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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), *Lactococcuslactis* (SL3), *Bifidobacterium breve* (BR3), and *Bifidobacterium lactis* (BL3) were evaluated for their anti-*C. difficile* activity. Co-culture incubation of *C. difficile* (10^6 and 10^{10} 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 MIC_{50} 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 signicantly decreased recurrence rates (16.7%) compared to vancomycin and placebo (50%). The *S. boulardii* given with the low dose vancomycin or metronidazole was not signicantly 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

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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-dominat 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 Duoalc 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 rhmannos* 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 medium 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 Na_2HPO_4 (Sigma, St. Louis, MO, USA), 1.0 g/L KH_2PO_4 (Sigma), 0.25 g/L D-cycloserine (Sigma), 0.1 g/L $MgSO_4$ (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 10^6 CFU/ml for the mono-culture experiments and 10^6 or 10^{10} CFU/ml for the co-culture experiment. The test strains were inoculated at the concentration of 10^8, 10^9, or 10^{10} 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 (EC_{50}) 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 determination. All test CFSs were subjected to two-fold serial dilutions series with RCM broth respectively. Ten microliters each of the diluted solutions were put into 96 micro-titre plate wells. Then 90 μl of *C. difficile* cell suspension adjusted to 10^6 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 *Bac*Light kit™ (L7012, Invitrogen, USA). MIC_{50} 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 *Bac*Light 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 amplications. 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) of all the test strains (Fig. 1). At the lower concentration of *C. difficile* (10^6 CFU/ml), SL3 showed the strongest inhibitory activity (EC_{50} = 8.82 × 10^6 CFU/ml), whereas BR3 showed the lowest activity (EC_{50} = 1.64 × 10^9 CFU/ml). Similarly, at the higher concentration of *C. difficile* (10^{10} CFU/ml), SL3 showed the strongest inhibitory activity (EC_{50}: 5.08 × 10^8 CFU/ml), whereas

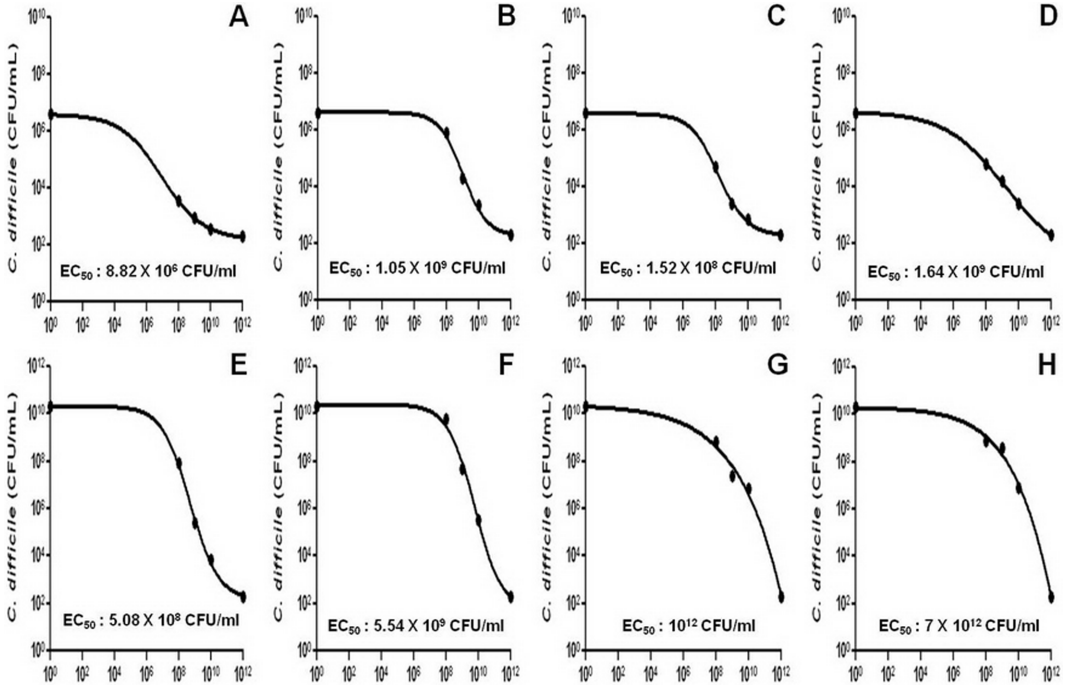

Fig. 1. EC_{50} of SL3, LR5, BL3 and BR3 co-cultured with *Clostridium difficile* KCCM 5009 in RCM broth anaerobically for 6 hr at 37°C. SL3 (A), LR5 (B), BL3 (C), and BR3 (D) at 10^8, 10^9, or 10^{10} CFU/ml were co-cultured with *C. difficile* at 10^6 CFU/ml for a simulation of disease prevention and SL3 (E), LR5 (F), BL3 (G), and BR3 (H) at 10^8, 10^9, or 10^{10} CFU/ml were co-cultured with *C. difficile* at 10^{10} CFU/ml for a simulation of disease treatment.

BR3 showed the lowest activity (EC_{50}: 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 MIC[a]	0.212	0.382	0.659	0.359
1 MIC[b]	0.424	0.765	1.318	0.719
2 MIC[c]	0.849	1.530	2.636	1.438

[a]½ MIC: minimum inhibitory concentration required to inhibit the growth of 0~50% of organisms.
[b]1 MIC (or MIC_{50}): minimum inhibitory concentration required to inhibit the growth of 50~90% of organisms.
[c]2 MIC (or MIC_{90}): 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	Cell percentage (%)												
		Control	SL3			LR5			BL3			BR3		
			½ MIC[a]	1 MIC[b]	2 MIC[c]	½ MIC[a]	1 MIC[b]	2 MIC[c]	½ MIC[a]	1 MIC[b]	2 MIC[c]	½ MIC[a]	1 MIC[b]	2 MIC[c]
0	Live	85.3	86.5	86.5	86.5	89.5	89.5	89.5	92.3	92.3	92.3	89.7	89.7	89.7
	Dead	14.7	13.5	13.5	13.5	10.5	10.5	10.5	7.7	7.7	7.7	10.3	10.3	10.3
3	Live	90.1	54	38.6	22.9	64.1	51.5	42.8	68.8	46.5	44.2	57.9	40.2	39.6
	Dead	9.9	46	61.4	77.1	35.9	49.5	57.2	31.2	53.5	55.8	42.1	59.8	60.4
24	Live	95.3	62.6	3.9	0	16.1	3.9	0.3	25.4	13.2	0.1	42.9	41.3	0.3
	Dead	4.7	37.4	96.1	100	83.9	96.1	99.7	74.6	86.8	99.9	57.1	58.7	99.7

[a]½ MIC: minimum inhibitory concentration required to inhibit the growth of 0~50% of organisms.
[b]1 MIC (or MIC_{50}): minimum inhibitory concentration required to inhibit the growth of 50~90% of organisms.
[c]2 MIC (or MIC_{90}): minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

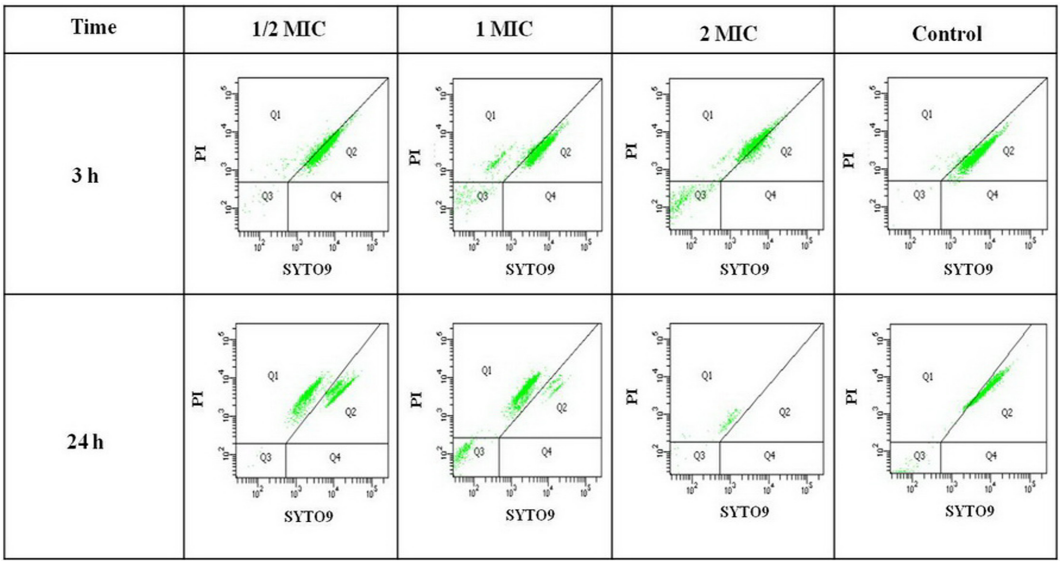

Fig. 2. Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *Lc. lactis* SL3 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, Q1: dead cell, and Q3: injured cell and debris.

bial activity against some Gram-positive pathogens such as *Listeria monocytogens* 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. rhmannos* 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_{90} of 0.256 mg/L) (24). The inhibitory spectra of lactococcal bacteriocins, other

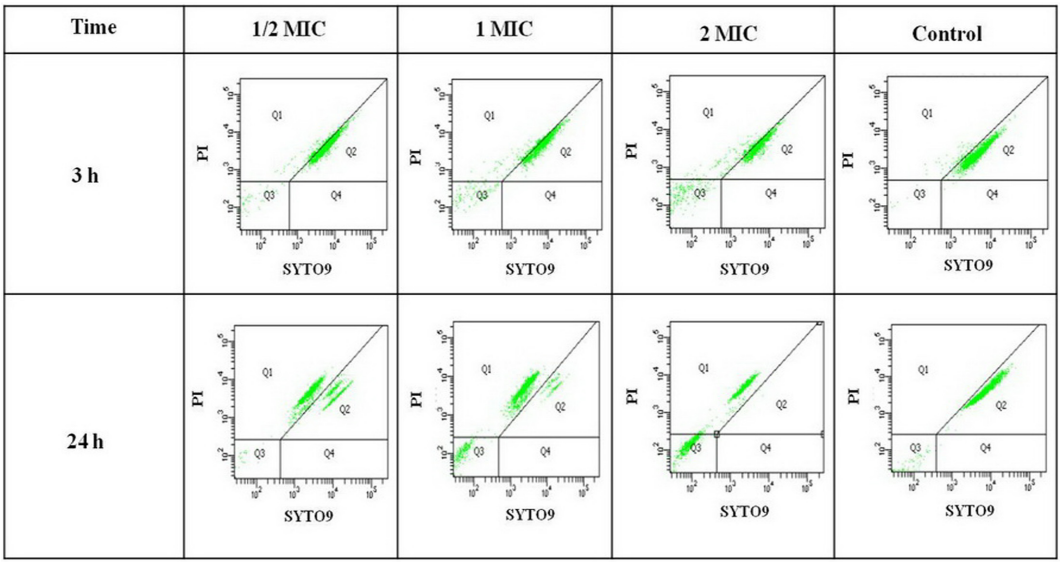

Fig. 3. Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *L. rhamnosus* LR5 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell,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

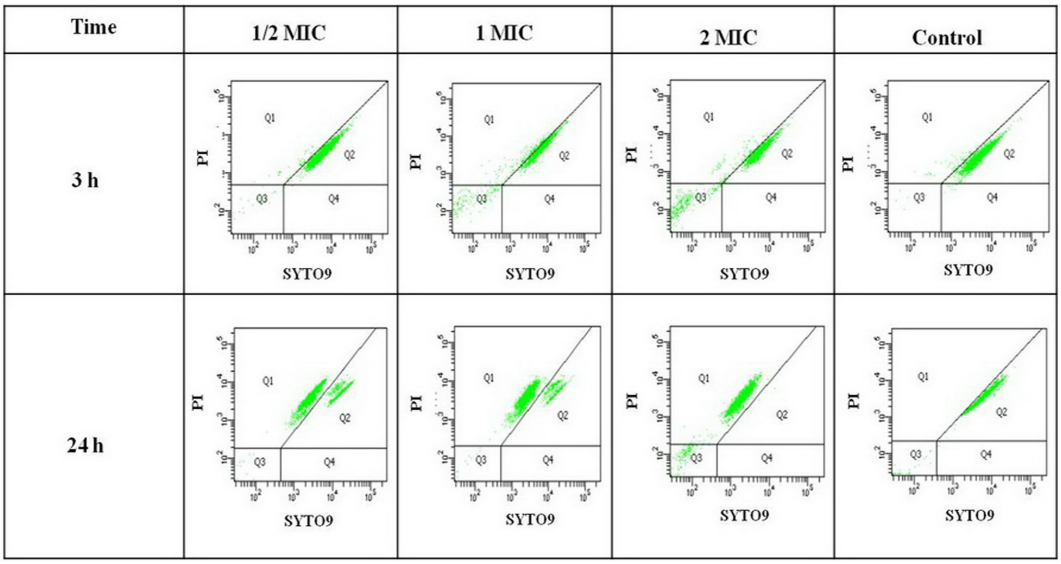

Fig. 4. Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *B. lactis* BL3 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell, 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 *Bac*Light kit and subjected to FCMs in order to determine the proportion of live and dead *C. difficile* cells in the sam-

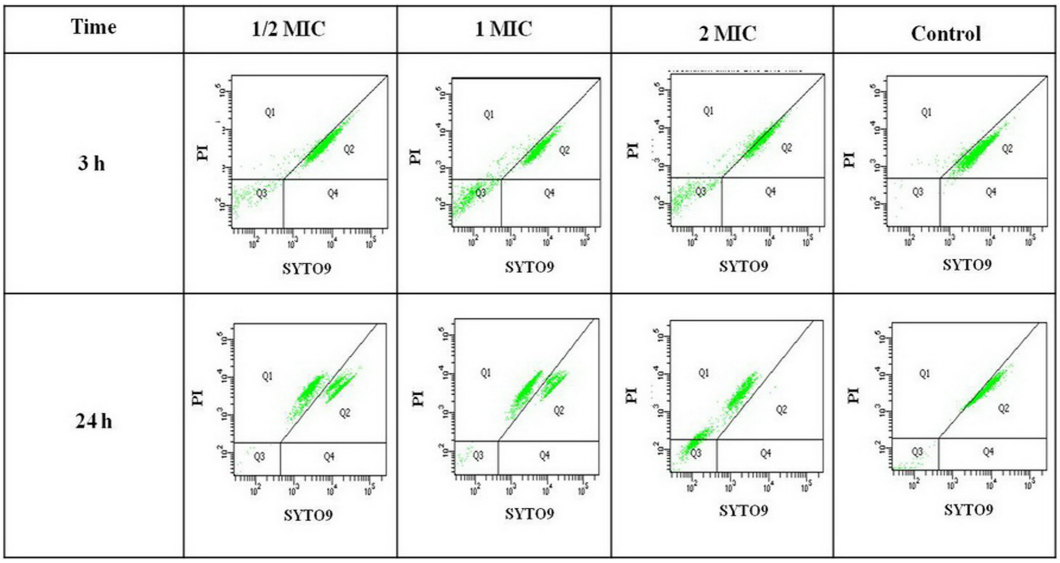

Fig. 5. Flow cytometric analysis of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *B. breve* BR3 for 24 hr. All bacteria were previously stained with STYO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. Q2: live cell,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

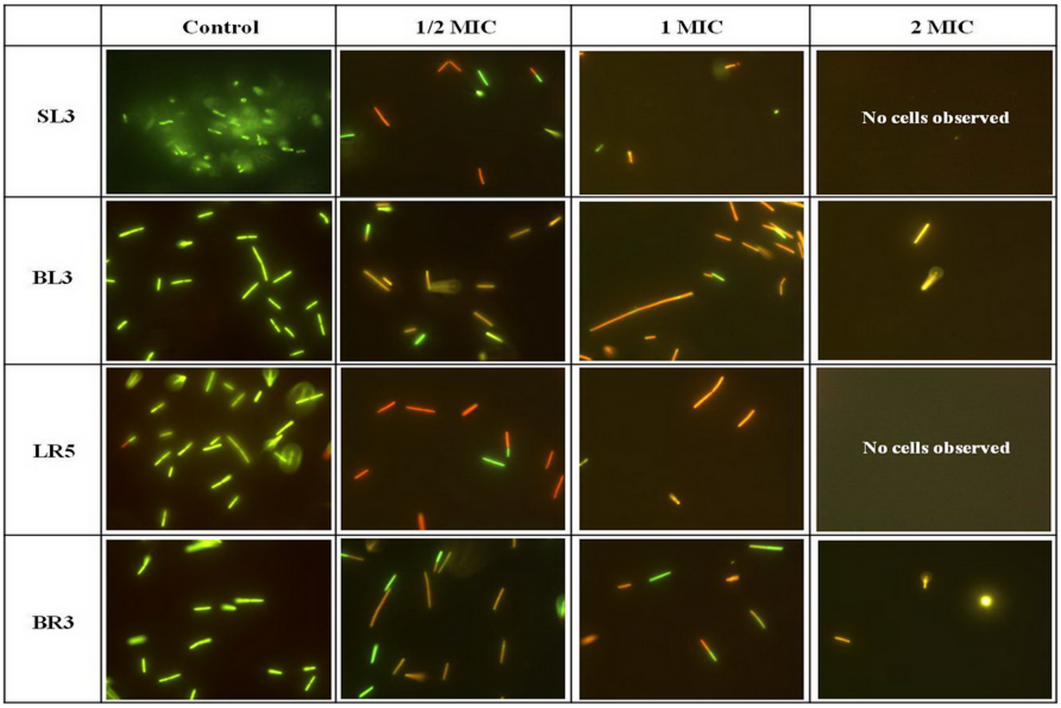

Fig. 6. Fluorescent microscopic images of *C. difficile* incubated with ½, 1, and 2 MIC cell-free supernatants of *Lc. lactis* SL3, *L. rhamnosus* LR5, *B. lactis* BL3, and *B. breve* BR3. *C. difficile* cells were stained with Live/Dead Baclight kit after incubation with the cell-free supernatants. Live cells: green, dead cells: red, yellow and orange: injured.

ples. 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 tendancy 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. rhmannos* 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 sprase, patients with CDI are known to excrete ~10^4~10^7 *C. difficile* (g/faeces) (28). Our results indicate that the use of *Lc. lactis* SL3 or its culture supernatant may be an efficient way for the control of CDI. In conclusion, this study may provide a basis for the use of probiotic LAB for treatment of CDI.

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