In Vitro Evaluation of Antimicrobial Activity of Lactic Acid Bacteria against Clostridium difficile

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Clostridium difficile infection (CDI) has become a significant threat to public health. Although broad-spectrum antibiotic therapy is the primary treatment option for CDI, its use has evident limitations. Probiotics have been proved to be effective in the treatment of CDI and are a promising therapeutic option for CDI. In this study, 4 strains of lactic acid bacteria (LAB), namely, Lactobacillus rhamnosus (LR5), Lactococcus lactis (SL3), Bifidobacterium breve (BR3), and Bifidobacterium lactis (BL3) were evaluated for their anti-C. difficile activity. Co-culture incubation of C. difficile (10⁶ and 10¹⁰ CFU/ml) with each strain of LAB indicated that SL3 possessed the highest antimicrobial activity over a 24-hr period. The cell-free supernatants of the 4 LAB strains exhibited MIC₅₀ values between 0.424 mg/ml (SL3) and 1.318 (BR3) mg/ml. These results may provide a basis for alternative therapies for the treatment of C. difficile-associated gut disorders.

Key words: Clostridium difficile infection, lactic acid bacteria, antimicrobial activity, cell-free supernatant

INTRODUCTION

Clostridium difficile is an infectious Gram-positive spore forming, toxin-producing bacterium and causes mild to severe gastrointestinal disorders such as diarrhea and colitis. C. difficile infection (CDI) has become a significant threat to public health, and its outbreak rate has been constantly increasing. The use of broad-spectrum antibiotic therapy is known to be responsible for the development of CDI. For example, one of the main predisposing factors for the acquisition of C. difficile-associated diarrhoea (CDAD) is antibiotic therapy. Current treatments for CDAD include oral metronidazole and vancomycin (1,2). However, metronidazole would be the drug of choice because of the danger of colonization of the gut with vancomycin-resistant enterococci (3) or the spread of vancomycin resistance to pathogenic bacteria within the hospital (4). Antibiotics are known to disturb the normal intestinal microbiota, leading to depletion of the epithelial barrier and subsequent colonization of pathogenic bacteria (5,6). The development of alternative therapies based on bacterial replacement is considered important due to the rapid emergence of antibiotic-resistant pathogenic strains and the adverse consequences of antibiotic therapies on the protective microbiota (7).

Probiotics have been defined as ‘Living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition’ (8). Among the health benefits provided by probiotics, antimicrobial activity against pathogenic microbes has been drawing much attention due to a growing concern about the widespread of antibiotic resistance. The activity of probiotic lactic acid bacteria is in part mediated through secretion of antimicrobial compounds, i.e organic acids, hydrogen peroxide, and bacteriocin (6,9,10). Probiotics have been proven to be effective in the treatment of CDI and suggested as a promising therapeutic option for CDI. Patients treated with the high dose vancomycin and S. boulardii had significantly decreased recurrence rates (16.7%) compared to vancomycin and placebo (50%). The S. boulardii given with the low dose vancomycin or metronidazole was not significantly protective against CDI (11). However, this finding was in contrast with the result from a prior clinical trial where the same strain S. boulardii was tested as adjunct to standard vancomycin or metronidazole therapy (12). Several other trials for CDI were terminated early due to slow enrollment rates and the resulting small study sizes (15~25 patients) precluded any statistical conclusions (13-15). Therefore, more research is needed encompassing large, standardised...
clinical trials with different probiotic strains.

LAB strains have shown to have protective effects on acute traveler’s diarrhea, antibiotic associated diarrhea (AAD), and rotavirus diarrhea (16, 17). The possible mechanisms underlying the probiotic effects include competition for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation (18). Recently two multi-species probiotic products, Duolac7 and Duolac Gold, have been tested to evaluate their efficacy in diarrhea-dominated irritable bowel syndrome (IBS) patients. The Duolac7 study showed that consumption of the study product was helpful in improving stool frequency, which was probably through stabilization of the intestinal microbiota (19). On the other hand, consumption of Duolac Gold for 4 weeks improved the symptoms of IBS such as abdominal pain and bloating and the improvements of the symptoms were positively correlated with increases in the levels of some strains of the study product (unpublished result). These results indicate that the LAB strains in the products are likely to have potential normalizing the intestinal microbiota, which motivated us to investigate if the strains are able to antagonize CDI. In addition, Lactococcus lactis SL3, isolated from a dairy product, was previously found to have antimicrobial activity.

The aim of this study was to evaluate anti-C. difficile activity of Lactococcus lactis SL3, Lactobacillus rhamnosus LR5, Bifidobacterium breve BR3, and Bifidobacterium lactis BL3 selected from the strains of Duolac7 and Duolac Gold based on their inhibitory activity in a C. difficile-infected cell line experiment. In addition, Lactococcus lactis SL3 was also included in this study since it was previously found to exhibit anti-C. difficile activity in a spot-on-the-lawn assay (unpublished result).

MATERIAL AND METHODS

Microorganisms. Lactococcus (Lc.) lactis SL3 (KCTC 11904BP), Lactobacillus (L.) rhamnosus LR5 (KCTC 12202BP), Bifidobacterium (B.) breve BR3 (KCTC 12201BP), and B. lactis BL3 (KCTC 11904BP) were tested in this study, and Clostridium (C.) difficile KCTC 5009 was obtained from Korean Collection for Type Cultures (Daejeon, Korea). The four LAB strains and C. difficile KCTC 5009 were cultured in reinforced clostridium medium (RCM; BD Diagnostics, Sparks, MD) broth anaerobically at 37°C until the stationary phase (18–24 hr) for the four LAB strains or 24–72 hr for C. difficile. L. rhamnosus LR5 and Lc. lactis SL3 were enumerated on MRS agar (BD Diagnostics, Sparks, MD), and B. breve BR3 and B. lactis BL3 were on BL agar (BD Diagnostics). C. difficile cells were counted on selective clostridium agar containing 32.0 g/L Proteose peptone (BD Diagnostics), 20.0 g/L Agar (Junsei, Japan), 6.0 g/L D-(-)-fructose (Duksan, Korea), 2.0 g/L sodium chloride (Samchun chemical, Korea), 5.0 g/L Na2HPO4 (Sigma, St. Louis, MO, USA), 1.0 g/L KH2PO4 (Sigma), 0.25 g/L D-cycloserine (Sigma), 0.1 g/L MgSO4 (Sigma), 0.03 g/L neutral red (Sigma-Aldrich, St. Louis, MO, USA) and 16.0 mg/L cefoxitin (Sigma) for C. difficile. Bacterial cell numbers were determined in triplicate by cultivating anaerobically at 37°C.

Preparation of cell-free supernatants (CFSs). The CFSs of the test strains was obtained by centrifuging the cultures, followed by filtration through a 0.2 μm sterile syringe filters (Pall, USA). The resultant filtrate (20 ml) was then transferred to a 3 K MWCO Amicon Filter (Millipore, France), and spun at 5,000 × g until the volume was concentrated to 2 ml per sample.

The protein concentrations were determined by using a Bradford assay kit (Bio-Rad Laboratories, USA). Bovine serum albumin was used as the standard. All reactions were carried out in triplicate.

Co-culture experiments. C. difficile KCTC 5009 was grown with or without each of the test strains in RCM broth. Experiments were performed under anaerobic conditions at 37°C. The inoculum concentration of C. difficile was 106 CFU/ml for the mono-culture experiments and 108 or 1010 CFU/ml for the co-culture experiment. The test strains were inoculated at the concentration of 108, 109, or 1010 CFU/ml. At time points (0, 6, 12, and 24 hr), 1 ml sample was taken and cell concentration was determined by plating on agar medium and incubating anaerobically at 37°C for 18 hr (test strains) or 24 hr (C. difficile). The results of the inhibitory activity from the co-culture experiment were presented based on the 50% efficacy concentration (EC50) of the test strains using GraphPad Prism (version 4.0, GraphPad Software, San Diego, CA).

Determination of minimum inhibitory concentration (MIC) and in vitro bacterial killing curves. The MIC of the CFSs for the test strains was determined in RCM broth, according to the standard broth micro-dilution method (20). The filter concentrates of the culture supernatant of the test strains were from 2.875 to 6.791 mg/ml for MIC determinations. All test CFSs were subjected to two-fold serial dilutions series with RCM broth respectively. Ten microliters of each of the diluted solutions were put into 96 micro-titre plate wells. Then 90 μl of C. difficile cell suspension adjusted to 105 CFU/ml were added to the wells which was then incubated for 24 hr at 37°C. The lowest concentration that completely inhibited microbial growth as determined by optical density measurements at 600 nm was taken to be the MIC. Samples were taken at various times (0, 3, and 24 hr) and subjected to viable cell count using RCM agar or LIVE/DEAD BacLight kit™ (L7012, Invitrogen, USA). MIC50 was defined as the lowest concentration of filter con-
centrate for all test strains at which 50% or more inhibition by the culture supernatants occurred. Fifty micro-liters of the suspensions were then dropped onto quadrants of RCM agar plate. Once dried, the plates were incubated at 37°C for 24 hr to determine viable counts. Results were expressed as CFU/ml.

In vitro killing curves of filter concentrates of the culture supernatant of the test strains were determined for *C. difficile*, at ½ MIC, 1 MIC, and 2 MIC.

**Staining of bacterial cells.** Cells collected at the time points (0, 3, and 24 hr) were adjusted to be 10^6~10^7 CFU/ml with phosphate buffered saline (PBS, pH 7.2), treated with the reagents in the BacLight kit according to the recommendation by the manufacturer and gently shaken for 15 min in dark condition. First labeling was confirmed by the use of a fluorescent microscope (CC-12, Olympus, Japan) set to accept fluorescence intensity at a wavelength (emission 1; green and emission 2; red).

**Flow cytometric measurements (FCMs).** FCMs were performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA.) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube. Cells were diluted to approximately 10^6 cells per ml and delivered at the low flow rate, corresponding to 150 to 500 cells per sec. FSC, SSC, and three fluorescence signals were measured. A band pass filter of 530 nm (515 to 545 nm), 585 nm (564 to 606 nm), or 670 nm was used to collect the green fluorescence (FL1), yellow-orange fluorescence (FL2), or the red fluorescence (FL3), respectively. FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected by using logarithmic amplifications. A combination of FSC and SSC was used to discriminate bacteria from background.

**RESULTS AND DISCUSSION**

**Microbial inhibition.** Co-cultures of a test strains with *C. difficile* is used to demonstrate the potential of various probiotic bacteria to decrease the number and virulence of *C. difficile* (21,22). The inoculum concentration of the test strains were 10^8, 10^9, and 10^10 CFU/ml, and the inoculum concentrations of *C. difficile* were 10^6 and 10^10 CFU/ml. Samples were withdrawn at 6 hr intervals after the start of culture incubation. In the co-culture test, the growth of *C. difficile* at 10^6 and 10^10 CFU/ml, was inhibited by the higher concentration (10^10 CFU/ml), whereas BR3 showed the lowest activity (EC50: 5.08 x 10^8 CFU/ml), whereas
BR3 showed the lowest activity (EC50: 7 × 10^{12} CFU/ml). The difference in the anti-C. difficile activity between the strains tested might be due to the nature of the antimicrobial compounds they produced. The anti-C. difficile activity of LR3, BL3, and BR3 may be mediated mainly by the organic acid production, i.e. lactic acid and acetic acid. In fact, the decline in pH arising from the production of organic acid from LAB is a well-known factor to inhibit certain strains. Non-dissociated form of, for example, lactic acid triggers to lower internal pH of the bacterial cell, which causes collapse in electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bactericidal effect (23). The strongest anti-C. difficile activity by exerted SL3 was likely to be mediated through the action of a bacteriocin since we previously observed its antimicro-

Table 1. Determination of MICs of the cell-free supernatants prepared from Lc. lactis SL3, L. rhamnosus LR5, B. lactis BL3, and B. breve BR3

| Protein concentrations (mg/ml) | SL3 | LR5 | BR3 | BL3 |
|-------------------------------|-----|-----|-----|-----|
| 10 × Concentrate              | 6.791 | 6.118 | 5.271 | 2.875 |
| ½ MICa                        | 0.212 | 0.382 | 0.659 | 0.359 |
| 1 MICb                        | 0.424 | 0.765 | 1.318 | 0.719 |
| 2 MICc                        | 0.849 | 1.530 | 2.636 | 1.438 |

*½ MIC: minimum inhibitory concentration required to inhibit the growth of 0~50% of organisms.
*1 MIC (or MIC50): minimum inhibitory concentration required to inhibit the growth of 50~90% of organisms.
*2 MIC (or MIC90): minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

Table 2. Summary of C. difficile survival rates following incubation at ½, 1, and 2 MIC cell-free supernatants of Lc. lactis SL3, L. rhamnosus LR5, B. lactis BL3, and B. breve BR3.

| Time (hr) | Cell status | Control | SL3 | LR5 | BL3 | BR3 |
|-----------|-------------|---------|-----|-----|-----|-----|
|           | Live        | 85.3    | 86.5| 86.5| 86.5| 89.5|
|           | Dead        | 14.7    | 13.5| 13.5| 13.5| 10.5|
| 3         | Live        | 90.1    | 54  | 38.6| 22.9| 64.1|
|           | Dead        | 9.9     | 46  | 61.4| 77.1| 35.9|
| 24        | Live        | 95.3    | 62.6| 3.9 | 0   | 16.1|
|           | Dead        | 4.7     | 37.4| 96.1| 100 | 83.9|

*½ MIC: minimum inhibitory concentration required to inhibit the growth of 0~50% of organisms.
*1 MIC (or MIC50): minimum inhibitory concentration required to inhibit the growth of 50~90% of organisms.
*2 MIC (or MIC90): minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

Fig. 2. Flow cytometric analysis of C. difficile incubated with ½, 1, and 2 MIC cell-free supernatants of Lc. lactis SL3 for 24 hr. All bacteria were previously stained with SYTO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, Q1: dead cell, and Q3: injured cell and debris.
bial activity against some Gram-positive pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*, which was pH-independent and protease-sensitive (data not shown).

**Determination of MIC and in vitro bacterial killing curves.** The CFSs of *Lc. lactis* SL3, *L. rhamnosus* LR5, *B. breve* BR3, or *B. lactis* BL3 were 10-fold concentrated, which were then used to determine the MICs of the test strains against *C. difficile*. The relationship between ½, 1, and 2 MICs of each strain indicates the determination was carried out precisely (Table 1). The MIC$_{50}$ determined ranged from 0.424 to 1.318 mg/ml. Among the test strains, *Lc. lactis* SL3 showed the strongest anti-*C. difficile* activity (MIC$_{50}$; 0.424 mg/ml), followed by *L. rhamnosus* LR5 (0.719 mg/ml), *B. lactis* BL3 (0.765 mg/ml), and *B. breve* BR3 (1.318 mg/ml). The MIC$_{50}$ of SL3 was comparable to that reported for purified nisin (MIC$_{50}$ of 0.256 mg/L) (24). The inhibitory spectra of lactococcal bacteriocins, other

| Time | 1/2 MIC | 1 MIC | 2 MIC | Control |
|------|---------|-------|-------|---------|
| 3 h  | ![Plot](image1.png) | ![Plot](image2.png) | ![Plot](image3.png) | ![Plot](image4.png) |
| 24 h | ![Plot](image5.png) | ![Plot](image6.png) | ![Plot](image7.png) | ![Plot](image8.png) |

**Fig. 3.** Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *L. rhamnosus* LR5 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, Q1: dead cell, and Q3: injured cell and debris.

| Time | 1/2 MIC | 1 MIC | 2 MIC | Control |
|------|---------|-------|-------|---------|
| 3 h  | ![Plot](image9.png) | ![Plot](image10.png) | ![Plot](image11.png) | ![Plot](image12.png) |
| 24 h | ![Plot](image13.png) | ![Plot](image14.png) | ![Plot](image15.png) | ![Plot](image16.png) |

**Fig. 4.** Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *B. lactis* BL3 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, Q1: dead cell, and Q3: injured cell and debris.
than nisin, produced by different species and strains of lactococci were described before (25, 26). Nisin has been known for its inhibition of clostridial spores for a long time (27). Since *Lc. lactis* SL3 showed an anti-*C. difficile* activity as effective as purified nisin, the responsible substance for the activity needs to be characterized.

*C. difficile* cells after incubation with ½, 1, and 2 MIC of the CFS of each test strains for 24 hr were stained with the BacLight kit and subjected to FCMs in order to determine the proportion of live and dead *C. difficile* cells in the sam-

| Time | 1/2 MIC | 1 MIC | 2 MIC | Control |
|------|---------|-------|-------|---------|
| 3 h  | ![Chart](chart1.png) | ![Chart](chart2.png) | ![Chart](chart3.png) | ![Chart](chart4.png) |
| 24 h | ![Chart](chart5.png) | ![Chart](chart6.png) | ![Chart](chart7.png) | ![Chart](chart8.png) |

**Fig. 5.** Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *B. breve* BR3 for 24 hr. All bacteria were previously stained with SYTO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, Q1: dead cell, and Q3: injured cell and debris.

**Fig. 6.** Fluorescent microscopic images of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *Lc. lactis* SL3, *L. rhamnosus* LR5, *B. lactis* BL3, and *B. breve* BR3. *C. difficile* cells were stained with Live/Dead Baclight kit after incubation with the cell-free supernatants. Live cells: green, dead cells: red, yellow and orange: injured.
ple. The results are summarized in Table 2. When treated with the CFS of SL3 at ½ MIC condition, the percentage of live C. difficile cells was 62.6% (Fig. 2). At 1 MIC, the level of live C. difficile cells were largely decreased and determined to be only 3.9%. Similarly, 2 MIC condition resulted in no viable C. difficile cells. On the other hand, viable cell counts of C. difficile after treatment with the CFS of SL3 were determined on agar plate, and showed a similar tendency toward decrease as observed in FCMs, in good agreement with each other (Data not shown). The analysis of FCM revealed that the proportion of live C. difficile cells were also decreased when treated with LR5 (Fig. 3), BL3 (Fig. 4), or BR3 (Fig. 5). However, a shift from live to dead cell occurred more quickly by SL3 than the others (Table 2).

A large change in the proportion of live and dead C difficile cells was also observed under fluorescent microscope (Fig. 6). When C. difficile cells were treated with the CFSs for 24 hr in ½, 1, and 2 MIC conditions, most of C. difficile the cells were observed to be severely damaged (orange) or dead (red). By contrast, the majority of C. difficile cells in the control (no treatment with CFSs) was found to be live (green).

Taken together, we showed that Lc. lactis SL3, L. rhamnos LR5, B. breve BR3, and B. lactis BL3 possess anti-C. difficile activity, among which SL3 appeared to have the strongest activity. Although the information regarding the level of C. difficile in the gut of patients suffering from CDI is sparse, patients with CDI are known to excrete ~10^4~10^7 C. difficile cells per day (Fig. 6). When C. difficile cells were treated with the CFSs for 24 hr in ½, 1, and 2 MIC conditions, most of C. difficile the cells were observed to be severely damaged (orange) or dead (red). By contrast, the majority of C. difficile cells in the control (no treatment with CFSs) was found to be live (green).

The Use of Probiotic Lactic Acid Bacteria for the Treatment of Clostridium difficile Infection

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