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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), anticolitis 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; Albesharat et al., 2011). In fact, human breast milk, as

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the first source of nutrition for the neonate, not only 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% $CaCO_3$ (wt/vol). All plates were anaerobically incubated at 37°C for 48 h. The isolates yielding a dissolving circle of $CaCO_3$ 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).

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	AGGGAGGATGAGTGATTTGC
IL-10-R	AACTGGGAGGAACACTGACC
TNF-α-F	TTTGATCCCTGACATCTGGA
TNF-α-R	GGCCTAAGGTCCACTTGTGT
β-2-microglobulin-F	GGCTATCCAGCGTACTCCAAA
β-2-microglobulin-R	CGGCAGGCATACTCATCTTTTT

[1]F= forward; R = reverse.

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 1×10^8 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