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Study of probiotic potential of four wild *Lactobacillus rhamnosus* strains

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ABSTRACT

The four wild *Lactobacillus rhamnosus* strains were examined in vitro for resistance to simulated gastro and intestinal juices, adhesion to HT-29 cells, antagonistic activity against enteric pathogens and immunomodulating activity. The strains *L. rhamnosus* SB5L, J5L and IN1L were able to survive in simulated gastro juice while the strain *L. rhamnosus* SB31L lost viability exposed to simulated gastro juice for 3 h. The four strains had high viability in simulated small intestinal juice with little loss (<1.0 cycle reduction). The strains SB5L, J5L and IN1L antagonized against *Escherichia coli* ATCC 25922, *Salmonella enterica serovar Typhimurium* ATCC 14028, *Shigella sonnei* ATCC 25931. The strain *L. rhamnosus* IN1L had the highest adhesive capability to HT-29 cells in vitro (251 bacteria cells per 100 HT-29 cells) compared to the other three *L. rhamnosus* strains. The live bacteria, cell wall and DNA of the four *L. rhamnosus* induced the secretion of pro-inflammatory cytokines IL-12 (p70), IFN-γ and TNF-α by human peripheral blood mononuclear cells (PBMCs). The levels of IL-12 (p70), IFN-γ and TNF-α produced by stimulated PBMCs were significantly higher ($P < 0.05$) than those of the control. Those data indicated that the four *L. rhamnosus* strains have the potential as the probiotic for human being use, although further studies are still needed.

1. Introduction

Lactobacilli are members of the normal indigenous flora of the human gastrointestinal tract and are frequently used in the production of fermented food. Consumption of lactobacilli can exert beneficial effects on the host, such as enhancing immunity [1], preventing against pathogen infection and acute diarrhoea [2,3], reducing risks of cancer [4] and so on. Many different strains and species of lactobacilli, which confer health benefits on a host, have been used commercially as probiotics [5]. To match the increasing demand for functional food containing probiotics, new strains having probiotic potential have been screened out [6,7].

Survival ability in gastrointestinal conditions, adhering ability on intestinal epithelial cells and antagonistic activity against pathogens are the common criteria for screening strains with probiotic potential [6–8]. In order to survive and colonize in the gastrointestinal tract, probiotic bacteria should be tolerant to low pH (pH 2.5–3.5) and pepsin of the stomach, bile salts and pancreatin in the upper part of the intestine, and have the ability to adhere to intestinal mucus [9]. Adherent strains are preferred, since their establishment in the intestines seems to be necessary for the probiotic effects to be exert [10]. A good adherence capacity of probiotic lactobacilli can promote the gut residence time, pathogen exclusion, and interaction with host cells for the protection of epithelial or immune modulation [11]. Probiotic bacteria could inhibit enteropathogenic bacteria invading and infecting intestinal epithelial cells by producing antibacterial substance (organic acid or proteinaceous substance) [12,13]. Several lactic acid bacteria isolated from traditional fermented food by our lab could antagonize some spoilage and pathogenic bacteria due to production of class IIa bacteriocin [14].

The immunomodulating activity was recognized as the important property of probiotics [15–17]. Both live and inactivated lactobacilli showed immunoregulating activity [18]. In fact, some macromolecular compounds in cell wall (including peptidoglycan, lipoteichoic acids and capsular polysaccharides) and chromosomal DNA of lactobacilli play key role in immunoregulation [19]. Lactobacilli could induce immunocompetent cells, such as peripheral blood mononuclear cells (PBMC), to produce cytokines including IL-10, IL-12,

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TNF-α, IFN-γ and so on [20]. IL-12 and IFN-γ are important cytokines implicated in innate defense mechanisms in response to bacteria. IL-12 potently stimulates cytotoxic T cells and NK cells, and enhances the production of Th1 cytokines (IFN-γ), which can activate specific immune responses, and has reported anti-tumour activity [1].

In this study, four *Lactobacillus rhamnosus* strains, isolated from traditional fermented food in Gansu and infant feces in Harbin, China, were assessed in vitro for their viability in simulated gastrointestinal conditions, adhesion to HT-29 cells, antagonistic activity against pathogens and the immunomodulating activity. The objective of the study was to find novel lactobacilli having potential as probiotics.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. rhamnosus strains tested in this study were isolated from traditional fermented food in Gansu and infant feces in Harbin, China, using spread plate technique. The strains were identified according to the results of Gram stain reaction, catalase reaction and carbohydrate fermentation patterns using Biolog System (Biolog Inc, Hayward, CA, USA). The detailed information about the strains was listed in Table 1.

No commercial starter cultures were used in the preparation of the traditional fermented foods, instead, previously fermented product was used as source of the natural microflora into the freshly food material. The fermentation of the food occurs at room temperature in the household. The traditional food has a long history of safe use and has been important main component of local peoples' daily diet.

The strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 18 h in an anaerobic incubator before use. The colony forming units (CFU/ml) of the strains were determined by plating serial 10-fold dilution into MRS-agar.

Indicator bacteria used for antimicrobial assays were *Escherichia coli* ATCC 25922, *Salmonella enterica serovar Typhimurium* ATCC 14028, *Shigella sonnei* ATCC 25931, which were cultured in tryptone soy broth or agar (TSB or TSA) in aerobic condition at 37 °C for 18 h.

2.2. Collection of live cells of bacterial strains

Bacterial cells from 18 h four *L. rhamnosus* strains cultures were harvested by centrifugation (5000 × *g*, 10 min, 4 °C; Micro fuge-18, Low Temperature Superspeed Centrifuge, American Beckman Company) and washed twice with PBS buffer (pH 7.2) before being resuspended in RPMI1640 medium (Thermo Scientific HyClone, Thermo Fisher Scientific Inc. USA) at a concentration of 2×10^9 CFU/ml.

2.3. Preparation of cell wall and genomic DNA of the lactobacilli

The cell wall of the four *L. rhamnosus* strains was prepared according to the method described by Tuo et al. [21]. Briefly, after 18 h incubation the bacteria cells from 50 mL lactobacilli culture were harvested by centrifugation (5000 × *g*, 10 min, 4 °C), washed twice with PBS (pH 7.2). The pelleted cells were suspended in 10 ml purified water and broken immediately with three or four volumes of SiO_2 beads (diameter, 0.05–0.1 mm) in a vortex mixer at maximal speed for 30 min at 4 °C. Unbroken bacterial cells and SiO_2 beads were separated from the suspension by centrifugation (2000 × *g*, 15 min, 4 °C). The homogenate was centrifuged (30,000 × *g*, 30 min, 4 °C) to pellet cell wall. Cell wall material was resuspended in 2 ml of purified water. The protein content of the cell wall extracts was determined by the Lowry method [22].

Genomic DNA of the four *L. rhamnosus* strains incubated for 18 h at 37 °C was purified by phenol–chloroform–isoamyl alcohol extraction method [23]. The concentration and purity of all DNA preparations were determined by measuring absorbance at 230 nm, 260 nm and 280 nm (OD_{230}, OD_{260} and OD_{280}). Only the DNA with the $OD_{260/280}$ ratio >1.8 and $OD_{260/230} \geq 2$ was used.

The cell wall and DNA extracted from the four strains were diluted in RPMI1640 medium and stored at −20 °C.

2.4. Survival under conditions simulating the human GI tract

The resistance of the examined *L. rhamnosus* strains in simulating stomach juice and small intestinal juice was tested as described by Maragkoudakis et al. [12]. Briefly, bacterial cells from 18 h lactobacilli cultures were harvested by centrifugation (5000 × *g*, 10 min, 4 °C), washed twice with PBS buffer (pH 7.2), then resuspended in PBS solution, pH 2.0, containing pepsin (3 mg/ml) (simulating gastric juice), or in PBS solution, pH 8.0, containing pancreatin (1 mg/ml) and bile salts (0.3%, w/v) (simulating small intestinal juice). Resistance was assessed in terms of viable colony counts and enumerated after incubation at 37 °C for 0, 1 and 3 h in simulating gastric juice, and 0 and 4 h in small intestinal juice, reflecting the time spent by food in the stomach and small intestine, respectively. The assay was performed in triplicate.

2.5. Antimicrobial activity against pathogens

The four *Lactobacillus* strains were tested for antimicrobial activity against *E. coli*, *S. typhimurium*, and *S. sonnei*. Fresh 18 h lactobacilli MRS culture supernatants were collected by centrifugation (15,000g, 15 min, 4 °C). The antimicrobial activity was tested using well diffusion assays.

In the well diffusion assays, 1×10^5 CFU/ml of the pathogenic strains were incorporated into soft agar (1%, v/v) plates of TSA. Lactobacilli supernatant samples (50 μl) were pipetted into holes drilled into the agar. The plates were then incubated at 37 °C. Antimicrobial activity was recorded as growth free inhibition zones around the well. MRS broth adjusted at pH 6.5 served as control. All tests for each strain were performed in triplicate.

2.6. Adhesion to HT-29 cells

The adhesion ability of the four *Lactobacillus* strains was assessed according to Gopal et al. [24]. The human colon carcinoma cell line (HT-29) was obtained from the Cancer Institute of the Chinese Academy of Medical Science. HT-29 cells were routinely grown in RPMI1640 (Thermo Scientific HyClone, USA), supplemented with 10% (v/v) heat-inactivated foetal calf serum (Hanzhou Sijiqing Co. Ltd, China), 1% (v/v) antibiotics (10,000 IU/ml penicillin and 10,000 μg/ml streptomycin, Beijing Solarbio Co. Ltd, China). Cells were cultured at 37 °C in a tissue culture incubator (5% CO_2). For the adhesion assay, monolayers of HT-29 cells were prepared on glass coverslips which were placed in six-well tissue culture plates (Corning Inc, USA). The HT-29 monolayers on glass coverslips were

Table 1
Strains of *Lactobacillus rhamnosus* used in this study.

Strains	Species	Origin[a]
SB5L	*L. rhamnosus*	Traditional fermented yak's milk food, Subei, Gansu
SB31L	*L. rhamnosus*	Traditional fermented yak's milk food, Subei, Gansu
J5L	*L. rhamnosus*	Traditional fermented vegetable juice, in Lanzhou, Gansu
IN1L	*L. rhamnosus*	Infant feces in Harbin, Heilongjiang

[a] The fermented foods were made by the local people using traditional method under local climate and environmental conditions; infant feces were from healthy baby, who was natural birth, one month old and not taking antibiotics.