A novel method for screening of potential probiotics for high adhesion capability

Xin Wang,*† Qinglong Wu,‡ Kan Deng,* Qiang Wei,* Ping Hu,* Jingjing He,* Huan Liu,* Yong Zheng,* Hua Wei,§ Nagendra P. Shah,‡¹ and Tingtao Chen*¹

*Institute of Translational Medicine, and

†Institute of Life Science, Nanchang University, Nanchang, Jiangxi 330031, PR China

‡Food and Nutritional Science, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong

§State Key Laboratory of Food Science and Technology, Nanchang University, Jiangxi 330047, China

ABSTRACT

To screen for potential probiotics with high adhesion capability, a chemostat model-based cultured human feces and denaturing gradient gel electrophoresis methods were applied, and the adhesion capability of the isolates was evaluated in vitro and in vivo. *Lactobacillus plantarum* (HM218749), *Lactobacillus reuteri* (EU547310), and *Enterococcus faecalis* (HM218543) were isolated from the slime on the chemostat wall, as these organisms could grow better at 37°C in an anaerobic environment and could resist harsh conditions (pH 1.5 and 0.30% bile salt). *Lactobacillus plantarum*, *L. reuteri*, and *E. faecalis* could adhere to HT-29 cells and reduce the adhesion of *Shigella dysenteriae* 2457, *Staphylococcus aureus* Cowan1, *Enterobacter sakazakii* 45401, and *Escherichia coli* 44102 to HT-29 cells. Moreover, the animal experiment showed that *L. plantarum* could adhere to mice intestine, increasing the number of lactobacilli and decreasing the number of enterococci.

Key words: probiotics, denaturing gradient gel electrophoresis, *Lactobacillus plantarum*, chemostat

INTRODUCTION

Increased awareness of health and busy lifestyles of consumers have created a strong and dynamic probiotics market (Granato et al., 2010). Probiotics, which are defined as “live microorganisms which when administered in adequate amounts, confer a health benefit on the host,” are popular areas of study for microbiologists and nutritionists (Diplock et al., 1999; Hill et al., 2014). In addition to survival during gastrointestinal transit, the ability of probiotics to adhere to the intestinal

mucosa is one of the main selection criteria for probiotics (McNaught and MacFie, 2001; Ouwehand et al., 2002). Many health effects, including antagonism against pathogens, modulation of the immune system, and enhanced healing of damaged intestinal mucosa, are considered related to the adhesion of probiotics to intestinal mucosa, although strong adhesion of probiotics may only sometimes be associated with transient colonization (Johansson et al., 1993; Elliott et al., 1998).

Different probiotics exhibit different colonization capabilities and adhesion processes involve nonspecific adhesion (depending on the sticky substance, such as proteinaceous compounds, teichoic and lipoteichoic acids, peptidoglycans, and exopolysaccharides) and specific adhesion (involving interaction with the mucus layer of the gastrointestinal tract or with the proteins of the extracellular matrix through cell envelope and secreted proteins; Juntunen et al., 2001; Siciliano and Mazzeo, 2012). In our previous studies (Chen et al., 2011c, 2014a), we used chemostat models of the human large bowel, in which sterile medium enters and exits at a fixed rate and physicochemical variables (e.g., pH, temperature, and medium components) do not change, and a steady state or stable flora is usually achieved for 7 to 10 d after inoculation. In those experiments we found that some human fecal microflora could nonspecifically adhere to the chemostat wall and form a thick film, indicating their high adherence capability. Hence, a chemostat was designed to obtain a viscous membrane on a glass surface, and probiotic characteristics of the bacteria that were identified as having potential adhering ability were assessed both in vitro and in vivo.

MATERIALS AND METHODS

Chemostat

The chemostat was composed of units based on the same concept as that developed previously for the fecal

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¹Corresponding authors: chentingtao1984@163.com and npshah@hku.hk

or colonic system (Gibson et al., 1988; Chen et al., 2014a). The culture medium was (g/L): pectin (Sigma, St. Louis, MO), 0.6; xylan (Sigma), 0.6; arabinogalactan (Sigma), 0.6; amylopectin (Sigma), 0.6; Lintner starch (Sigma), 5.0; casein (Sigma), 3.0; peptone (Sigma), 3.0; K_2HPO_4 (Aoboxing, Beijing, China), 2.0; $NaHCO_3$ (Aoboxing), 0.2; NaCl (Aoboxing), 4.5; $MgSO_4 \cdot 7H_2O$ (Aoboxing), 0.5; $CaCl_2 \cdot 2H_2O$ (Aoboxing), 0.45; cysteine (Sigma), 0.4; $FeSO_4 \cdot 7H_2O$ (Aoboxing), 0.005; hemin (Sigma), 0.01; bile salts (Sigma), 0.05; Tween 80 (Aoboxing), 2 mL; vitamin solution (Sigma), 1 mL. One milliliter was pumped into the vessel. Culture fluid (500 mL) was stirred, sparged with gas mixture (85% N_2 , 5% H_2 , 10% CO_2), and kept at 37°C and between pH 6.4 and 6.6. Fresh medium was pumped into the culture vessel at 35 mL/h (a dilution rate of 0.07/h) and the excess medium was passed from the chemostat to a collecting vessel via a side arm (Carman et al., 2004).

Eight adult volunteers signed informed consent and donated their feces, which were stored at -70°C (Chen et al., 2014a). Before inoculation into the chemostat, feces were thawed at room temperature for 1 h and uniformly suspended in prereduced and anaerobically sterilized diluent (culture medium) at a feces-to-diluent ratio of 1:4 and pooled together for the inoculation. The chemostat was inoculated with 50 mL of the suspension containing 10 g of feces on d 1, 3, and 5 by injecting through the septum of the lid of the vessel.

The chemostat was run for 31 d after inoculation to ensure a steady state was reached. From d 23 to 31, the thick film on the glass wall of the chemostat was aseptically collected and used for denaturing gradient gel electrophoresis (DGGE) analysis and cell culture experiments.

Cultivable Cell Counts and Identification by Colony and Bacterial Morphology

Bacterial counts were enumerated as previously reported (Harmsen et al., 2000). Diluted aliquots were replica-plated onto brain heart infusion (BHI; BHI; Difco Laboratories, Detroit, MI) agar (an enriched non-selective medium for the total bacterial count), de Man, Rogosa, Sharpe (MRS; Difco) agar (for *Lactobacillus*), Slanetz-Bartley medium agar (for *Enterococcus*), yeast potato dextrose (YPD; Difco) agar (for fungi), and MacConkey agar (for *Escherichia coli*; Difco). Plates were then incubated anaerobically in the anaerobic system (full of 85% N_2 , 5% H_2 , 10% CO_2) at 37°C for 24 to 36 h followed by counting colonies from plates showing 25 to 250 colonies. The microbes were identified accord-

ing to their colony and bacterial morphology, as well as based on their nutrients and oxygen requirements.

DGGE Analysis

The DNA was isolated by a bead-beating method (GC clamp sequence: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGGCACGGGGGG) and the bacterial primers (V3-R518, ATTACCGCGGCTGCTGG; V3-F357-GC, GC clamp: TACGGGAGGCAGCAG), *Lactobacillus* primers (Lac1, AGCAGTAGGGAACTCTTCCA; Lac2GC, GC clamp: ATTYCACCGCTACACATG), and fungal primers [FF390(F), CGATA-ACGAACGAGACCT; FR1 (R) GC clamp: AICCATTCAATCGGTAIT] were used for the DGGE analysis (Chen et al., 2011b).

The bands of interest in DGGE gels were excised using a sterile blade and incubated overnight at 4°C in Tris-EDTA buffer (pH 8.0) to allow diffusion of DNA for further amplifications. The PCR products for sequencing were purified using the QIA quick PCR purification kit and subcloned with the pMD18-T vector system I (Takara, Dalian, China) according to the manufacturer's instructions. The transformants were randomly picked and sequenced by Invitrogen (Shanghai, China).

Acid, Salt, Temperature, and Oxygen Tolerance of Isolates

The isolates were grown in corresponding media at 37°C overnight and subcultured 3 times, then the cultures were centrifuged at $4,500 \times g$ for 10 min at 4°C to obtain the cell pellets of the isolates. For acid tolerance, each isolate was diluted 1:100 (vol/vol) in PBS at pH 1.5, 2.5, 3.5, 4.5, and 7.0 and incubated for 4 h. For bile salt tolerance, freshly prepared cultures were inoculated into corresponding media containing 0.1 to 0.3% (wt/wt) bile salts and incubated at 37°C for a further 4 h. Growth at different temperatures (25, 30, 37, 42, and 46°C) and that under anaerobic or aerobic conditions were also evaluated. All the operations were carried out in the anaerobic system and all bacterial colonies were enumerated using the plate count method (Chen et al., 2014b).

HT-29 Cell Cultures and Adherence Assay

The HT-29 cells were stored in a liquid nitrogen tank with 90% fetal bovine serum (Hyclone Laboratories, Ogden, UT) and 10% dimethyl sulfoxide. The cells were cultured in complete RPMI-1640 medium (Hyclone

Laboratories) supplemented with 10% (vol/vol) fetal bovine serum in an incubator (HEPA class 100, Thermo Scientific, Waltham, MA) with 5% CO₂ and 95% air at 37°C. The culture medium was changed daily until HT-29 monolayers were at 80 to 90% confluence.

Adherence of probiotics to HT-29 cells was examined as described previously (Zhang et al., 2010). The HT-29 monolayers, which were prepared on glass coverslips and placed in 6-well plates (Corning Inc., Corning, NY), were washed twice with PBS (pH 7.4). A 1-mL aliquot of probiotics (8 log cfu/mL) and 1 mL of the cell line culture medium were added to each well of the tissue culture plate and the plate was incubated at 37°C in 5% CO₂. After 2 h of incubation, monolayers were washed 4 times with sterile PBS, fixed with methanol for 30 min, Gram stained, and examined microscopically. An adherence index was determined from 20 random microscopic fields of adhering probiotics per 100 HT-29 cells. Each adherence assay was carried out in triplicate.

Inhibition of Adhesion of Foodborne Pathogens to HT-29 Cells

The ability of probiotics to inhibit adhesion of foodborne pathogens to HT-29 cells was assayed following the method reported previously (Zhang et al., 2010). The Luria-Bertani medium (for laboratory strains *Shigella dysenteriae* 2457, *Staphylococcus aureus* Cowan1, *Enterobacter sakazakii* 45401, and *Escherichia coli* 44102) and YPD (for laboratory strain *Candida albicans* SC5314) medium were used to culture the pathogens at 37°C overnight and subcultured for 3 times; Approximately 3×10^5 HT-29 cells per well were seeded in a 12-well plate (Corning Inc.), culture medium was changed daily, and HT-29 monolayers at 80 to 90% confluence were washed twice with PBS (pH 7.4) before the experiments. In the exclusion assay, HT-29 monolayers were inoculated with 300 mL of the suspension containing probiotics (7 log cfu/well) in RPMI-1640 medium and incubated for 1 h at 37°C in 5% CO₂. The HT-29 monolayers were then inoculated with 100 mL of foodborne pathogens (7 log cfu/well) in RPMI-1640 medium and incubated for another 1 h.

In all experiments, nonadhering cells were removed by washing 4 times with sterile PBS and then treated with 0.5 mL of 0.5% (vol/vol) Triton X-100 (Amresco, Solon, OH) for 5 min in an ice water bath. The cells were then serially diluted and plated onto LB plates for counting foodborne pathogens. The ability of probiotics to exclude foodborne pathogens was determined by comparing adhesion of foodborne pathogens in the presence of probiotics to that of foodborne pathogens.

The number of foodborne pathogen cells prevented from adhering to HT-29 cells was then expressed as a percentage value. All experiments were independently carried out 3 times and each assay was performed in triplicate.

Animal Model and Experimental Design

Six- to 8-wk-old specific-pathogen-free BALB/c female mice (18 ± 2 g) were purchased from the Hebei Municipal Center for Disease Control and Prevention (Hebei, China). After 1 wk of adjustment in the Laboratory Animal Center of Nanchang University, the feces of mice ($n = 8$) were collected before experiments (control stage); then probiotics were fed for 2 wk (treatment stage), followed by collection of feces 2 wk after stopping feeding of the probiotics (recovery stage). An aliquot of the feces was used for microbial count and the rest of the samples were stored at -20°C for the DGGE analysis.

Statistical Analysis

Data of viable cell counts were submitted to ANOVA, and then significant differences between means were determined by the nonparametric Student-Newman-Keuls separation test with significance level ($P < 0.05$) or very significant level ($P < 0.01$).

RESULTS AND DISCUSSION

Isolation of Bacteria from the Chemostat Wall Using Viable Cell Count and DGGE

To isolate bacteria with strong adherence ability to the human intestine, human feces were obtained from human volunteers and cultured in chemostat for 31 d to allow the formation of a microbial layer on the chemostat wall. Then the bacterial layer was aseptically collected and smeared on to BHI, MRS, and YPD plates and incubated at 25 or 37°C under anaerobic conditions. Forty-one different colonies (16 from BHI, 16 from MRS, and 9 from YPD) were picked based on their cell and colony morphologies and their identity was determined using DGGE method (Chen et al., 2011a). In principle, the same bacteria exhibited the same band number and band location in DGGE gel; hence, the microbes corresponding to lanes 5, 7, and 14 as shown in Figure 1A, lanes 5, 11, and 14 as shown in Figure 1B, and lanes 5 and 7 as shown in Figure 1C were sent for sequencing (Shenggong Biotech, Shanghai, China). Three different bacteria, namely *Lactobacillus plantarum* (HM218749, on BHI and MRS

Figure 1. Classification of bacteria using specific primers in denaturing gradient gel electrophoresis (DGGE) gel. (A) Bacterial DGGE gel; M = mixture of all the bacteria; 1–10 = strains isolated from de Man, Rogosa, Sharpe (MRS) medium; 11–16 = strains isolated from brain heart infusion medium. (B) *Bacillus* DGGE gel; M = mixture of all the bacteria; 1–10 = strains isolated from MRS medium; 11–16 = strains isolated from BHI medium. (C) Fungal DGGE gel; M = mixture of all fungi; 1–9 = strains isolated from yeast potato dextrose medium.

plates), *Lactobacillus reuteri* (EU547310, on BHI and MRS plates), and *Enterococcus faecalis* (HM218543, on BHI and YPD plates), were also identified (Table 1).

Evaluation of Acid, Salt, Temperature, and Oxygen Tolerance of Isolates

It is crucial for probiotics to be viable to exert their beneficial effects; hence, tolerance to acid and bile salt

has become an important selection criterion for probiotic organisms (Jensen et al., 2012). It is anticipated that foods will stay in the stomach for 2 to 3 h at low pH (about 3.0) and in the intestine at high salt concentrations (about 0.3%). As shown in Figure 2, the isolated *L. plantarum*, *L. reuteri*, and *E. faecalis* could tolerate a low pH of 1.5 and exhibited high biomass at 7 to 8 log cfu/mL for up to 4 h. The tolerance of isolates to bile salt was inferior compared with that of

Table 1. Identification of the microorganisms isolated from the chemostat

Strain no.	Closest relative	Similarity (%)	GenBank No.	Sequence length ¹ (bp)
1	<i>Lactobacillus plantarum</i>	99	HM218749.1	1,450
2	<i>Lactobacillus reuteri</i>	98	EU547310.1	1,450
3	<i>Enterococcus faecalis</i>	99	HM218543.1	1,450

¹Sequenced length of 16S rDNA V3 region.

Figure 2. The probiotic characteristics of *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Enterococcus faecalis*. (A) pH tolerance; (B) bile salt tolerance; (C) temperature tolerance; (D) oxygen tolerance. Error bars represent SE.

pH tolerance, and their biomass at 0.3% bile salt was at 3 to 4 log cfu/mL. Additionally, a temperature of 37°C and anaerobic conditions were suitable for all isolates.

Evaluation of Isolates for Ability to Adhere to Intestinal Cells and Exclude Pathogens

Adhesion to mammalian epithelial cells is a key process for bacteria to colonize the gastrointestinal tract. Adhesion of pathogens allows release of enzymes and

toxins initiating necrotic processes against target cells, thereby facilitating invasion (Jankowska et al., 2008), and probiotics could competitively bind to adhesion sites and produce components with antimicrobial activity to exclude pathogens from the gastrointestinal tract (Baccigalupi et al., 2005; Collado et al., 2006).

From Figure 3A, all the isolates exhibited strong adherence ability to HT-29 cells; *L. plantarum* possessed the highest adherence at 1,100 cfu/100 cells. With regard to antiadherence property to pathogens, *L.*

Figure 3. Adherence capability of the *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Enterococcus faecalis* on HT-29 cell line (A) and the antiadherence property of the isolates against pathogens (*Shigella dysenteriae*, *Staphylococcus aureus* Cowan 1, *Enterobacter sakazakii*, *Escherichia coli*, and *Candida albicans*) on HT-29 cell line (B). Error bars represent SE.

plantarum could also significantly lower the adherence of *S. dysenteriae* 2457, *Staphylococcus aureus* Cowan 1, *E. sakazakii* 45401, and *E. coli* 44102 ($P < 0.01$). It is important to note that all the isolates enhanced,

rather than lowered, the growth of fungal pathogen *C. albicans* SC5314. It may be that *C. albicans* SC5314 possessed different binding sites on to HT-29 cells compared with *L. plantarum*, thus *L. plantarum* exhib-

Figure 4. The microbial diversity in mice intestine. (A) The effect of *Lactobacillus plantarum* on the microbial number of lactobacilli, enterobacteria, and enterococci in control stage (I; before probiotics feeding); treatment stage (II; probiotics feeding for 2 wk); and recovery stage (III; stop probiotics feeding for 2 wk); error bars represent SE. (B) The effect of *L. plantarum* on microbial species in control stage (I; before probiotics feeding); treatment stage (II; probiotics feeding for 2 wk); and recovery stage (III; stop probiotics feeding for 2 wk). Bands in the white boxed area belong to *L. plantarum* and represent their existence in mice intestinal tract.

ited little antiadherence property against *C. albicans* SC5314.

Evaluation of the Adherence of Isolates in Mice Intestines

The human intestinal tract is a nutrient-rich environment packed with up to 100 trillion microbes (Whitman

et al., 1998), and the microbial community has been considered as a factor that could influence predisposition to specific diseases in the context of certain hosts (Ley et al., 2006). In our previous studies, *L. plantarum* could strongly adhere to HT-29 cells (Figure 3), glass, stone, wood, and plastic; thus, *L. plantarum* was chosen for evaluating its adherence ability using a mice model. Before feeding *L. plantarum* to mice, it appeared that 3

of 8 mice (mice 1, 3, and 5) were positive for this strain (Figure 4); however, during the treatment stage, the *L. plantarum* not only occupied the dominant position among *Lactobacillus* species in the intestine of the mice but also enhanced the microbial diversity, which could benefit host health. Two weeks after feeding without *L. plantarum*, this strain still exhibited dominance in the intestine of 6 of 8 mice, showing much more abundance than the control (Figure 4). In addition, the viable cell count results indicated that the *L. plantarum* could increase the number of *Lactobacillus* species and lower the number of *Enterococcus* species, suggesting a positive effect on host health (Cross, 2002).

CONCLUSIONS

In the present study, we developed a chemostat model to screen organisms for strong adherence ability, and *L. plantarum* was isolated using this model and its probiotic characteristics were determined. The organism showed tolerance to low pH and high concentrations of salt bile and possessed high adherence capability and antiadherence property against foodborne pathogens both in vivo and in vitro.

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