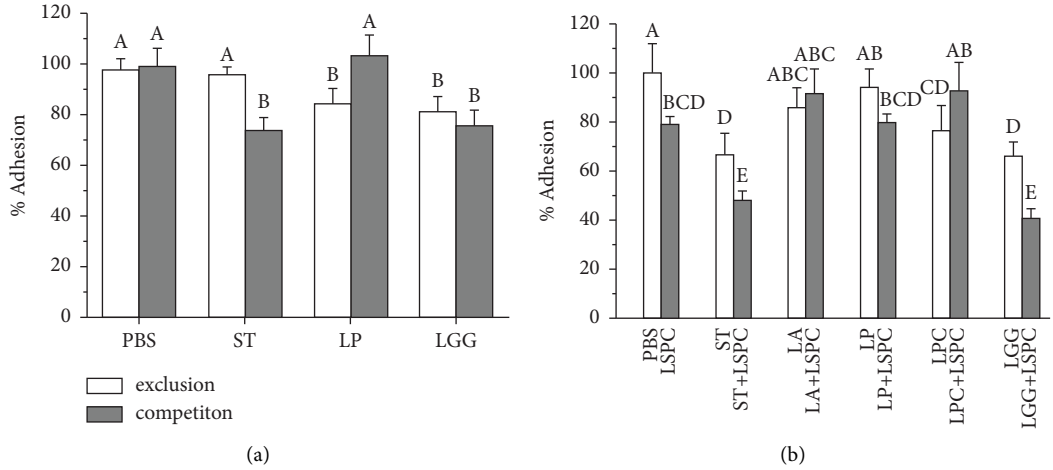

Figure 5: The inhibition of ETEC adhesion to IPEC-J2 by LSPC and probiotic treatments. Columns with different letters are significantly different between groups ($P < 0.05$), while no significant difference was observed with the same letters.

Table 3: Results of full factorial design.

Number	LGG (CFU/mL)	PA (μg/mL)	Adhesion rate (%)
1	0	0	100.004
2	10^8	0	53.807
3	10^9	0	5.536
4	0	200	62.982
5	10^8	200	29.145
6	10^9	200	4.534
K1	81.493	53.116	
K2	41.476	32.220	
K3	5.035		
R	76.458	20.895	

Table 4: Variance analysis.

Factor	DEVSQ	DOF	*F*-ratio	*P* value
d	32770.213	1	1874.7949	0.000
A	17550.198	2	502.02636	0.000
B	1964.7572	1	112.40442	0.000
A * B	1004.9526	2	28.746839	0.000
Error	209.7523	12		

3.4.2. Effects of LSPC on Zeta Potential of IPEC-J2 Cell. It has been reported that bacterial adhesion to the cell surface is the result of the interaction between bacteria and cell membrane, and the main driving forces include hydrophobic interaction and electrostatic interaction (Elbourne et al. 2019). The binding of a charged ion or molecule to the cell surface induced a change in the Zeta potential of the cell. Therefore, the interaction between the LSPC and the IPEC-J2 cell members was investigated using Zeta potential measurements. As shown in Figure 6(a), the Zeta potential of IPEC-J2 (2×10^5 cells/mL) was -8.79 ± 1.05 mV, while the Zeta potential of 50 μg/mL, 100 μg/mL, and 200 μg/mL LSPC solutions was -13.93 ± 1.32, -12.30 ± 0.85, and -13.93 ± 1.32 mV, respectively. The Zeta potential value of cells containing LSPC was significantly higher than that of blank cells ($P < 0.05$), but there was no marked difference compared with LSPC. In addition, the absolute value of the Zeta potential in cells containing LSPC decreases as the culture time is extended and gradually recovers to the level of the blank cell. After incubating cells with 50, 100, and 200 μg/mL of LSPC for 20 h, the absolute values of Zeta potential changed to 10.03 ± 0.470, 10.67 ± 0.32, and 7.61 ± 0.64 mV, respectively. Interestingly, the Zeta potential of IPEC-J2 cells and LSPC with different concentrations showed no obvious change with the extension of culture time (Figure 6(b)). These phenomena may indicate that the variation of the Zeta potential value after the addition of the LSPC may be caused by the interaction of the charged particles of the LSPC with the cell membrane [32]. As the culture time was extended, the cell potential value slowly recovered to its original level, possibly as a result of the introduction of charged particles into the cell.

3.4.3. Effect of LGG and LSPC on Changes in Cell TEER Values. Transepithelial electrical resistance (TEER) is the interpolation between the transepithelial electrical resistance and the cellular bypass resistance [33]. TEER values can also be used as a marker of cell permeability. The adhesion of pathogenic bacteria to intestinal epithelial cells can lead to a decrease in the TEER value of host cells [34]. The changes in cellular TEER values during the 6 h incubation of LSPC and LGG with IPEC-J2 cells are shown in Figure 6(c), the TEER values of LSPC, LGG, and LSPC + LGG cells increased significantly ($P < 0.05$) after 2 h of incubation, and the effect of LGG alone on cellular TEER was greater than that of the LSPC and LSPC + LGG groups. As the incubation time was extended to 4 h, the increase rate in TEER values in the LSPC and LSPC + LGG groups became faster, with no significant difference from the LGG treated alone ($P > 0.05$). It was shown that LSPC with moderate concentrations of LGG can significantly increase the TEER value of IPEC-J2 and protect the integrity structure of monolayer cells, thus increasing the fixed resistance of cells to pathogenic bacteria.

3.4.4. Effects of LSPC and Probiotics Combination on Anti-Inflammatory Factors. ETEC infection can promote the expression of proinflammatory cytokines [3]. To explore the

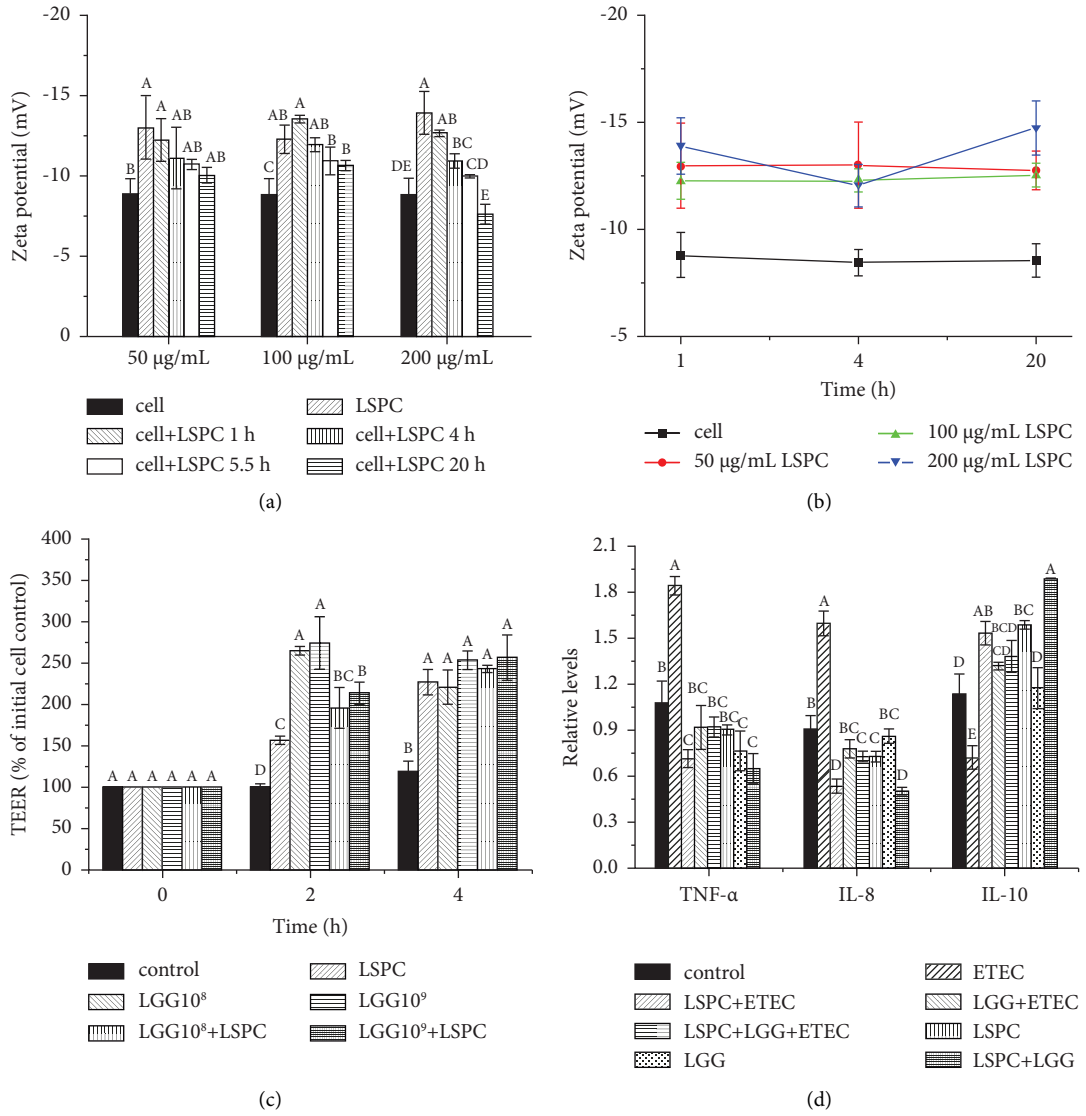

Figure 6: Effects of LSPC on the Zeta potential (a, b), TEER value (% of cell control) (c), and cell immunity (d) of IPEC-J2 cell. Columns with different letters are significantly different between groups ($P < 0.05$), while no significant difference was observed with the same letters.

effect of LGG and LSPC on cellular immunity, the mRNA levels of inflammatory factor (TNF-α), proinflammatory factor (IL-8), and anti-inflammatory factor (IL-10) were analyzed by RT-qPCR method. It can be seen from Figure 6(d) that after ETEC infection, the level of cellular inflammation intensified, as evidenced by the increased mRNA levels of the TNF-α and IL-8 in IPEC-J2 cells, which were 1.84 and 1.60 times higher than those in the control group, and the mRNA levels of IL-10 were obviously downregulated ($P < 0.05$). Interestingly, when ETEC was incubated with LSPC, LGG, and LSPC-LGG, the mRNA levels of TNF-α, IL-8, and IL-10 were almost restored to normal, though the modulating ability of the LGG-LSPC combination was slightly improved or close to the level of LGG or LSPC alone. This implied that both LGG and LSPC can reduce the cellular inflammatory response induced by ETEC and even elevate the level of cellular immunity. When the cells were incubated with LSPC, LGG, and LSPC-LGG for 2 h, the mRNA level of IL-10 was upregulated and the gene levels of TNF-α and IL-8 were downregulated. It is worth mentioning that the combination of LGG and LSPC has the most positive regulation levels of proinflammatory and anti-inflammatory factors. These phenomena implied that LGG and LSPC may possess a synergistic effect in regulating inflammation levels.

3.4.5. Effect of LSPC on the Production of Short-Chain Fatty Acids by Different Probiotic Bacteria. SCFAs in the organism are organic acids with carbon atoms of less than 6 produced by the degradation of carbohydrates by intestinal microorganisms. There was abundant evidence to show that SCFAs, butyric acid (BA) in particular, played an important role in the impairment of diseases which influence the gut barrier, the inflammatory tone, and the metabolic homeostatic control in different tissues [35]. As shown in Table 5,

Table 5: Effect of LSPC on the SCFAs (μmol/mL) produced by different probiotics.

Group	Propanoic acid (PA)	Isobutyric acid (IBA)	Butyric acid (BA)	Isovaleric acid (IVA)	Valeric acid (VA)
Blank	—	—	—	—	—
LGG	27.72 ± 1.93a	15.71 ± 0.84bc	17.78 ± 1.95b	10.18 ± 0.79bc	12.25 ± 1.01d
LGG + LSPC	28.26 ± 1.47a	15.78 ± 1.31bc	28.97 ± 1.99a	11.23 ± 1.26b	13.23 ± 0.24d
LA	28.90 ± 1.48a	16.34 ± 2.59bc	17.64 ± 0.45b	8.97 ± 1.30bc	18.25 ± 1.40c
LA + LSPC	30.95 ± 2.79a	15.49 ± 0.9bc	23.48 ± 3.00ab	10.36 ± 1.06bc	26.45 ± 1.26a
ST	20.12 ± 1.56b	11.19 ± 1.54c	18.41 ± 1.95b	6.33 ± 0.89c	6.98 ± 0.28e
ST + LSPC	27.25 ± 1.19a	17.90 ± 1.60a	28.72 ± 2.07a	13.00 ± 1.41b	17.26 ± 1.23c
LPC	29.44 ± 2.59a	18.21 ± 2.17a	20.22 ± 4.74b	20.21 ± 1.16a	28.34 ± 1.42a
LPC + LSPC	30.92 ± 0.73a	16.87 ± 0.76bc	17.97 ± 0.18b	20.29 ± 2.25a	22.86 ± 1.04b
LP	31.20 ± 1.48a	19.34 ± 1.69a	21.18 ± 1.68b	20.82 ± 1.31a	11.60 ± 0.81d
LP + LSPC	29.21 ± 0.57a	18.54 ± 1.48a	18.37 ± 1.84b	20.46 ± 2.89a	8.18 ± 0.31e

*Columns with different letters are significantly different between groups ($P < 0.05$), while no significant difference was observed with the same letters.

after the addition of LSPC, the total SCFA contents in LGG, LA, and ST significantly increased compared to the LSPC-free group ($P < 0.05$), where VA decreased in LPC and LP and increased by 8.20 μmol/mL in LA. Notably, the BA content in the LGG-LSPC and ST-LSPC groups increased by 11.19 and 10.31 μmol/mL, respectively, compared with that of the probiotic-treated group alone. These results implied that the effects of LSPC on SCFAs' production by probiotic bacteria were related to the probiotic species. Overall, LSPC increased the intestinal short-chain fatty acid content more significantly than the other probiotics used in the study.

4. Discussion

A previous study found that PA addition can enhance the antibacterial activity of probiotics against ETEC, confirming the potential effect of PA and probiotics combination on the prevention and treatment of ETEC diarrhea [22, 24]. The key step in bacterial infection is pathogenic adhesion to host cells by adhesions (except for Enterinvasive *E.coli*), and anti-pathogenic adhesion is an effective way to prevent or treat bacterial infection [26]. Both probiotics and polyphenolic compounds have been shown to effectively inhibit the adhesion of *E. coli* pathogenic bacteria such as ETEC in gut epithelial cells [39]. Polyphenols can selectively promote the growth and adhesion of probiotics, while probiotics (e.g., *Lactobacillus*) can promote the antiadhesive effect of polyphenols on pathogenic bacteria [19, 26]. In this work, the mode of action, duration of treatment, and interaction between LSPC and probiotics on the antiadhesion of ETEC on IPEC-J2 cells were investigated, and the mechanism of their antiadhesive effect was initially explored.

Cell adhesion experiment indicated that LSPC had an effective antiadhesive effect (exclusion mode) on ETEC, and it was proportional to the duration of cell pre-incubation, which corresponded to the effect of LSPC on the Zeta potential of cells. This might be explained by the polyhydroxyl groups of LSPC dissociating to charged molecules or ions, and these charged molecules or ions may cause changes in Zeta potential when they bind to the cell surface through electrostatic or hydrophobic interactions [40]. In addition to acting on the host cell, preincubation of LSPC with ETEC significantly reduced the rate of ETEC adhesion to the cell, suggesting that LSPC, like other polyphenols, may reduce the amount of adhesion of pathogenic bacteria by binding to adhesins or toxins on the surface of the bacterial membrane. For probiotics, the exclusion and competition action modes are considered to be more effective in inhibiting the colonization of pathogenic bacteria; however, this is influenced by probiotic species [27]. Among the tested probiotics, only LGG could effectively inhibit the adhesion to ETEC on cells in both competitive and repulsive ways. It suggests that LGG may inhibit the adhesion of pathogenic bacteria by competing with ETEC for nutrients, cell membrane surface receptor binding sites, and elevating the immune response.

The antiadhesive effect of probiotics promoting polyphenolic compounds against pathogenic bacteria has been widely demonstrated. The boost is mutual: probiotics can increase the bioavailability of polyphenolic compounds such as proanthocyanidins, which, in turn, can regulate the gut flora by promoting probiotic growth and inhibiting harmful bacteria [26, 38]. The interaction between LSPC and LGG can effectively enhance the immune defense of host cells: LSPC and LGG, alone or in combination, can inhibit the expression levels of proinflammatory factors (TNF-α and IL-8) and increase the expression levels of anti-inflammatory factors (Il-10), with the best effect of LSPC and LGG in combination. On the other hand, the invasion by pathogenic bacteria in the host cell can disrupt the structural integrity of the cell, causing changes in cellular transmembrane resistance and tight-binding proteins. TEER is a functional parameter of epithelial tightness, and a healthy barrier function possesses a high TEER, and ETEC invasion will decrease intestinal epithelial cell integrity and TEER values [16, 39]. In this work, an increase in TEER values was observed in cells with either LGG or LSPC alone or in combination, suggesting that LGG and LSPC may be able to resist cell-damaging ETEC colonization by modulating the membrane barrier function of the intestinal epithelium.

SCFAs are degradation products of undigested carbohydrates by intestinal microorganisms, which could reshape the gut ecology, induce immunomodulatory and antibiotic activity, and mediate inflammatory signaling cascade during

gut inflammation [40]. Banerjee et al. found that probiotics seem to improve other characteristics such as SCFAs production [11, 12]. The increased secretion of SCFAs in the intestine acidifies the colonic environment, promotes the absorption of minerals, and combats the invasion of pathogenic bacteria [35, 41]. Of all the SCFAs generated, the butyric acid is the main source of energy supply for colon cells and has a strong protective effect against colonic diseases [42]. This was also confirmed in our results, where LSPC significantly increased the total amount of SCFAs and butyric acid produced by LGG and ST and decreased the rate of ETEC adhesion compared to other probiotics, and the antiadhesive effect was proportional to the duration of action. In addition, SCFAs also attenuated the host inflammatory response such as the reduction of IL-1β, IL-6, and TNF-α [43]. The abovementioned results may imply that the inhibition of ETEC adhesion by LSPC with specific probiotics (e.g., LGG) may also act by increasing the SCFA content, especially butyric acid.

5. Conclusions

ETEC adhesion to gut epithelial cells is a prerequisite for diarrhea. This study investigated the antiadhesive effect and mechanism of LSPC in combination with probiotics on ETEC. Results indicated that the antiadhesive effect of LSPC on ETEC was as expected. Coincubation of ST with ETEC showed an obvious antiadhesion effect on ETEC, and LGG also can reduce ETEC's adhesion, with the adhesion rate of ETEC slightly higher in exclusion ($81.43 \pm 6.52\%$) than the mode in competition ($75.60 \pm 6.52\%$). It was worth noting that when LGG or ST was combined with LSPC, a stronger antiadhesion effect on ETEC was observed. In addition, LGG-LSPC recovered cellular transmembrane resistance and enhanced the expression of anti-inflammatory factor (IL-10, 1.89-fold), as well as reduced the cellular inflammatory factor levels (TNF-α and IL-8) induced by ETEC infection. The gas chromatography analysis confirmed that LSPC, as a prebiotic, increased the total amount of short-chain fatty acids (especially, butyric acid) produced by probiotics. In conclusion, the combination of LGG and LSPC may inhibit the adhesion and colonization to host cells by enhancing cellular immune defense, cellular barrier function, and maintenance of intestinal health, which may serve as a reference for later *in vivo* studies [44–47].

Data Availability

The data used to support the findings of the study are available from the corresponding author upon request.

Disclosure

A preprint has previously been published by Tang and Li et al.

Conflicts of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Authors' Contributions

Tang Cuie conceptualized and visualized the study, proposed the methodology, validated and investigated the study, performed the formal analysis, and wrote the original draft. Li Donghui curated the data, validated and investigated the study, and performed the formal analysis. Xie Bijun, Li Yan, and Sun Zhida supervised the study and reviewed and edited the manuscript. Cuie Tang and Donghui Li are the co-first authors.

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Supplementary Materials

The graphical abstract is shown in the supplementary material. (*Supplementary Materials*)

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