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Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*

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ABSTRACT

In the present study, 78 lactic acid bacteria (LAB) strains were screened for the antagonistic activity against *Campylobacter jejuni* and 15 LAB strains with high bactericidal capacity were selected for further study through the analysis of the inhibition zone of LAB spent culture supernatant on the growth of *C. jejuni*. Among these strains, four isolates, *Lactobacillus plantarum* N8, N9, ZL5 and *Lactobacillus casei* ZL4 exhibited high adhesion ability to HT-29 cells. All the cell free supernatant (CFS) of these four strains contained high concentration of organic acid and their inhibition effects against *C. jejuni* were pH sensitive. Furthermore, these four strains could strongly antagonize the adhesion and invasion of *C. jejuni* to HT-29 cells and showed good tolerance to artificial gastric and small intestinal juices. This study suggests that *Lactobacillus* strains N8, N9, ZL4 and ZL5 could be used as potential probiotics in food applications against *C. jejuni* infection.

1. Introduction

Campylobacter infections usually lead to watery or bloody diarrhea and abdominal pain and have been identified as the leading bacterial pathogen causing human enterocolitis in many countries (Dasti, Tareen, Lugert, Zautner, & Gross, 2010). In some developed countries, *Campylobacter* infections induce diarrheal disease 7–12 times as frequently as infections with *Salmonella* species, *Shigella* species, or *Escherichia coli* O157:H7 (Blaser, Wells, Feldman, Pollard, & Allen, 1983; Slutsker et al., 1997). Furthermore, *Campylobacter* infections might cause serious sequel – the Guillain–Barré syndrome (GBS), a demyelinating disorder resulting in acute neuromuscular paralysis (Altekruse, Stern, Fields, & Swerdlow, 1999). *Campylobacter jejuni* is now recognized as one of the main causes of bacterial food-borne disease in many developed countries (Moore et al., 2005). At present, antibiotics are commonly used as the treatment of diseases caused by *Campylobacter*. However, the increasing incidence of antibiotic-resistant *Campylobacter* strains suggests that novel alternative approaches are necessary to be developed (Alfredson & Korolik, 2007).

Lactobacilli are inhabitants of the gastrointestinal tract of healthy humans and play a pivotal role in normal gut function and maintaining host health. Beneficial effects conferred by lactobacilli include inhibition of microbial pathogens, such as *Salmonella* (Bernet-Camard et al., 1997), *E. coli* (Mangell et al., 2002) and *Listeria monocytogenes* (de Waard, Garssen, Bokken, & Vos, 2002; Wang et al., 2014). Some studies have also evidenced the protective effect of lactobacilli in preventing *Campylobacter* infections. Chaveerach, Lipman, and van Knapen (2004) reported that probiotic bacteria isolated from conventional chicken had potential inhibitory activity against this pathogen, and the culturability of *Campylobacter* was under the detection limit after 48 h of incubation with *Lactobacillus* (P93). The efficacy of mixed probiotic preparations in inhibiting pathogen shedding and colonization of *C. jejuni* in chicks has also been reported (Morishita, Aye, Harr, Cobb, & Clifford, 1997; Willis & Reid, 2008). Another study described a 55% reduction in the invasion of *C. jejuni* into human intestinal epithelial cells after treatment with *Lactobacillus helveticus* R0052, which also suggested that competitive exclusion could contribute to protection by adherent probiotics (Wine, Gareau, Johnson-Henry, & Sherman, 2009). Moreover, Wagner, Johnson, and Kurniasih Rubin (2009) reported that lactobacilli and bifidobacteria could supplement the colonization resistance of a model

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human microbiota against *C. jejuni* enteric persistence in immunodeficient and immunocompetent mice. However, the studies directly focused on the ability of potential *Lactobacillus* strains to inhibit *C. jejuni*, especially the antagonistic activity of *Lactobacillus* strains to reduce adhesion and invasion of *C. jejuni* in human intestinal epithelial cells are still not enough.

In this study, to explore new potential probiotics of antimicrobial activity against *C. jejuni*, 78 LAB were screened and four *Lactobacillus* strains with high adhesion capacity to human intestinal epithelial cells were selected for further study on their antimicrobial properties. With characterizing by *in vitro* assay, these selected *Lactobacillus* strains may be considered as new anti-campylobacter agent and applied to food to provide an efficient method for inhibiting *C. jejuni*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

78 LAB were isolated from fermented pickles, health infant feces and fermented dairy products. *Lactobacillus rhamnosus* GG (LGG, ATCC 533103), *C. jejuni* NCTC 11168 (ATCC 700819), *C. jejuni subsp. jejuni* (ATCC 33291), *C. jejuni subsp. jejuni* (ATCC BAA-1153), *L. monocytogenes* (ATCC 19114), Enteroinvasive *E. coli* (ATCC 43893), *Enterococcus faecalis* (ATCC 19433) and other 11 strains were obtained from the Culture and Information Center of Industrial Microorganisms of China Universities, Jiangnan University (CICIM-CU). All *Lactobacillus* strains were cultured in deMan, Rogosa and Sharpe (MRS) broth at 37 °C for 20 h. *C. jejuni* strains were incubated in Mueller-Hinton broth (Oxoid, UK) and cultured on Columbia blood agar base plates (Oxoid) supplemented with 6% sheep blood and *C. jejuni* selective supplement for 48 h at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) in anaerobic jars.

For adhesion and inhibition assays, *Lactobacillus* strains cultured for 20 h were harvested and washed twice (5000 × *g*, 10 min, 4 °C) with 0.1 mol/L sterile phosphate-buffered saline (PBS, pH7.2), and finally resuspended in RPMI-1640 medium (HyClone, USA) at a concentration of 10^8 CFU/mL. *C. jejuni* strains cultured on Columbia blood agar base plates were harvested and washed twice with PBS (4000 × *g*, 15 min, 4 °C), the concentration of *C. jejuni* cultures (ATCC 700819 only or equal mixture of ATCC 700819, ATCC 33291 and ATCC BAA-1153) was adjusted to 10^8 CFU/mL with RPMI-1640 medium.

2.2. Growth inhibition assay

To detect the antimicrobial activity of the selected LAB on *C. jejuni* NCTC 11168 and other pathogenic bacteria, the oxford cup method was used. *Campylobacter* cultures (250 μl, 10^8 CFU/mL) were plated on charcoalcefoperazone − deoxycholate (CCDA) agar (Oxoid, UK). Other pathogenic bacteria were plated on tryptone soy yeast extract (TSYE, *L. monocytogenes*), lysogeny broth (LB, Enteroinvasive *E. coli*) or MRS (*E. faecalis*). Then 100 μL cell free culture supernatant (CFS) from the 20 h LAB liquid culture (8000 × *g*, 20 min, 4 °C) which filtered with 0.22 μm pore size filter was added into the Oxford cup. Sterilized MRS broth (pH 3.6) was used as a negative control and gentamicin (320 μg/mL; Sangon, Shanghai, China) was used as the positive control. Plates were incubated for 48 h at 37 °C under microaerophilic conditions and the diameter of the inhibition zone around each well was measured. Assays were performed in triplicate. The inhibition values were classified into four categories corresponding to +++ (>13 mm diameter), ++ (10–13 mm diameter), + (8–10 mm diameter) and − (<8 mm diameter).

Experiments were performed to examine the sensitivity of *Lactobacillus* strains CFS antimicrobial activity to heat (100 °C, 20 min), pH (pH was adjusted to 6.5) and enzymes. To assess the effect of enzymes on the inhibitory effect of *Lactobacillus* strains CFS on *C. jejuni* growth, the antimicrobial activity of CFS were tested after incubated at 37 °C for 1 h with trypsin (200 μg/mL; Sangon), proteinase K (200 μg/mL; Sangon) or catalase (1 mg/mL; Sangon).

The growth of *C. jejuni* co-cultured with *Lactobacillus* strains was determined by the following method according to Sgouras (Sgouras et al., 2004). The fresh *C. jejuni* NCTC 11168 cells (10^7 CFU/mL) suspended in antibiotic-free brain heart infusion broth (BHIB) containing 5% serum were incubated under microaerophilic conditions for 48 h at 37 °C in the presence of a 10% volume of live lactobacilli cells (10^7 CFU/mL). The viability of *C. jejuni* NCTC 11168 was evaluated from the number of viable CFUs of *C. jejuni* NCTC 11168 cultured as described above on *C. jejuni*-selective plates.

2.3. Organic acid analysis

The organic acids in *Lactobacillus* strains CFS were determined by HPLC as previously described by Lin et al. (2009) with some modifications. Following protein precipitation with 50% H_2SO_4, supernatant was filtered through a 0.22 μm pore size filter and diluted 10-fold with ddH_2O_2. 10 μL diluted supernatant were injected into a 250 × 4.6 mm Synergi Hydro-RP column. Elution was performed at 30 °C with 5% CH_3OH and 0.05% H_3PO_4 (pH 2.3) at a flow rate of 0.8 mL/min. Organic acids were determined by optical density (OD) measurements at 210 nm. Lactic acid, acetic acid, malic acid, citric acid, succinic acid, and fumaric acid were used as the standard.

2.4. Cell lines and adhesion assay

The HT-29 cell line was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (HyClone, USA) and 100 U/mL penicillin–streptomycin at 37 °C in an incubator with 5% CO_2. For adhesion assays, HT-29 cells were seeded (4×10^5 cells/well) into six-well tissue culture plates (Gibco, USA). The plates were cultured until monolayers of cells were formed in each well. Prior to adherence assays, HT-29 monolayers were washed twice with PBS. 1 mL lactobacilli suspension (10^8 CFU/mL in RPMI-1640) and 1 mL antibiotic-free RPMI-1640 were added to each well and incubated at 37 °C in 5% CO_2. After a 2-h incubation, cells were washed twice with PBS, fixed with methanol, Gram-stained and then examined microscopically under oil immersion. The adherence index was evaluated in 20 random microscopic fields of adhering bacteria per 100 cells. Adherence assays were performed in triplicate.

2.5. Spectrophotometric hydrophobicity assays

Hydrophobicity interaction was carried out by the method of Kos et al. (2003) with modifications. *Lactobacillus* strains cultured for 24 h were harvested and re-suspended in 0.1 mol/L KNO_3 (pH 6.2) to 10^8 CFU/mL. The absorbance of the cell suspension was measured at 600 nm (A_0). 1 mL of dimethylbenzene was added to 3 mL of cell suspension. After a 10-min pre-incubation at room temperature, the two-phase system was mixed by vortexing for 1 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of bacterial adhesion to dimethylbenzene (H%) was represented by the following formulae:

$$H\% = (1 - A_1/A_0) \times 100\%$$