Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk

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ABSTRACT

Lactobacillus plantarum WLPL04, a specific strain isolated from human breast milk, was investigated for its survival capacity (acid and bile salt tolerance, survival in simulated gastrointestinal tract, inhibition of pathogens, antibiotic susceptibility, yield of exopolysaccharides) and probiotic properties (antiadhesion of pathogens, protection from harmful effect of sodium dodecyl sulfate, and antiinflammatory stress on Caco-2 cells). The results showed that *Lb. plantarum* WLPL04 had broad-spectrum activity against gram-positive strains (*Listeria monocytogenes* CMCC54007, *Bacillus cereus* ATCC14579, and *Staphylococcus aureus* CMCC26003) and gram-negative strains (*Pseudomonas aeruginosa* MCC10104, *Shigella sonnei* ATCC25931, *Enterobacter sakazakii* ATCC29544, *Salmonella typhimurium* ATCC13311, and *Escherichia coli* O157:H7). Antibiotic susceptibility tests showed that *Lb. plantarum* WLPL04 was susceptible to 8 of 14 antibiotics (e.g., erythromycin and nitrofurantoin) and resistant to 6 of 14 antibiotics (e.g., kanamycin and bacitracin). *Lactobacillus plantarum* WLPL04 was able to survive at pH 2.5 for 3 h and at 0.45% bile salt for 12 h, suggesting that it can survive well in the gastrointestinal tract. In addition, the exopolysaccharide yield of *Lb. plantarum* WLPL04 reached 426.73 ± 65.56 mg/L at 24 h. With strategies of competition, inhibition, and displacement, *Lb. plantarum* WLPL04 reduced the adhesion of *E. coli* O157:H7 (35.51%), *Sal. typhimurium* ATCC 13311 (8.10%), and *Staph. aureus* CMCC 26003 (40.30%) on Caco-2 cells by competition, and subsequently by 59.80, 62.50, and 42.60%, respectively, for the 3 pathogens through inhibition, and by 75.23, 39.97, and 52.88%, respectively, through displacement. *Lactobacillus plantarum* WLPL04 attenuated the acute stress induced by

sodium dodecyl sulfate on Caco-2 cells and significantly inhibited the expression of inflammatory cytokines (IL-6, IL-8 and tumor necrosis factor- α) on Caco-2 cells but increased IL-10 expression in vitro compared with the *Salmonella*-treated group. In summary, *Lb. plantarum* WLPL04 from breast milk could be considered as a probiotic candidate for dairy products to promote human health.

Key words: *Lactobacillus plantarum* WLPL04, stress resistance, antiadhesion, antiinflammatory

INTRODUCTION

Numerous reports have demonstrated that probiotics from humans or fermented food can improve host health by maintaining the balance of flora in the gastrointestinal tract (GIT), preventing pathogen invasion, and activating the immune system (Parvez et al., 2006; Jankovic et al., 2010). Recently, *Lactobacillus plantarum* has been the subject of much scientific work due to its beneficial effects on the host, broad distribution in nature (e.g., pickles, sausage, and sourdough), and high commercial value for dairy products (da Silva Sabo et al., 2014; Kwak et al., 2014). Generally, most isolates of *Lactobacillus plantarum* from fermented food were evaluated for resistance to acidic environments and to bile salt juice, antagonism against pathogens, and adhesion on epithelial cells in vitro. Other works have focused on its immune-regulatory property (Zago et al., 2011), anticolic effect (Lee et al., 2015), and potential beneficial adverse effect on inflammatory bowel disease (Mileti et al., 2009) in vitro.

Fermented vegetables (e.g., kimchi and pickles) containing *Lactobacillus plantarum* are routinely consumed by many people in China, Korea, and other Asian countries. It is hypothesized that this microorganism might exist in the mammary gland and breast milk of pregnant women who continuously consume foods fermented by *Lactobacillus plantarum* (Maldonado et al., 2010; Al-besharat et al., 2011). In fact, human breast milk, as the first source of nutrition for the neonate, not only

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provides protection against different infectious diseases (Lara-Villoslada et al., 2007), but is also a reservoir for probiotics, such as bifidobacteria and lactic acid bacteria (LAB; Martín et al., 2003; Solís et al., 2010). A few reports have shown that LAB isolated from human milk are able to modulate both natural and acquired immune responses in mice and humans (Díaz-Ropero et al., 2007; Olivares et al., 2007), possess potent antibacterial activities to reduce the incidence and severity of infections (Olivares et al., 2006), exert efficacy to treat infectious mastitis (Arroyo et al., 2010), and play important roles in the infant (Maldonado et al., 2010).

In our previous work (Li et al., 2014b), we demonstrated that metabolites of *Lactobacillus plantarum* R315 exert antioxidant and antibacterial activities, and that *Lactobacillus plantarum* ZDY2013 exhibits high stress resistance and possesses antibacterial properties. Moreover, we isolated several strains from breast milk and partially identified them as LAB by 16S rDNA sequencing. Based on a protocol of in vitro antagonism tests against pathogens and challenging microorganisms in simulated GIT, to further determine whether *Lb. plantarum* WLPL04 inhibits pathogen adhesion on epithelium cells or affects inflammatory bowel disease by foodborne pathogenic bacteria, we used competition, inhibition, and displacement assays to ascertain the ideal strategy for *Lb. plantarum* WLPL04 protection, and measured cytokines at the transcriptional level to understand the antiinflammatory effect of *Lb. plantarum* WLPL04 on Caco-2 cells.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Human breast milk samples were collected in sterile tubes by manual expression using sterile gloves after cleaning nipples and surrounding skin with sterile water and discarding the first drops. Samples were properly diluted and plated on de Man, Rogosa, and Sharpe (MRS) agar (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) plates containing 3% CaCO₃ (wt/vol). All plates were anaerobically incubated at 37°C for 48 h. The isolates yielding a dissolving circle of CaCO₃ were evaluated by Gram staining and microscopic observation to identify potential strains of LAB. Those strains were further screened by determination of the halo against *Escherichia coli* O157:H7 and *Listeria monocytogenes*. The selected isolates were then amplified by PCR with 16S rDNA universal primers 27F and 1492R (shown as in Table 1). The amplicons were sequenced by Sangon Biotech Ltd. (Shanghai, China) and compared with sequences in the National

Center for Biotechnology Information (NCBI) database by using the BLAST algorithm to determine their classification (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Antimicrobial Activity

The antimicrobial activity of isolates against 8 foodborne pathogens was investigated by agar diffusion assay (Chen et al., 2014). *Listeria monocytogenes* CMCC54007, *Pseudomonas aeruginosa* MCC10104, *Bacillus cereus* ATCC14579, *Shigella sonnei* ATCC25931, *Enterobacter sakazakii* ATCC29544, *Salmonella typhimurium* ATCC13311, *Staphylococcus aureus* CMCC 26003, and *E. coli* O157:H7 were used as indicator strains to evaluate the antimicrobial spectrum of isolates. Cell-free supernatant of *Lactobacillus* strains was harvested by centrifugation (7,500 × *g*, 10 min, 4°C) and filtered through a 0.22-μm Millipore filter (Merck Millipore Ltd., Tullagreen, Ireland). Indicator strains were cultured overnight to approximately 1 × 10⁸ cfu/mL, and 100 μL of each strain was spread on Luria-Bertani agar plates. Then, 200 μL of the lactobacilli cell-free supernatant was added to an Oxford cup (a stainless cylinder of outer diameter 7.8 ± 0.1 mm, inner diameter 6.0 ± 0.1 mm, and height 10.0 ± 0.1 mm), which was placed on the agar. After incubation for 12 h, the diameter of the inhibition zone around the cup was measured. Three independent replicates were conducted for each experiment.

Tolerance to Acid and Bile Salt

Tolerance to acid and bile was assayed as reported previously (Huang et al., 2015) with minor modifications. To determine acid tolerance, overnight cultures of *Lb. plantarum* WLPL04 were inoculated in MRS broths (1%, vol/vol), which were adjusted to pH 4.5, 3.5, and 2.5, with HCl (1.0 M). Cultures inoculated in nonacidi-

Table 1. Primers used in this study

Primer ¹	Oligonucleotide sequence (5'–3')
27F	AGAGTTTGATCCTGGCTCAG
1492R	TACGGCTACCTTGTTACGACTT
IL-6-F	AGCAAAGCAAAGAAACCGAT
IL-6-R	CAGCTCTGAGATGGCTTCAG
IL-8-F	AGGACAAGAGCCAGGAAGAA
IL-8-R	CAGAGCTGCAGAAATCAGGA
IL-10-F	AGGAGGATGAGTGATTGTC
IL-10-R	AACTGGGAGGAACACTGACC
TNF-α-F	TTTGATCCCTGACATCTGGA
TNF-α-R	GGCCTAAGGTCCACTTGTGT
β-2-microglobulin-F	GGCTATCCAGCGTACTCCAAA
β-2-microglobulin-R	CGGCAGGCATACTCATCTTTTT

¹F= forward; R = reverse.

fied MRS (pH 6.5) served as controls. Tolerance to bile salt was tested by inoculating the cultures into MRS broth (1%, vol/vol) supplemented with 0, 0.15, 0.30, and 0.45% (wt/vol) of oxgall (YuanYe Bio-Technology Ltd., Shanghai, China). Cultures were then incubated at 37°C for 0, 3, 6, 12, and 24 h. The acid and bile salt tolerance of the cells were assessed by enumerating viable cells on MRS agar plates. The experiments were performed in triplicate.

Resistance to GIT

The survival of *Lb. plantarum* WLPL04 during passage through a chemically simulated GIT was determined as reported by Nikolic et al. (2012), with some modifications. Briefly, cells of *Lb. plantarum* WLPL04 from 24-h MRS cultures were harvested by centrifugation ($7,500 \times g$, 10 min, 4°C), washed twice with 0.85% NaCl, resuspended in gastric juice (**GJ**; 125 mM NaCl, 7 mM KCl, 45 mM NaHCO₃, 0.30% pepsin adjusted to pH 2 with HCl) containing 10% (wt/vol) skim milk, and incubated for 90 min at 37°C in aerobic conditions. Afterward, bacterial suspensions were centrifuged ($7,500 \times g$, 10 min), resuspended in duodenal juice (**DJ**; 1% bovine bile adjusted with 10 M NaOH to pH 8.0), and incubated for 10 min at 37°C in an anaerobic chamber. Then, the cells were centrifuged again, resuspended in intestinal juice (**IJ**; 0.30% bovine bile, 0.1% pancreas acetone powder porcine Type I (Sigma, St. Louis, MO), pH 8.0], and incubated for 120 min at 37°C in anaerobic conditions. Two samples were collected during the IJ challenge, after 60 and 120 min. Viable cell counts were obtained from the initial cultures and after each of the GJ, DJ, and IJ challenges. Cells were serially diluted in 0.01 M PBS and plated on MRS agar. Plates were incubated for 48 h at 37°C, and results were expressed

as log colony-forming units per milliliter. Experiments were carried out in triplicate.

Production of Exopolysaccharides

The exopolysaccharides (**EPS**) from cell-free culture supernatant were extracted and detected by methods previously reported (Li et al., 2014a). Briefly, *Lb. plantarum* WLPL04 was inoculated (1%) in 100 mL of MRS broth and cultured anaerobically at 37°C. Bacterial cells were removed by centrifugation at $4,000 \times g$ for 10 min. The EPS in the cell-free culture supernatant was precipitated with ethanol (80% vol/vol) overnight and then recovered by centrifugation at $8,000 \times g$ for 20 min, at 4°C. The precipitate was collected and dissolved in distilled water. Proteins were removed with Sevag solution (chloroform:*n*-butanol = 4:1) followed by centrifugation ($8,000 \times g$, 10 min). Then, the collected EPS was dialyzed against distilled water for 24 h at 4°C. The concentration of EPS was determined by the phenol-sulfuric acid assay method (Dubois et al., 1956) using glucose as standard.

Antibiotic Susceptibility Assay

The resistance of *Lb. plantarum* WLPL04 to antibiotics was determined by the disc diffusion method (Tan et al., 2013). In brief, *Lb. plantarum* WLPL04 was grown in MRS broth overnight at 37°C in anaerobic conditions, and 100 µL of each culture (10^7 cfu/mL) was swabbed on MRS agar plates. Antibiotic discs (Bio-Kont Co. Ltd., Wenzhou, China) were placed on the surface of inoculated plates. The concentrations of the tested antibiotics are as in Table 2. The plates were incubated anaerobically at 37°C for 24 h. The inhibition zone after incubation was measured and interpreted as

Table 2. The antibiotic susceptibility of *Lactobacillus plantarum* WLPL041 (means $\pm$ SD of 3 trials)

Class	Antibiotic	Content	Diameter of inhibition zone (mm)	Sensitivity ¹
Macrolide	Erythromycin	15 µg	23.84 $\pm$ 0.19	S
	Rifampicin	5 µg	0.00 $\pm$ 0.05	R
Aminoglycoside	Streptomycin	10 µg	3.84 $\pm$ 0.34	I
	Gentamicin	10 µg	4.07 $\pm$ 0.14	I
	Kanamycin	30 µg	0.00 $\pm$ 0.05	R
	Nitrofurantoin	300 µg	24.47 $\pm$ 0.93	S
Tetracyclines	Tetracycline	30 µg	18.04 $\pm$ 0.09	I
Amphenicols	Chloramphenicol	30 µg	23.87 $\pm$ 0.24	S
β -Lactams	Amoxicillin	10 µg	23.14 $\pm$ 0.12	S
	Penicillin	10 U	23.34 $\pm$ 0.17	S
Glycopeptide	Bacitracin	0.04 U	0.00 $\pm$ 0.05	R
	Polymyxin B	30 µg	0.00 $\pm$ 0.05	R
Quinolones	Ciprofloxacin	5 µg	0.00 $\pm$ 0.05	R
	Nalidixic acid	30 µg	0.00 $\pm$ 0.05	R

¹S = susceptible; I = intermediate; R = resistance.

susceptible, intermediate, or resistant according to the instructions for the antibiotic discs (Bio-Kont Co. Ltd.)

Adhesion Assay

Cell Culture. The human intestinal epithelial cell line Caco-2 (purchased from the cell bank of Chinese Academy of Sciences, Shanghai, China) was used to assess the adhesion ability of selected strains. The Caco-2 cells were in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (ExCell Bio Co. Ltd., Shanghai, China) and a standard mixture of antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin; Beijing Solarbio Science and Technology Co. Ltd.) in an atmosphere of 5% (vol/vol) CO₂ at 37°C. The Caco-2 cells were maintained in 75-cm² cell culture flasks and differentiated into enterocytes after 15 to 20 d; the medium was replaced every 2 d.

Adhesion. Adhesion assays were carried out in a 35-mm-diameter 6-well plate. A Caco-2 cell monolayer was washed twice with PBS (pH 7.4) to remove antibiotics before use. Then, 0.1 mL (about 10⁷ cfu/mL) of *Lb. plantarum* WLPL04 or pathogen suspension was added at a Caco-2 cell:bacteria ratio of 1:10. After 90 min of incubation at 37°C under 5% CO₂ atmosphere, wells were gently washed 3 times with PBS solution to remove unbound bacteria. Caco-2 cells and adhered bacteria were then detached with an EDTA-trypsin solution (Sigma-Aldrich), and the attached bacteria were counted by plating in MRS agar. Adhesion was expressed as the percentage of bacteria adhered with respect to total number of bacteria added. Assays were performed in triplicate.

Effects on Adhesion of Pathogens to Caco-2 Cells. The ability of *Lb. plantarum* WLPL04 to inhibit the adhesion of pathogens (*Sal. typhimurium* ATCC13311, *Staph. aureus* CMCC26003, and *E. coli* O157:H7) to Caco-2 cells was investigated according to the method reported by Dhanani et al. (2011). Briefly, 3 different types of experiments were performed: (1) competitive assay; that is, simultaneously incubating Caco-2 cells with *Lb. plantarum* WLPL04 and each of the pathogens for 90 min; (2) inhibition assay; that is, pre-incubating Caco-2 cells with the *Lb. plantarum* WLPL04 for 90 min, and then adding each of the pathogens and incubating for another 90 min; (3) displacement assay; that is, pre-incubating Caco-2 cells with each of the pathogens for 90 min, and then adding *Lb. plantarum* WLPL04 and further incubating for 90 min. Competitiveness was calculated as the percentage of adhesion of pathogens added in combination with *Lb. plantarum* WLPL04 relative to pathogen-bound bacteria in the absence of *Lb. plantarum* WLPL04 (con-

trol). The inhibition of the adhesion of pathogens was expressed as a percentage using the following formula: Inhibition of adhesion = 100 × (1 - T1/T2), where T1 and T2 are the percentage of adhesion by pathogen cells in the presence and absence of *Lb. plantarum* WLPL04, respectively. Displacement of pathogens was expressed as the percentage of adhesion by pathogen cells in the presence and absence of *Lb. plantarum* WLPL04 strain, as described above.

Protection Against Inflammatory Stress

Inflammatory stress was induced by treating the Caco-2 cells with SDS, which was added into DMEM at a final concentration of 0.05% (wt/vol). *Lactobacillus plantarum* WLPL04 (10⁷ cfu/mL) was resuspended in DMEM and added into each well of a 96-well plate. The residual cell viability at 24 h was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Presti et al., 2015). Cells were washed with sterile PBS, and then 0.05% (wt/vol) MTT in DMEM was added into each well and incubated for 4 h. After incubation, the medium was removed and replaced with dimethyl sulfoxide, and shaken for 10 min. The formazan produced was measured as absorbance at 490 nm (A₄₉₀) by using a microplate reader. The results were calculated as (A₄₉₀ of treated cells - A₄₉₀ untreated cells)/A₄₉₀ untreated × 100.

Quantification of Cytokine in Caco-2 Cultures

The levels of IL-6, IL-8, and tumor necrosis factor α (TNF-α) as pro-inflammatory cytokines and IL-10 as anti-inflammatory cytokine in Caco-2 cells were determined as follows: 1 × 10⁶ cells were treated with 1 mL of DMEM containing *Sal. typhimurium* ATCC 13311 (10⁷ cfu/mL) alone or with *Lb. plantarum* WLPL04 (10⁷ cfu/mL) for 4 h. Untreated Caco-2 cells were used as control. At the end of incubation, the medium was removed and replaced with fresh DMEM containing antibiotic, and cells were incubated for a further 20 h (Finamore et al., 2014). The transcription level of cytokines in Caco-2 cells was evaluated by quantitative PCR. Total RNA was isolated from cells using TRIzol Reagent (Trans, Beijing, China). Single-strand cDNA was synthesized from the total RNA using the PrimeScript RT reagent kit with gDNA Eraser for quantitative PCR (Takara, Shiga, Japan) according to the manufacturer's instructions. The PCR was conducted with a SYBR Premix Ex Taq II (TliRNaseH Plus; Takara) and ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA). The cycling profile used for quantitative PCR was as follows: a preheating step for enzyme activation at 95°C for 1 min, followed

by 40 cycles of 95°C for 15 s, 58°C for 60 s, and 72°C for 15 s. The relative transcription level was calculated using the $2^{-\Delta\Delta C_t}$ method (Feng et al., 2010). The β -microglobulin gene was used as a reference gene. Oligonucleotides specific to IL-6, IL-8, IL-10, and TNF- α (shown in Table 1) were used for quantitative PCR.

Statistical Analysis

Triplicate data were expressed as mean $\pm$ standard deviation. The SPSS 13.0 software (SPSS Inc., Chicago, IL) was used to perform statistical analyses by means of independent one-way ANOVA tests. The multiple comparison of each test was assessed by least significant difference (LSD) test ($P < 0.05$).

RESULTS

Isolation, Identification of *Lb. plantarum* WLPL04, and Antimicrobial Activity and Susceptibility

Eighteen isolates from human breast milk were identified as potential lactobacilli by organic acid yield testing in modified MRS plates containing CaCO_3 and microscopic observation. Those isolates were subjected to preliminary evaluation for their antimicrobial activity by using *E. coli* O157:H7 and *Listeria monocytogenes* as model strains of pathogens. Subsequently, one strain, named *Lb. plantarum* WLPL04, was screened out for its best antagonistic properties against *Listeria monocytogenes* CMCC54007 and *E. coli* O157:H7. The antimicrobial spectrum of *Lb. plantarum* WLPL04 was investigated by using 8 pathogens as targets. As shown in Figure 1, *Lb. plantarum* WLPL04 demonstrated its broad activity against all tested gram-positive (*L. monocytogenes* CMCC54007, *B. cereus* ATCC14579, and *Staph. aureus* CMCC26003) and gram-negative (*P. aeruginosa* MCCC10104, *Shigella sonnei* ATCC25931, *Ent. sakazakii* ATCC29544, *Sal. typhimurium* ATCC13311, and *E. coli* O157:H7) strains, with halos ranging from 12.6 ± 0.46 mm on *B. cereus* ATCC14579 to 33.07 ± 0.15 mm on *P. aeruginosa* MCCC10104.

Antibiotic susceptibility was an important index for the safety of LAB before application. As shown in Table 2, *Lb. plantarum* WLPL04 was susceptible to macrolides (e.g., erythromycin), nitrofurans (e.g., nitrofurantoin), amphenicols (e.g., chloramphenicol), and β -lactams (e.g., amoxicillin and penicillin); and showed intermediate sensitivity to tetracyclines and aminoglycoside (e.g., streptomycin and gentamicin). In contrast, *Lb. plantarum* WLPL04 was resistant to rifampicin, kanamycin, glycopeptide (e.g., bacitracin, polymyxin B), and quinolones (e.g., ciprofloxacin, nalidixic acid).

Figure 1. Antibacterial activity of supernatants of overnight culture of *Lactobacillus plantarum* WLPL04 against 8 foodborne pathogens. Data of triplicate trials are expressed as mean $\pm$ SD.

Growth and EPS Production of *Lb. plantarum* WLPL04

In our previous work (Li et al., 2014b), we determined that inhibition of pathogens might result not only from low pH but also from primary metabolite EPS. Thus, EPS yields, viable cell counts, and pH were chosen as parameters to monitor in the current study. As shown in Figure 2, *Lb. plantarum* WLPL04 reached stationary phase 12 h after inoculation; the cell count increased by 2.17 log cfu/mL from the initial inoculate of 6.53 log cfu/mL, and then maintained a stable biomass and low pH to 3.75 at 36 h. The EPS yield of *Lb. plantarum* WLPL04 peaked at 426.73 ± 65.56 mg/L at 24 h.

Figure 2. Kinetics of growth, pH value of fermentation culture, and exopolysaccharide (EPS) production by *Lactobacillus plantarum* WLPL04 in de Man, Rogosa, and Sharpe medium. Shown are the amounts of EPS produced (bar graph), bacterial cell counts ($\blacktriangle$), and pH of fermentation culture ($\bullet$). Data of triplicate trials are expressed as mean $\pm$ SD.

Acid and Bile Salt Tolerance and Survival in Simulated GIT

To evaluate the antistress ability of *Lb. plantarum* WLPL04, we determined the survival of *Lb. plantarum* WLPL04 under different acidic pH and bile salt conditions. As shown in Figure 3A, *Lb. plantarum* WLPL04 maintained stable growth at pH 4.5 and a static state at pH 3.5 from 0 to 24 h, but cell count by 2.34 log cfu/mL after 6 h at pH 2.5. At 6 h, *Lb. plantarum* WLPL04 was sensitive to pH from 4.5 to 2.5, with a decreased survival rate of cells from 7.69 to 4.22 log cfu/mL (Figure 3A inset).

The effect of bile salt on viable counts of *Lb. plantarum* WLPL04 is shown in Figure 3B; cell counts decreased slightly with 0.15% bile salt, and reduced by 0.83 and 1.17 log cfu/mL with 0.30 and 0.45% bile salts, respectively, for 6 h. A significant reduction ($P < 0.05$) in cell count occurred at 24 h in both the 0.30 and 0.45% bile salt treatments.

To further evaluate the survival capability of *Lb. plantarum* WLPL04, a simulated gastric-duodenal-intestinal transit system was set up. As shown in Figure 4, the count of *Lb. plantarum* WLPL04 decreased by 0.40 and 1.07 log cfu/mL, from initial values of 6.09×10^8 cfu/mL to 2.42×10^8 cfu/mL and 5.18×10^7 cfu/mL, respectively, after the GJ and DJ (1% bile salt) challenges. After exposure to IJ, which consisted of 0.30% bile and pancreatic enzymes, bacterial viability increased up to 1.30×10^8 cfu/mL.

Adhesion and Antiadhesion Against Pathogens

Adhesion and antiadhesion of pathogenic microorganisms are the most important biological properties for probiotic selection. Using the Caco-2 cell line as a model, we approached the antagonistic effect of adhesion of *Lb. plantarum* WLPL04 against *E. coli* O157:H7, *Sal. typhimurium* ATCC13311, and *Staph. aureus* CMCC26003 with 3 strategies—competition, inhibition, and displacement. As shown in Figure 5, under competition, the adhesion ratio of *E. coli* O157:H7, *Sal. typhimurium* ATCC13311, and *Staph. aureus* CMCC26003 was reduced by 35.51, 8.10, and 40.30%, respectively. Under inhibition, that ratio was reduced by 59.81, 62.50, and 42.60%, respectively, and under displacement, by 75.23, 39.97, and 52.88%, respectively.

Protection from Harmful Effects of SDS

Probiotics usually protect against oxidative stress. To investigate that potential in *Lb. plantarum* WLPL04, we induced an acute stress state of Caco-2 cells by SDS (a detergent) as a model for the protection of this

Figure 3. The survival capacity of *Lactobacillus plantarum* WLPL04 challenged for resistance to acid (A) and bile salt (B). Data of triplicate trials are expressed as mean $\pm$ SD. * $P < 0.01$; all conditions are compared with respective controls.

Figure 4. Survival of *Lactobacillus plantarum* WLPL04 (10% skim milk) through simulated gastrointestinal tract (GIT). Viable counts were obtained at each sampling point. GJ = gastric juice, DJ = duodenal juice, IJ = intestinal juice. Data of triplicate trials are expressed as mean $\pm$ SD. All data were compared with initial value; * $P < 0.05$.

Figure 5. Antiadhesion assays (competition, inhibition, and displacement) of *Lactobacillus plantarum* WLPL04 against *Escherichia coli* O157:H7, *Salmonella typhimurium* ATCC13311, and *Staphylococcus aureus* CMCC26003. Data of triplicate trials are expressed as mean $\pm$ SD.

Figure 6. Protective effect of *Lactobacillus plantarum* WLPL04 on Caco-2 cells in the presence of SDS as irritant agent after 24 h; nontreated cells were used as negative control. Residual cell viability was measured by MTT assay and expressed as percentage (%). Data of triplicate trials are expressed as mean $\pm$ SD. All the data were compared with nontreated group; ** $P < 0.01$.

strain. As shown in Figure 6, *Lb. plantarum* WLPL04 significantly improved the viability of Caco-2 cells ($P < 0.01$), by 8.79-fold, compared with the SDS-treated group, and thus prevented the adverse effect (oxidation damage on cells) of SDS.

Protection from Salmonella-Induced Inflammation

We investigated the antiinflammatory effect of *Lb. plantarum* WLPL04 on Caco-2 cells by measuring the expression level of mRNA of 4 cytokines, using *Salmonella typhimurium* ATCC13311 as stimulator. As shown in Figure 7, under stimulation of *Salmonella typhimurium* ATCC13311, mRNA expression of IL-6, IL-8, IL-10, and TNF- α showed a tendency to increase. When compared with the *S. typhimurium* ATCC13311-treated group, *Lb. plantarum* WLPL04 alone decreased the proinflammatory cytokines (IL-6, IL-8, and TNF- α) and increased the antiinflammatory cytokine IL-10 ($P < 0.01$). In contrast, a mixture of *Lb. plantarum* and *S. typhimurium* had the same potential to decrease the expression of all proinflammatory cytokines mentioned above but had no effect on IL-10.

DISCUSSION

Recently, *Lb. plantarum* has attracted increasing scientific work because of its wide range of health-

Figure 7. Effect of *Lactobacillus plantarum* WLPL04 on cytokine expression in Caco-2 cells: (A) IL-6, (B) IL-8, (C) IL-10, and (D) tumor necrosis factor- α (TNF- α). Nontreated cells were used as control. Groups of *Lb. plantarum* WLPL04 (WLPL04) and a mixture of *Lb. plantarum* WLPL04 and *Salmonella typhimurium* ATCC13311 (WLPL04 + S) were compared with *S. typhimurium* ATCC13311 (S). ** $P < 0.01$, # $P < 0.01$. Data of triplicate trials are expressed as mean $\pm$ SD.

promoting effects. In our previous work, *Lb. plantarum* isolated from homemade acid beans demonstrated high stress resistance at either the physiological or molecular level and possessed antibacterial and gut microbiota modulation properties (Huang et al., 2015). In the current study, *Lb. plantarum* WLPL04 was systematically evaluated for its in vitro resistance against extreme acid and bile salts, digestion of enzyme, antagonistic effect against the adhesion of 3 pathogens (*E. coli* O157:H7, *Sal. typhimurium* ATCC13311, and *Staph. aureus* CMCC26003), and antiinflammatory effect on *Sal. typhimurium*-induced cytokine expression in a Caco-2 cell line. Our results indicated that *Lb. plantarum* WLPL04 can pass through the gastric environment, survive well in the intestinal tract, and protect epithelial cells from infection by pathogenic microorganisms. In part, our findings are consistent with reports that *Lactobacillus amylovorus* inhibits the inflammation of intestinal Caco-2 cells triggered by enterotoxigenic pathogens (Finamore et al., 2014), and that *Lactobacillus paracasei* modulates *Salmonella*-induced inflammation of Caco-2 cells (Bermudez-Brito et al., 2015).

In the in vitro inhibition tests, we demonstrated that *Lb. plantarum* WLPL04 achieved ideal antagonistic effect against 8 common pathogens (i.e., *P. aeruginosa* MCC10104, *B. cereus* ATCC14579, *Sh. sonnei* ATCC25931, *Ent. sakazakii* ATCC29544, *Sal. typhimurium* ATCC13311, *Staph. aureus* CMCC 26003, and *E. coli* O157:H7, and *L. monocytogenes* CMCC54007), with maximum halo (0.33 cm) on *P. aeruginosa* MCC10104 and minimum halo (0.13 cm) on *B. cereus* ATCC14579. In a similar study, *Lb. plantarum* was found to inhibit *Sal. typhimurium*, *Staph. aureus*, *E. coli* O157:H7, and *L. monocytogenes*, with a maximum halo of 0.21 cm on *L. monocytogenes* and minimum halo of 0.08 cm on *Staph. aureus* (Li et al., 2015). Considering that antagonistic effects of *Lb. plantarum* WLPL04 might be different in vitro and in vivo, we assayed competition, inhibition, and displacement to ascertain the antiadhesion properties of *Lb. plantarum* WLPL04 against *E. coli* O157:H7, *Sal. typhimurium* ATCC13311, and *Staph. aureus* CMCC26003. We found that adhesion of all pathogens on Caco-2 could be inhibited by *Lb. plantarum* WLPL04. Both *E. coli* O157:H7 and *Staph. aureus* CMCC26003 showed the same inhibition style; namely, the ideal inhibition strategy was displacement by *Lb. plantarum* WLPL04, whereas for *Sal. typhimurium* ATCC13311, the ideal inhibition strategy was preincubation of *Lb. plantarum* WLPL04 before adding that pathogen. These results suggested that probiotics might present different antagonistic mechanisms against pathogens. Similar to

our work, *Lb. plantarum* CLC17 was shown to reduce the attachment of *E. coli* on Caco-2 cell line by competition, inhibition, and displacement, but displacement was the ideal strategy (García-Ruiz et al., 2014). Dhanani et al. (2011) reported that *Lb. plantarum* CS24.2 achieved reduction of adhesion of *E. coli* on Caco-2 cells by displacement (53%), competition (41%), and inhibition (2.5%), which is similar to our findings for *E. coli*. In contrast, *Lb. plantarum* CS24.2 resulted in a reduction of adhesion ratio for *Sal. typhimurium* on Caco-2 cells in the order displacement (87%), competition (56%), and inhibition (17.4%), which is different from our finding for *Sal. typhimurium*. One explanation for this discrepancy might be that strains of *Lb. plantarum* from different resources have different specificities against different pathogens (Dhanani et al., 2011; García-Ruiz et al., 2014). It was proposed that competition between lactobacilli and pathogens was due to steric hindrance and the relative position of the hydrophobic surface and adhesion receptors (Lee and Puong, 2002). Currently, several reports note that the antagonistic effect of probiotics against pathogens might be relevant to adhesion factors (e.g., elongation factor Tu, EF-Tu), antimicrobial compounds (e.g., lactic acid, carbon dioxide, and bacteriocins), or completion for the same adhesion receptors (Liévin et al., 2000; Šušković et al., 2010; Abedi et al., 2013).

Based on the high survival ratio in simulated GIT and challenge with 3 pathogens, we further determined that *Lb. plantarum* WLPL04 protected Caco-2 cells from acute inflammatory damage caused by SDS, which might be associated with antioxidant substances such as EPS. In our previous study, we confirmed that EPS yielded by *Lb. plantarum* R315 exhibited a stronger antioxidant effect in vitro compared with bifidobacteria (Li et al., 2014b). Considering that little information has been reported about the antiinflammatory effect of *Lb. plantarum* on Caco-2 cells, and *Sal. typhimurium* is a common pathogen yielding LPS, which induces the inflammatory reaction of epithelial cells, we tested the antiinflammatory potential of *Lb. plantarum* WLPL04 by monitoring the inflammatory response of Caco-2 cells. We found that expression of the antiinflammatory cytokine IL-10 was increased and expression of proinflammatory cytokines (IL-6, IL-8, and TNF- α) induced by *Sal. typhimurium* was significantly decreased ($P < 0.01$), suggesting a potential antiinflammatory property of *Lb. plantarum* WLPL04. Overall, our results are in agreement with previous works (Frick et al., 2007; Candela et al., 2008), which reported that commensal bacteria may protect intestinal epithelial cells from enteropathogens by inhibiting IL-8 production, which is involved in epithelial cell injury.

CONCLUSIONS

To our knowledge, this is the first report to study the antagonistic effect of *Lb. plantarum* against 3 pathogens via competition, inhibition, and displacement strategies, and to report the antiinflammatory effect on Caco-2 cells on the mRNA level. *Lactobacillus plantarum* WLPL04 exhibited strong antimicrobial activity against various pathogens, survived well in simulated gastrointestinal tract, and yielded a high amount of EPS. Together with the antibiotic sensitivity results and antiadhesion and antiinflammatory properties against pathogens, our results indicate that *Lb. plantarum* WLPL04 from breast milk might be a suitable candidate probiotic for promoting host health.

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