In vitro and in vivo examination of anticolonization of pathogens by *Lactobacillus paracasei* FJ861111.1

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ABSTRACT

Very limited information exists on the exclusion of pathogens by probiotics in the gut of the host challenged with pathogens. In this study, we tested probiotic characteristics in vitro and anticolonization ability of *Lactobacillus paracasei* FJ861111.1 in mice infected with selected pathogenic microorganisms. The in vitro results indicated that *L. paracasei* FJ861111.1 had a high survival in acidic conditions at pH 2.5 and bile salt concentration at 0.3%, and strong inhibition ability against common pathogens including *Shigella dysenteriae*, *Staphylococcus aureus*, *Cronobacter sakazakii*, *Escherichia coli*, and *Candida albicans*. The cell adhesion assays showed that *L. paracasei* FJ861111.1 exhibited strong adherence to HT-29 cells and excluded the adhesion of selected food-borne pathogens to HT-29 cells. The in vivo results showed that fermented milk with *L. paracasei* and viili (a Nordic yogurt product) significantly improved the population of total bacteria and of *Lactobacillus* in the feces of mice, and significantly inhibited the colonization of *C. albicans* to the intestines of mice post-*C. albicans* infection. Thus, it appears that this strain could be used as a probiotic organism for manufacturing functional fermented milk. **Key words:** *Lactobacillus paracasei*, probiotic characteristics, anticolonization, pathogenic infection

INTRODUCTION

Consumption of fermented milks has shown several health-promoting benefits, including modulation of gut

microbiota, lowering of blood pressure, and reduction in the risks of bladder cancer and colon cancer. Fermented milks have a long history of consumption by humans (Alatossava et al., 2013). Viili, a semi-solid yogurt-type product originating from the Nordic countries, has a gelatinous consistency and a sour taste due to microbial action of yeasts and lactic acid bacteria. The product has been popular for its various biofunctionalities, such as reducing blood cholesterol level, preventing carcinogens, and enhancing host immunity (Kitazawa et al., 1991; Nakajima et al., 1992; Wang et al., 2008; Chen et al., 2013; Wu et al., 2013). Among the microbial consortium, lactic acid bacteria played an important role in the process of viili fermentation in regard to milk acidification, texture, flavor, and aroma development.

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Diplock et al., 1999; Hill et al., 2014), and lactic acid bacteria strains with beneficial properties have been increasingly used as probiotic starters for food fermentations for enhancing immune function and preventing gastrointestinal infections (Wood, 1998; de Roock et al., 2010; Higgins et al., 2010). Hence, candidate strains with probiotic features are very promising for industrial milk fermentation, such as manufacturing value-added fermented milks (Botta et al., 2014). To deliver its beneficial effects in the intestine, probiotics must be capable of surviving in the gastrointestinal tract, including extreme acidic environment in the stomach and bile salt conditions in the small intestine, and adhering to intestinal epithelial cells (Chae et al., 2013; Lamari et al., 2014; Ranadheera et al., 2014). Moreover, probiotics have been shown to bring a positive balance in the gut microbiota after an imbalance caused by antibiotic treatment or infections due to pathogens and other stress (Zhao, 2010; Zhang et al., 2012). This involves nutrient absorption, immune response, and colonization resistance to pathogens (Belkaid and Hand, 2014; Chen et al., 2014a; Fung et al., 2014).

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However, very limited clinical intervention or practice to overcome such imbalance has been carried out, especially for restoring the microbiota after infections with pathogens. Recently, probiotics have been suggested for modulating the microbiota; however, no guidance for choosing probiotics exists because of their strain-specificity. *Lactobacillus paracasei* FJ861111.1 was previously isolated from viili and exhibited good properties for milk curdling and production of exopolysaccharides (Chen et al., 2013). In the present study, this strain was used to evaluate its acid and bile salt tolerance, antibacterial activities, inhibition of food-borne pathogens adhesion to HT-29 cells, and anticolonization effect in the gut of the mice post-*Candida albicans* infection. Another human-derived probiotic organism, *Bifidobacterium longum* ATCC15708, was included as a control due to its wide applications in food fermentation and as a probiotic organism (Gomes and Malcata, 1999).

MATERIALS AND METHODS

Bacterial Strains and Cultivation Conditions

Lactobacillus paracasei FJ861111.1 was previously isolated from viili (Chen et al., 2011a). In addition, *B. longum* ATCC15708 was used as a positive probiotic control in our study. *Bifidobacterium longum* and *L. paracasei* were cultivated in de Man-Rogosa-Sharpe (MRS) medium and incubated at 37°C for 24 h under anaerobic conditions. Pathogenic microorganisms including *Shigella dysenteriae* 301 and 2457, *Staphylococcus aureus* Cowan1 and CMC, *Cronobacter sakazakii* 45401 and 45402, *Escherichia coli* 44102, and *C. albicans* SC5314 were cultivated in Luria-Bertani (LB) medium and incubated at 37°C for 24 h under aerobic conditions. Organisms were propagated in corresponding media 3 times before making fermented milks and conducting experiments.

Acid and Bile Salts Tolerance Assay

Stock cultures of *L. paracasei* and *B. Longum* were propagated twice in MRS medium at 37°C for 24 h before the following assay. For the acid tolerance assay, the cultures were harvested by centrifugation at $4,500 \times g$ and 4°C for 10 min, and bacterial cell pellet was then resuspended in PBS. The cell suspension was 100-fold diluted in PBS at various pH values, including 1.5, 2.5, 3.5, 4.5, and 7.0, and incubated at 4°C for 4 h. For the bile salt tolerance test, fresh cultures were inoculated into MRS medium containing 0.1 to 0.5% (wt/wt) bile salts and incubated at 37°C for 4 h. After incubation, the viable cells of *L. paracasei* and *B. longum* were examined using plate count method (Chen et al., 2014b).

Antimicrobial Activity of *L. paracasei* and *B. longum* Agar Diffusion Assay

The agar diffusion assay was carried out to examine the antimicrobial activity of *L. paracasei* and *B. longum* as previously described (Chen et al., 2014b). Cultures of *L. paracasei* and *B. longum* grown anaerobically for 24 h in MRS medium were centrifuged to obtain supernatants. Overnight (12 h) cultures of the pathogenic microorganisms including *Sh. dysenteriae* 301 and 2457, *Staph. aureus* Cowan1 and CMC, *Cr. sakazakii* 45401 and 45402, *E. coli* 44102, and *C. albicans* SC531 were spread on the surface of the LB agar plates. Aliquots (200 μ L) of the supernatant was loaded into an Oxford cup (outer diameter = 7.8 ± 0.1 mm, inner diameter = 6.0 ± 0.1 mm, and height = 10.0 ± 0.1 mm), which was placed on the surface of the agar. The size of the inhibition zone was measured until the formation of clear zone around the Oxford cup. The experiment was carried out in duplicates.

Adherence of *L. paracasei* and *B. longum* to HT-29 Cells

Adherence of *L. paracasei* and *B. longum* to HT-29 cells was examined as described previously (Zhang et al., 2010). The cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% (vol/vol) fetal bovine serum (Hyclone) and incubated at 37°C in a CO₂ incubator (HEPA class 100, Thermo Fisher Scientific, Waltham, MA). The medium was changed daily until HT-29 monolayers were at 80 to 90% confluence. The HT-29 monolayers prepared on glass coverslips and placed in 6-well plates (Corning Inc., Corning, NY) were washed twice with PBS buffer (pH 7.4). One milliliter of the bacterial cultures (~ 8 log cfu/mL) and 1 mL of cell line culture medium were added to each well of the tissue culture plate, and the plates were incubated at 37°C in 5% CO₂. After 2 h of incubation, monolayers were washed 4 times with sterile PBS followed by fixing cell lines with methanol for 30 min. Nonadhering bacterial cells were washed away during this procedure. Gram staining was then carried out to stain the bacterial cells, which were examined under a microscope, then 100 HT-29 cells were randomly selected and the total number of adhering bacterial cells was counted as the adherence index. Each adherence assay was carried out in triplicate.

Inhibition of Food-borne Pathogens Adhering to HT-29 Cells

The ability of *L. paracasei* and *B. longum* to inhibit food-borne pathogens adhering to HT-29 cells was

assayed according to the method reported previously (Zhang et al., 2010). Approximately 3×10^5 HT-29 cells per well were seeded in a 12-well plate (Corning Inc.). The medium was changed daily and HT-29 monolayers at 80 to 90% confluence were washed twice with PBS (pH 7.4) before conducting this experiment. In the exclusion assay, HT-29 monolayers were inoculated with 300 μ L of cell suspension of *L. paracasei* and *B. longum* (~ 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 μ L of food-borne pathogens, respectively (~ 7 log cfu/well), in RPMI-1640 medium and incubated for another 1 h.

After incubation, nonadhering bacterial cells were removed by washing 4 times with sterile PBS, and cells 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 mixtures were then serially diluted and plated onto LB agar plates for counting food-borne pathogens. As *L. paracasei* and *B. longum* were not able to grow in LB medium, the cell counts generated from LB agar plates were for pathogens. The ability of *L. paracasei* and *B. longum* to inhibit pathogens adhering to HT-29 cells was determined by comparing the number of pathogens adhering HT-29 cells in the presence or absence of *L. paracasei* or *B. longum* for the co-cultivation. Three independent experiments were carried out and each assay was performed in triplicate.

Preparation of Fermented Milks Using *L. paracasei*, *B. longum*, and Viili Starter

The viili starter was a generous gift from Cheng Luo (University of Tartu, Tartu, Estonia). To prepare various fermented milks, *L. paracasei* (5%, vol/vol), *B. longum* (5%, vol/vol), and viili starter (5%, wt/vol) were inoculated in 10% (wt/vol) in sterile skim milk and incubated at 25°C for 20 h followed by feeding

these 3 fermented milks to mice. The fermented milks were kept in a refrigerator and were used within 1 wk of preparation.

Animal Experiment Design

The animal experiment was approved by the Ethics Committee of Nanchang University (Jiangxi, China). Six- to 8-wk-old SPF BALB/c female mice (18 ± 2 g) were purchased from the Hebei Center for Disease Control and Prevention (Hebei, China). After 1 wk of acclimatization in the Laboratory Animal Center at Nanchang University, the animals were randomly divided into 4 groups ($n = 3$) and received 1 of the 4 fermented milks: (1) *Bifidobacterium* group (*B. longum*-fermented milk); (2) *Lactobacillus* group (*L. paracasei*-fermented milk); (3) viili group (viili starter-fermented milk); and (4) control group (sterile milk). The timeline of the animal experiment is shown in Figure 1.

Mice were gavaged with 1 mL of fermented milk or sterile milk each day during the second week. After that, each mouse was gavaged with 1 mL of *C. albicans* (8 log cfu/mL) for 3 d in total, followed by a 7-d recovery period. Feces were collected at d 0 (control stage) as a baseline, d 7 (after acclimatization), 14 (after feeding diets 1–4), 17 (after feeding *C. albicans*), 18 (1 d post-*C. albicans* infection), 21 (3 d post-*C. albicans* infection), and 24 (after recovery). Upon receipt of the feces, plate counting of each sample was carried immediately (Chen et al., 2014a).

Enumeration of Fecal Microorganisms

Microbial counts were enumerated according to previous studies (Chen et al., 2011b, 2014b). Briefly, serial dilutions of fecal samples were prepared in prereduced anaerobically sterilized (media) (PRAS) diluent, which were then plated on brain-heart-infusion agar plates,

Figure 1. Animal experimental design. Time points (7 points) for collecting feces are indicated in the figure. Mice were divided into 4 groups and received 4 different diets: *Bifidobacterium* group (*B. longum*-fermented milk), *Lactobacillus* group (*L. paracasei*-fermented milk), viili group (viili starter-fermented milk), and a control group (sterile milk). Dietary treatments were carried out in mice in the morning, whereas the fecal sample collection was performed in the evening.

MRS agar plates, or yeast extract-peptone-dextrose agar plates and then incubated anaerobically at 37°C for 24 to 36 h for counting colonies. Brain-heart-infusion agar, an enriched nonselective medium for the isolation and cultivation of most anaerobic microorganisms and other fastidious microorganisms, was used to count the total microorganisms in our study; MRS agar, an enriched selective medium intended for the isolation and cultivation of *Lactobacillus*, was used for counting gut lactobacilli. Yeast extract-peptone-dextrose agar was used for counting *C. albicans*.

Statistical Analysis

Data were analyzed and their means are presented in Figures 2 to 7 (SE measurement). Statistical analysis was performed in which ANOVA was calculated and the independent *t*-test was used to test for significance between groups ($P < 0.05$ or $P < 0.01$). All analyses were carried out with using the statistics programs of SPSS 13.0 software (SPSS Inc., Chicago, IL).

RESULTS

Acid Tolerance, Bile Salt Tolerance, and Antipathogenic Activity of *L. paracasei* FJ861111.1

The populations of *L. paracasei* and *B. longum* after exposure to acid and bile salt are shown in Figure 2 and Figure 3. As shown in the figures, the suitable condition for both *L. paracasei* and *B. longum* was pH 7.0 without

Figure 2. Tolerance of *Lactobacillus paracasei* FJ861111.1 and *Bifidobacterium longum* ATCC15708 to different acidic conditions. Error bars represent standard errors.

bile salts supplemented to the medium. When pH was lowered and the bile salt concentration was increased, an obvious reduction in the viabilities of both *L. paracasei* and *B. longum* was observed. In addition, the population of *L. paracasei* was 7.5 log cfu/mL at pH 2.5 and 6.0 log cfu/mL in bile salt concentration of 0.5%; values of 7.8 log cfu/mL at pH 2.5 and 6.0 log cfu/mL in bile salt concentration of 0.3% may suggest high survival of these organisms during passage through the gastrointestinal tract. Moreover, the viabilities of these 2 organisms under the same condition were not significantly different.

The antibacterial activity of *L. paracasei* and *B. longum* is shown in Figure 4. In general, the antibacterial activity of *L. paracasei* was weaker than that of *B. longum*. It was found that *L. paracasei* could inhibit all the tested pathogens, especially *Staph. aureus* CMC (18 mm) and *Cr. sakazakii* 45401 (16 mm). This may be beneficial for food preservation and may likely kill the pathogens in the gut of the host.

Adherence Capacity and Inhibition of Pathogens Adhering to HT-29 by *L. paracasei* FJ861111.1

The adherence index of *L. paracasei* and *B. longum* to HT-29 cell is shown in Figure 5. The adhered cells of *L. paracasei* to HT-29 cell were 1,173 cfu/100 cells, which was similar to the adherence index of human-derived *B. longum*.

The result of inhibition of pathogens adhering to HT-29 by *L. paracasei* and *B. longum* is shown in Figure 6. In general, *L. paracasei* could inhibit most of the tested pathogens adhering to HT-29 cells, including *Sh. dysenteriae* 301 (49.5%), *Sh. dysenteriae* 2457 (60.5%), *Staph. aureus* Cowan1 (32.3%), *Staph. aureus* CMC (3.2%), *Cr. sakazakii* 45401 (21%), *Cr. sakazakii* 45402 (24.7%), *E. coli* 44102 (19.3%), and *C. albicans* SC5314 (14.6%). It appears that *L. paracasei* exhibited higher ($P < 0.01$) inhibition of *Sh. dysenteriae* adhering to intestinal epithelial cells than that of *B. longum*.

Counts of Total Bacteria, *Lactobacillus*, and *C. albicans* in Mice Before and After Feeding Fermented Milks and Before and After *C. albicans* Infection

The populations of total bacteria, *Lactobacillus*, and *C. albicans* cells in the feces of mice before and after *C. albicans* infection are shown in Figure 7. A significant increase was seen in the viable count of total bacteria ($P < 0.01$) at d 24 in both the *Lactobacillus* group and the viili group compared with that of the control group (Figure 7A). Little changes in the population of total bacteria were observed at d 24 among the *Bifidobacterium*, *Lactobacillus*, and viili groups compared with that

at d 0 (Figure 7A). However, the administration of *C. albicans* had a significant reduction in the population of total bacteria in the control group. This suggests that *L. paracasei*, viili starter, and *B. longum* were able to sustain the population of total bacteria after the invasion of *C. albicans*.

Similarly, the population of *Lactobacillus* in *Bifidobacterium*, *Lactobacillus*, and viili groups were significantly ($P < 0.01$) improved at d 24 compared with that of control group (Figure 7B). Also, a significant reduction ($P < 0.01$) in the counts of gut *Lactobacillus* was noted in both the viili and control groups at d 24 compared with that at d 0. This indicates less growth-promoting effect of viili starter-fermented milk than *L. paracasei*- and *B. longum*-fermented milk on the gut *Lactobacillus* in mice (Figure 7B).

Interestingly, it was found that all the fermented milk could inhibit the colonization of *C. albicans* in the gut of mice. This was evident by the significant ($P < 0.01$) reduction in the counts of *C. albicans* in the feces of mice at d 24 administrated with fermented milk (Figure 7C). Among the 3 fermented milks, *L. paracasei*-fermented milk exhibited the best scavenging ability to exclude the *C. albicans* cells, from 7.5 (in the control group) to 6.4 log cfu/mL at d 24 (Figure 7C).

DISCUSSION

Currently, most of studied probiotics, such as *Lactobacillus rhamnosus* GG and *Lactobacillus casei* strain Shirota, are from the *L. casei* group, including *L. casei*, *L. paracasei*, *L. rhamnosus*, and *Lactobacillus zeae*. It has been demonstrated that certain strains of *L. paracasei* exhibited important characteristics, for example good survival at low pH, tolerance to bile salts, reducing serum cholesterol level, and improving sensory properties in fermented milk (Caridi, 2002; Chiang and Pan, 2012; Costa et al., 2014). In our previous study, *L. paracasei* FJ861111.1, isolated from viili, could acidify milk rapidly and produce a high quantity of exopolysaccharides (Chen et al., 2013). This suggests that this organism could be a potential functional starter for manufacturing fermented milks.

It is crucial for probiotics to deliver their beneficial effects on the host as living cells. Thus, tolerance to gastric acid and bile salts becomes important selection criterion for screening probiotic organisms (Jensen et al., 2012). It appears that *L. paracasei* FJ861111.1 showed a strong capability to survive in the acidic condition of pH 2.5 and high bile salt concentration of 0.3%; these conditions are similar in the stomach and small intestine of humans (Figure 2 and Figure 3). In addition, high viability of *L. paracasei* FJ861111.1

at pH 4.5 suggests its high viable count during milk fermentation to deliver its beneficial effects to human upon consumption.

Adhesion to intestinal epithelial cells is another key criterion for probiotics so that they can colonize in the intestine for exerting their probiotic effects. Adhesion of pathogens allows the release of enzymes and toxins initiating necrotic processes directly into the target cell, thereby facilitating the invasion (Jankowska et al., 2008). A hidden but novel mechanism for probiotics to exclude pathogens from intestine is through the competition for adhesion sites on the surface of intestine and the production of antimicrobial components (Bacigalupi et al., 2005; Collado et al., 2006). Interestingly, a strong adherence of *L. paracasei* FJ861111.1 to HT-29 cells was observed when compared with human-derived *B. longum* ATCC15708 (Figure 5). This could be the base for *L. paracasei* FJ861111.1 to survive and colonize in human intestine to deliver its antiadhesion property against food-borne pathogens (Figure 4) and antibacterial activities (Figure 6). Thus, it was concluded that the viili-derived *L. paracasei* FJ861111.1 could be a good candidate as probiotics based on in vitro assays.

To examine in vivo anticolonization of pathogens by *L. paracasei* FJ861111.1, *C. albicans* was chosen for infecting mice due to inhibition of *C. albicans* adhering to HT-29 cell observed in the in vitro assay (Figure 6). Interestingly, this organism could significantly increase the population of total bacteria and *Lactobacillus* in the feces of mice post-*C. albicans* infection (d 24; Figure 3A and 3B). Moreover, the administration of *L. paracasei* FJ861111.1 before *C. albicans* infection was able to efficiently lower the counts of *C. albicans* in the feces of mice postinfection (Figure 7C). This suggests that this organism could exclude this pathogen from the gut of the host through its anticolonization ability.

Our intestinal tract is a nutrient-rich environment packed with up to 100 trillion microbes (Whitman et al., 1998). The diversity of microbiota in human gut is the result of co-evolution between microbiota and its hosts (Ley et al., 2006). It has been demonstrated that the imbalance of gut microbiota is closely associated with the development of specific diseases in certain host contexts (Ley et al., 2006). An examination of the viabilities of gut microbiota showed that the number of total bacteria and *Lactobacillus* in mice at d 17 post-*C. albicans* infection exhibited a little change compared with that at d 0, 7, and 14 before infection, whereas a significant reduction was observed in the control group compared with the *Bifidobacterium*, *Lactobacillus*, and viili groups (Figure 7A). In addition, a significant reduction in the counts of *C. albicans* (d 24) was noted; this contributed to the conclusion that *L. paracasei*

Figure 3. Tolerance of *Lactobacillus paracasei* FJ861111.1 and *Bifidobacterium longum* ATCC15708 to bile salts. Error bars represent standard errors.

FJ861111.1 was able to exclude pathogens from the gut and may regulate gut microbiota from imbalance to balance state.

CONCLUSIONS

Lactobacillus paracasei FJ861111.1 showed several important probiotic characteristics, including tolerance

Figure 5. Adhesion capacity of *Lactobacillus paracasei* FJ861111.1 and *Bifidobacterium longum* ATCC15708 to HT-29 cells. Error bars represent standard errors.

to acid and bile salts, adhesion to HT-29 cells, antipathogenic activity, and inhibition of pathogens adhering to HT-29 cells. Consumption of *L. paracasei* FJ861111.1-fermented milk increased the population of gut bacteria and *Lactobacillus* and reduced the counts of *C. albicans* in the feces of mice postinfection. However, no obvious changes in diversity of gut *Lactobacillus* in mice before and post-*C. albicans* infection was observed. We also concluded that *L. paracasei* FJ861111.1 could efficiently exclude pathogens from the gut postinfection.

Figure 4. Antibacterial activities of *Lactobacillus paracasei* FJ861111.1 and *Bifidobacterium longum* ATCC15708 against selected food-borne pathogens. The value of *L. paracasei* FJ861111.1 was compared with that of *B. longum* ATCC15708. Error bars represent standard errors. * $P < 0.05$; ** $P < 0.01$; all other values, $P > 0.05$.

Figure 6. Inhibition of food-borne pathogens adhering to HT-29 cells by *Lactobacillus paracasei* FJ861111.1 and *Bifidobacterium longum* ATCC15708. Error bars represent standard errors. * $P < 0.05$; ** $P < 0.01$; all other values, $P > 0.05$.

Figure 7. Viable counts of total bacteria (A), *Lactobacillus* (B), and *Candida albicans* (C) in feces from mice before and post-*C. albicans* infection. Viable counts from *Bifidobacterium* group, *Lactobacillus* group, and viili group were compared with those with the control group. Error bars represent standard errors. * $P < 0.05$; ** $P < 0.01$; all other values, $P > 0.05$.

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