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Screening of probiotic lactobacilli for inhibition of *Shigella sonnei* and the macromolecules involved in inhibition

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ABSTRACT

A total of 91 lactobacilli were screened for antimicrobial activity against *Shigella sonnei*. Agar-well assay showed that 16 lactobacilli displayed strong antibacterial activity against *S. sonnei*. The nature of these antimicrobial agents were investigated and shown to be dependent on their production of organic acids. Adhesion tests showed that 6 lactobacilli demonstrated good adherence to HT-29 cells, of these *Lactobacillus johnsonii* F0421 were selected for acid and bile salt tolerance properties. We further research on *L. johnsonii* F0421 inhibition of *S. sonnei* adhesion to HT-29 cells. The result showed that *L. johnsonii* F0421 exhibited significant inhibitory activity and excluded, competed and displaced adhered *S. sonnei* by 48%, 38% and 33%, respectively. In order to elucidate the inhibitory functions of macromolecules involved in *L. johnsonii* F0421, the cells were treated with 5 M LiCl, 0.05 M sodium metaperiodate and heating and assayed for inhibition activity. The results suggested a role of S-layer proteins on *L. johnsonii* F0421 cells in inhibition of the adhesion process, but carbohydrates do not seem to be involved. SDS-PAGE analysis confirmed the presence of S-layer proteins with dominant bands of approximately 40 kDa. In addition, 100 μg/well of S-layer proteins from *L. johnsonii* F0421 cells were effective in inhibiting adhesion of *S. sonnei* to HT-29 cells. These findings suggest that *L. johnsonii* F0421 possesses the capacity for inhibition of *S. sonnei* activity as well as probiotic properties, which could serve as a potential novel and effective probiotic strain for use in the food industry.

1. Introduction

Lactobacilli isolated from the human intestinal tract and fermented dairy products are generally regarded as safe (GRAS) and have been extensively exploited for their probiotic properties [1,2]. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a benefit on the host [3]. *Lactobacillus* and *Bifidobacterium* constitute the most frequently used genera. Most of the current commercial probiotic strains have not been selected for specific applications but rather are selected on the basis of their technological potential. Nevertheless, it is well known that probiotic effects are strain specific, therefore explicit strains may be selected for use-specific processes, where designated applications of probiotics may exert their maximal positive impact [4].

Several mechanisms have been suggested for the inhibitory activity of lactobacilli against Gram-negative pathogens, including production of organic acids and bacteriocin, and competition for adhesion sites with pathogens [5,6]. These properties provide a rationale for selection of lactobacilli to prevent infection from pathogens. Lactobacilli are also reportedly antagonistic against pathogens belonging to the genera *Salmonella*, *Escherichia* and *Candida* [6–8]. However, the capacity of lactobacilli for inhibition of *Shigella sonnei* in vitro has received little attention [9]. *Shigella* (i.e. *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *S. sonnei*) is a major cause of gastrointestinal illness throughout the world and in the United States, *Shigella* (mostly *S. sonnei*) ranks third among bacterial food borne pathogens (after *Campylobacter* and *Salmonella*) in the number of gastrointestinal cases according to the Centers for Disease Control and Prevention [10].

The substances associated with lactobacilli adhesion to host epithelial cells include collagen binding proteins, S-layer proteins and carbohydrates [11–13]. These different substances may play a critical role in the inhibition of pathogens [14,15] by competition for explicit binding sites. Furthermore, Horie et al. [16] reported that not only did *Lactobacillus crispatus* JCM 5810 inhibit the adhesion of *Escherichia coli* to host epithelial cells but also S-layer

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proteins were effective and this effect was ascribed to competition for the same binding sites in the extracellular matrix.

The aim of this study was to screen lactobacilli for inhibition of *S. sonnei* through antimicrobial activity against *S. sonnei*, adhesion to HT-29 cells and tolerance to simulated GI tract. Such information will provide a basis for the selection of probiotics with the ability to competitively exclude intestinal pathogens. In addition, a preliminary characterization of the macromolecules involved in inhibition of *S. sonnei* adhesion was also conducted. The identification of lactobacilli that demonstrate this capacity could serve as potential novel probiotic strain for use in the food industry.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The ninety-one lactobacilli used in this study were isolated from 22 human fecal samples using LAMVAB agar [17] and were subjected to a preliminary identification on the basis of Gram staining and catalase reaction. *L*actobacillus *rhamnosus* ATCC 53103 (LGG) was used as the reference strain. All lactobacilli were stored in our lab and cultured in deMan, Rogosa and Sharpe (MRS) (Difco) broth in aerobic conditions at 37 °C. The indicator bacteria used for antimicrobial assays was *S. sonnei* ATCC 25931 and was cultured in tryptone soy broth or agar (TSB or TSA) in aerobic conditions at 37 °C, provided by Microbiological Laboratory of Clinic Detection Center of Heilongjiang (Harbin, China).

2.2. Screening lactobacilli for anti-S. sonnei activity

Briefly, approximately 1×10^7 CFU of indicator strains were incorporated into a soft agar (1% w/v) plate of TSA. The cell-free culture supernatants (CFCS) (50 μl) from lactobacilli culture was transferred to holes (5 mm diameter) drilled into the agar. The plates were then incubated at 37 °C for 18 h, and antimicrobial activity was recorded as growth-free inhibition zones (diameter) around the well. MRS adjusted to pH 6.5 served as a control.

To test for the sensitivity of CFCS antibacterial activity for selected lactobacilli to Proteinase K, Proteinase K was added to 1 ml CFCS at a final concentration of 1 mg/ml at 37 °C for 3 h. The heat stability of the antibacterial activity was examined by incubating 1 ml of CFCS at 100 °C for 15 min. To determine the organic acid function, 1 ml CFCS was adjusted to pH 6.5 and then 1 ml CFCS of lactobacilli was retained as an untreated filtrate. MRS adjusted to pH 6.5 served as a control. The anti-*S. sonnei* activity of all samples was measured using Agar-well assay [18]. All experiments were performed three times independently and each assay was performed in duplicate.

2.3. Screening lactobacilli for adherence activity and Biolog System identification

Adherence of lactobacilli to HT-29 cells was examined as described previously [19]. The HT-29 monolayers, which were prepared on glass cover slips and placed in six-well plates (Corning Inc, NY, USA), were washed twice with phosphate-buffered saline (PBS, pH 7.4). Two ml of lactobacilli (10^8 CFU/ml) in complete RPMI-1640 medium were added to each well and incubated at 37 °C in 5% CO_2 for 2 h. Monolayers were then washed four times with sterile PBS, fixed with methanol for 30 min, stained with Gram stain, and examined microscopically. An adherence index was determined from 20 random microscopic fields of adhering lactobacilli per 100 HT-29 cells. Each adherence assay was conducted in triplicate. The selected lactobacilli were identified by carbohydrate fermentation patterns using Biolog System (Biolog, Hayward, CA).

2.4. Screening lactobacilli for the tolerance to simulated human GI tract

Resistance of lactobacilli to pepsin under low pH was tested as described previously [20]. Briefly, bacterial cells from overnight (18 h) were harvested and centrifuged at 4 °C for 10 min (6000 × g) and washed twice with PBS buffer (pH 7.3). Simulated gastric juice was prepared by suspending pepsin (0.3 mg/ml; Sigma) in PBS (pH 2.0). Subsequently, 0.2 ml of washed lactobacilli cell suspensions in PBS were inoculated into 1 ml simulated gastric juice and 0.3 ml NaCl (0.5% w/v), mixed and incubated at 37 °C. Total viable counts were evaluated at 0, 1 and 3 h for testing the transit tolerance in gastric juice and, in this way, reflecting the time spent by food in the stomach.

Tolerance to small intestine conditions was tested in PBS solution (pH 8.0), containing pancreatin (0.1 mg/ml, Sigma) and 0.3% (w/v) Oxgall (Sigma). Washed lactobacilli cell suspensions (2%) were added to this small intestine media condition and incubated at 37 °C. Total viable counts were evaluated at 0 h and 4 h for testing the transit tolerance through the small intestine that would represent the time spent by food in the small intestine [21]. All experiments were performed three times independently and each assay was performed in duplicate.

2.5. Lactobacillus strain F0421 inhibition of S. sonnei adherence to HT-29 cells and effect of chemical and physical treatments

The ability of *Lactobacillus* strain F0421 to inhibit *S. sonnei* adhesion to HT-29 cells was assayed following methods reported previously by our research group [9]. The F0421 strain was subjected to different pretreatments before testing the capacity for inhibition of *S. sonnei* adhesion. The F0421 strain cells were centrifuged and washed twice with PBS (pH 7.3) and suspensions were divided into aliquots. After centrifugation, the supernatants were removed and the bacterial pellets were resuspended in the following pretreatment solutions: 5 M LiCl [22], and citric acid-phosphate buffer (pH 4.5) containing 0.05 mM sodium metaperiodate [23]. These two mixtures were incubated at 37 °C for 1 h. After incubation, cells were harvested by centrifugation, washed, and re-suspended in PBS. A third treatment was also included by heating cells suspended in PBS at 100 °C for 10 min. Control treatments consisted of incubating the bacteria in PBS. All treated and untreated lactobacilli as assessed for their ability to exclude, compete and displace the adhered of *S. sonnei* to HT-29 cells were tested as described previously [9]. All experiments were performed three times independently and each assay was performed in triplicate.

2.6. Dose-response testing of Lactobacillus strain F0421 and involvement of S-layer protein exclusion S. sonnei adherence to HT-29 cells

To assess the effect of F0421 dose on *S. sonnei* adhesion, three different concentrations (from 10^6 to 10^8 CFU/well) of F0421 strain were tested in three independent experiments. To assess the effect of F0421 S-layer proteins on the adhesion of *S. sonnei*, cells were incubated with 100 μg/well of S-layer proteins, and the concentration of *S. sonnei* was adjusted in all experiments to 10^7 CFU/well. Wells with *S. sonnei* alone served as controls. The method for evaluating exclusion *S. sonnei* adherence to HT-29 cells was as described previously.

2.7. SDS-PAGE analysis of the S-layer proteins from Lactobacillus strain F0421 cells

Lactobacilli cells were treated with 5 M LiCl as described above. Ten ml of supernatant containing S-layer proteins were