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

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## 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* 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 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

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(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 nonselective 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 according 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: CGCCCGGGGCGCGCCCC-GGGCGGGGCGGGGGCACGGGGGG) and the bacterial primers (V3-R518, ATTACCGCGGCTGCTGG; V3-F357-GC, GC clamp: TACGGGAGGCAGCAG), *Lactobacillus* primers (Lac1, AGCAGTAGGGAA TCTTCCA; Lac2GC, GC clamp: ATTYCACCGCTA-CACATG), and fungal primers [FF390(F), CGATA-ACGAACGAGACCT; FR1 (R) GC clamp: AICCATT CAATCGGTAIT] 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 × *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% $CO_2$ 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,

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