Lactobacillus acidophilus modulates the virulence of Clostridium difficile

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ABSTRACT

Clostridium difficile is a spore-forming, toxin-producing, anaerobic bacterium that colonizes the human gastrointestinal tract. This pathogen causes antibiotic-associated diarrhea and colitis in animals and humans. Antibiotic-associated diseases may be treated with probiotics, and interest is increasing in such uses of probiotics. This study investigated the effect of Lactobacillus strains on the quorum-sensing signals and toxin production of C. difficile. In addition, an in vivo experiment was designed to assess whether Lactobacillus acidophilus GP1B is able to control C. difficile-associated disease. Autoinducer-2 activity was measured for C. difficile using the Vibrio harveyi coupled bioluminescent assay. Cell extract (10 μg/mL) of L. acidophilus GP1B exhibited the highest inhibitory activity among L. acidophilus strains on the quorum-sensing signals and toxin production of C. difficile. Survival rates at 5 d for mice given the pathogen alone with L. acidophilus GP1B cell extract or L. acidophilus GP1B were 10, 70, and 80%, respectively. In addition, the lactic acid-produced L. acidophilus GP1B exhibits an inhibitory effect against the growth of C. difficile. Both the L. acidophilus GP1B and GP1B cell extract have significant antipathogenic effects on C. difficile.

Key words: Clostridium difficile ribotype 027, Lactobacillus acidophilus GP1B, quorum sensing, autoinducer-2

INTRODUCTION

Clostridium difficile is a spore-forming, gram-positive, anaerobic bacillus that produces 2 exotoxins (toxin A and toxin B). Toxin A and B, encoded by tcdA and tcdB, are expressed in late-log and stationary growth phases. The 19.6-kbp pathogenicity locus is formed by tcdA and tcdB together with 3 accessory genes: tcdC, tcdE, and tcdR. This organism is the most common cause of infectious diarrhea in hospitalized patients, but a highly virulent strain (ribotype 027) has emerged, which is responsible for community-acquired infections in younger and lower-risk populations (McDonald et al., 2005). Thus, C. difficile-associated disease (CDAD) is increasing in incidence and severity. Moreover, its treatment may be becoming more difficult. Recently, according to numerous studies, C. difficile has been isolated from raw foods intended for human consumption in the United States (Songer et al., 2009), Canada (Rodriguez-Palacios et al., 2007; Weese et al., 2009), and Europe (Bakri et al., 2009; Von Abercron et al., 2009; Jöbstl et al., 2010). Therefore, C. difficile is a potentially food-borne pathogen (Rodriguez et al., 2013).

Quorum sensing (QS) is a bacterial cell-to-cell communication mechanism that involves the production, detection, and response to extracellular signaling molecules called autoinducers. Autoinducers accumulate in the environment as the bacterial population density increases, and bacteria detect a threshold concentration of these autoinducers, which leads to altered gene expression. Using these signal-response systems, bacteria act as a collective unit; that is, a multicellular entity, as opposed to individual cells all performing individual functions. Quorum sensing is involved in biofilm formation, and virulence factor secretion (Kaper and Sperandio, 2005; Bai and Rai, 2011; Rutherford and Bassler, 2012). Many pathogens synthesize an extracellular signal named LuxS or autoinducer-2 (AI-2), such as Escherichia coli O157:H7, Clostridium perfringens, and C. difficile. Thus, AI-2 has been implicated in control of virulence in pathogenic bacteria (Sperandio et al., 1999; Ohtani et al., 2002; Lee and Song, 2005).

Lactic acid bacteria such as Lactobacillus are important microorganisms in the healthy human microbiota (Lenoir-Wijnkoop et al., 2007). Lactic acid bacteria are beneficial microorganisms that have been associated with several probiotic effects in humans and animals, such as reduction of acute diarrhea and allergy (Szajewska et al., 2001; Ouwehand, 2007), relief of inflammatory bowel disease (Ewaschuk and Dieleman, 2006; Limdi et al., 2006) and antibiotic-associated gastrointestinal symptoms (Lenoir-Wijnkoop et al., 2007; Guglielmetti
et al., 2011), antiinflammatory effect (Tedelind et al., 2007; Maslowski et al., 2009), reduction of potentially pathogenic bacteria (Savard et al., 2011), and immunomodulatory effects (Bahrami et al., 2011). However, the ways in which probiotic bacteria elicit their health effects are not fully understood.

Some evidence exists that the expression of the locus of the enterocyte effacement (LEE) pathogenicity island in E. coli O157:H7 is downregulated by probiotics, and this may be mediated by the interference of the AI-2 autoinducer signaling system (Medellin-Peña et al., 2007). Lactobacillus acidophilus and bifidobacteria also exert antagonistic effects on the growth of pathogens such as Staphylococcus aureus, Salmonella Typhimurium, Yersinia enterocolitica, C. perfringens, E. coli O157:H7, and Campylobacter (Zeinhom et al., 2012; Mundi et al., 2013). Lactobacillus acidophilus GP1B stain is an intestinal isolate from swine and known to tolerate acid, assimilate cholesterol, and produce a thermostable bacteriocin (Han et al., 2007). The interference of QS signal on C. difficile through probiotics is poorly studied.

The purpose of this study was to evaluate the inhibitory activity of AI-2, toxin production, and transcriptional levels for virulence-associated genes in C. difficile culture by L. acidophilus GP1B.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Clostridium difficile PCR ribotype 027 was obtained from the Department of Pathobiology, University of Guelph (Guelph, ON, Canada). The organism was grown in Brain Heart Infusion (BHI; Difco, Detroit, MI) supplemented with 0.5% yeast extract (Difco) and 0.1% l-cysteine (Sigma-Aldrich, St. Louis, MO) with overnight incubation under anaerobic conditions at 37°C. For preparation of spores, C. difficile was incubated anaerobically at 37°C for 10 d to induce sporulation. The spores were scraped from BHI agar plates with inoculating loops and transferred to PBS. The spore suspension was then heated at 60°C for 20 min to inactivate vegetative cells. Finally, spores were resuspended in PBS at a concentration of 10⁷ cfu and stored at −20°C. Lactobacillus acidophilus GP1B was cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco) at 37°C for 18 h.

Preparation of Samples

Total viable cell numbers of L. acidophilus GP1B were adjusted to approximately 10⁸ cfu/mL for testing. To obtain heat-killed cells, overnight cultures were first washed twice with sterile 0.85% NaCl and resuspended with 0.85% NaCl (~10⁸ cfu/mL). Then, the cells were prepared by heating the bacteria to 95°C for 1 h. For the preparation of cell extract (CE), overnight-cultured Lactobacillus were centrifuged (8,000 × g, 30 min, 4°C), and cell pellets were washed and resuspended in PBS before being disrupted ultrasonically for 10 min at 1-min intervals on ice using a sonicator (Sonic, VCX 500, Newton, CT). Cell debris was removed by centrifugation (14,000 × g, 30 min at 4°C), and the sample was then sterilized by filtration (0.22-μm pore size; Sartorius, Epsom, UK).

Growth Changes of C. difficile

Clostridium difficile was grown overnight at 37°C in BHI containing 0.5% yeast extract and 0.1% l-cysteine under anaerobic conditions, diluted 1:100 in the same fresh medium containing 5 to 40 μg/mL of CE of L. acidophilus GP1B, and then grown at 37°C under anaerobic conditions. Growth kinetic experiments were performed with BHI broth at 37°C under anaerobic conditions.

AI-2 Activity Assays

The bacterial supernatants were assayed using the techniques developed by Surette and Bassler (1998). Clostridium difficile was grown overnight at 37°C in BHI, diluted 1:100 in the same fresh medium containing 5, 10, 20, and 40 μg/mL of CE or cell-free supernatants of L. acidophilus GP1B, and then grown at 37°C. The supernatant was passed through a 0.2-μm-pore-size cellulose nitrate membrane filter (Sartorius, Göttingen, Germany). The reporter strain, Vibrio harveyi BB170, was grown overnight in autoinducer bioassay medium; adjusted to pH 7.5 with 3 M KOH, which after autoclaving was supplemented with 10 mL of sterile 1 M potassium phosphate (pH 7.0), 1 mL of 10 μg/mL of riboflavin, and 1 mL of 1 mg/mL thiamine per liter of medium; and then diluted 1:5000 into fresh autoinducer bioassay medium. The cell-free supernatant of C. difficile was added to the diluted autoinducer bioassay medium at a concentration of 10% (vol/vol). The AI-2 activity was normalized as 100% activity by the light production of V. harveyi BB152 (positive control) and E. coli DH5α negative control. The luminescence values were measured with a Synergy HT multimode plate reader (BioTek, Winooski, VT). Each experiment was conducted in triplicate.

RNA Isolation

To obtain RNA for identification of the genes affected by CE of L. acidophilus GP1B, C. difficile was grown...
overnight in BHI, diluted 1:100 in the same medium containing 10 μg/mL of CE of L. acidophilus GP1B, and grown for 6 h at 37°C. The cells were harvested by centrifugation for 10 min at 4°C and 12,000 × g. The total RNA was isolated using an RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer’s protocols. The RNA was DNase-digested using the RNase-Free DNase Set (Qiagen Inc.). The RNA was assayed for quality and quantity in a Take 3 plate in a Synergy HT multimode plate reader (BioTek). Only the RNA samples with 260nm/280nm ratio between 1.8 and 2.0 and 260nm/230nm ratio greater than 2.0 were used for the analysis.

**Real-Time Quantitative PCR for Gene Transcription Analysis**

The cDNA was prepared from 50 ng of total RNA using Maxime RT PreMix (Random Primer) for the qRT-PCR kit (Intron, Seongnam, Korea) in accordance with the manufacturer’s recommendations. The expression level of respective genes was measured by real-time PCR using 2 × Prime QMaster Mix (Kapa Biosystems, Boston, MA). The relative expression level was determined using a CFX96TM Real-Time system (Bio-Rad, Hercules, CA). The reaction parameters for real-time PCR analysis were 94°C for 5 min followed by 40 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 60 s, and a final elongation step at 72°C for 5 min. All reactions were repeated more than 3 times. The genes to be analyzed and the sequences of primers to be used are shown in Table 1. Primers were designed based on our assembly consensus sequences. Primers were designed by using PRIMER3 version 4.0.0 (http://www.broadinstitute.org/genome_software/other/primer3.html). The housekeeping gene used rpoA as a reference gene (Metcalf et al., 2010). For comparative quantification, C. difficile virulence gene expression in the presence of CE of L. acidophilus GP1B were normalized to that of the housekeeping genes (rpoA) and expressed as fold change relative to the expression level of undifferentiated C. difficile virulence genes. The sample delta threshold cycle (ΔCt) (SΔCt) value was calculated as the difference between the Ct values of C. difficile virulence genes (luxS, tcdA, tcdB, and txeR). The ΔCt value of undifferentiated C. difficile virulence genes were used as a control ΔCt (CΔCt) value. The relative gene expression levels between the sample and the control were determined using the formula 2−(SΔCt−CΔCt).

**Coculture of C. difficile and L. acidophilus GP1B**

Clostridium difficile growth in coculture with L. acidophilus GP1B was investigated. For each pair of species, 4 combinations of initial cell concentrations were assessed. Viable counts and pH were determined at 3 time intervals. All flasks contained 10⁴ cells of C. difficile. Serial dilutions of L. acidophilus GP1B cultures were adjusted to equal volume to contain 10⁴ to 10⁸ cfu/mL of Lactobacillus. Each combination of ratios of organisms was tested only once, giving a total of 9 trials. All cultures were grown with MRS broth at 37°C under anaerobic conditions.

**Animal Study**

The C57BL/6 female mice (5 to 7 wk old) were purchased from Jackson Laboratory. All mice (80 female mice) used in the experiments were housed in groups of 4 per cage under the same conditions. Mice were housed in sterile cages containing bedding, food pellets, and water. Once every 2 d, mice were aseptically moved to sterile cages. Cage changes were performed in a laminar flow hood by a worker wearing a clean smock and gloves. Animals experienced a cycle of 12 h of light and 12 h of darkness. Animal tests were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Chonnam National University (CNU IACUC-YB-2012-45).

An antibiotic mixture of kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/
mL) was prepared in sterile drinking water (all antibiotics were purchased from Sigma-Aldrich). The antibiotic cocktail was administered in the drinking water on d −6 to −3, and then the animals were switched to regular autoclaved drinking water on d −3. On d −1, a single dose of clindamycin (10 mg/kg) was administered intraperitoneally, and they were infected on d 0 with *C. difficile* by gavage. The animals of treated group received *L. acidophilus* GP1B cells (~10^8 cfu), *L. acidophilus* GP1B CE (10 mg/d) or heat killed *L. acidophilus* GP1B (~10^8 cfu) by gavage for 4 d from d 1 (day of challenge) to d 4. Mice were observed for symptoms, survival, and weight, all of which were recorded daily (Figure 1).

**Histopathological Analysis**

Histopathological analysis was performed to evaluate mucosal damage and inflammation induced by the toxins. Mice were pretreated with an antibiotic cocktail and infected with *C. difficile* spores (~10^7 cfu). On d 1 to 4, mice received *L. acidophilus* GP1B (as preparations of CE, live cells, or dead cells), or PBS, and some were slaughtered on d 2. Cecum and colon tissues were removed from mice, fixed in biopsy specimen-embedding cassettes using freshly made ice-cold 4% paraformaldehyde or Carnoy’s fixative, and incubated overnight at 4°C. After fixation, the samples were washed twice with PBS and dehydrated before processing for embedding and sectioning. Deparaffinized 6-μm-thick sections were stained with hematoxylin and eosin for histological analysis.

**Bacterial Enumeration in Colon**

Homogenized colon was serially diluted in anaerobic diluent. The anaerobic diluents consisted of 4.5 g of KH2PO4, 6.0 g of Na2HPO4, 0.5 g of L-cysteine, and
0.5 g of Tween 80 per liter of H₂O, presaturated and sealed with 100% CO₂ gas. Then, aliquots of the appropriate dilutions were spread onto one nonselective agar plate and 3 selective agar plates. The nonselective agar was standard-plate-count agar (Difco) for aerobes. The selective agars were modified MRS agar (pH 5.0) for Lactobacillus spp., BL-NPNL agar (Teraguchi et al., 1978) for Bifidobacterium spp., and C. difficile selective agar (CDSA, Oxoid, Basingstoke, UK) for C. difficile. Standard plate count agar plates were incubated in anaerobic conditions at 37°C for 48 h, and MRS, BL-NPNL, and CDSA plates were grown for 48 h in an anaerobic chamber (Concept 400, Ruskinn Technology, Leeds, UK). After incubation, each plate was examined for the presence of bacterial colonies. For Lactobacillus spp. and Bifidobacterium spp. identified, colony and cellular morphologies, Gram reaction, spore formation, aerobic growth, and 16s rDNA sequencing were conducted.

**Statistical Analyses**

The experiments were replicated 3 times. All data were expressed as mean ± standard error and were analyzed by one-way ANOVA using SAS (SAS Institute Inc., 2006). Differences among means were tested for significance ($P < 0.05$) by Tukey’s test for multiple comparisons. Survivals were calculated according to the Kaplan-Meier method (Kaplan and Meier, 1958). A $P < 0.05$ was considered statistically significant.

**RESULTS AND DISCUSSION**

**Growth and AI-2 Production by C. difficile**

As shown in Figure 2, optical density at 600 nm of C. difficile increased from 0.1 to 1.3 over an incubation time of 24 h. The AI-2 production of C. difficile was affected by the growth stage, and the maximum induction of AI-2 was exhibited at 9 h of incubation. Similar to other C. difficile strains (Carter et al., 2005; Lee and Song, 2005), the AI-2 production was maximally induced during the mid-exponential growth stage and decreased gradually (Figure 2).

**Inhibitory Effect of L. acidophilus GP1B on AI-2 Production**

To determine whether CE of L. acidophilus GP1B inhibit the AI-2 production of C. difficile, CE was added to the culture of C. difficile (9 h) at concentration of 0, 5, 10, 20, and 40 μg/mL. When used in 10, 20, and 40 μg/mL of CE, AI-2 production was found to reduce bioluminescence to approximately 54% of the level induced by wild-type V. harveyi BB170. A 5-μg/mL concentration of CE showed only 20% inhibition rate compared with the control (0 μg/mL; Figure 3A).

Figure 3B shows the inhibitory effect of the CE of L. acidophilus GP1B on the growth of C. difficile in BHI broth containing various concentrations for the CE of L. acidophilus GP1B (0, 5, 10, 20, and 40 μg/mL) at 37°C under anaerobic conditions. With addition of 40
μg of CE, the growth of C. difficile was slightly reduced, whereas, 5, 10, and 20 μg/mL of CE of L. acidophilus GP1B did not affect the bacterial growth. Therefore, the 10 μg/mL of CE was used in further investigations because 10 μg showed the most inhibition of AI-2 production without any disturbance in C. difficile growth.

To confirm the inhibition of AI-2 production, we compared the inhibition rate during incubation of C. difficile in BHI broth that was supplemented with 10 μg/mL of CE (Figure 4). The inhibition rate of AI-2 productions exhibited 65% at 6 h and 30% at 9 h compared with the control. Even though the maximum AI-2 production was at 9 h of incubation (Figure 2), the maximum inhibition rate was at 6 h of incubation.

It has been suggested that probiotics confer protection against enteric infections by inhibiting the growth of food-borne pathogens, including Salmonella enterica serovar Typhimurium, Staph. aureus, C. perfringens, C. difficile, and pathogenie E. coli (Silva et al., 1987; Chattean et al., 1993). In addition, single and combinations of probiotics have been shown to effectively inhibit pathogens (Chapman et al., 2012). It has been reported by other workers that L. acidophilus strains inhibit QS and thereby affect virulence of E. coli O157:H7 (Medel-lin-Peña et al., 2007; Kim et al., 2012; Zeinhom et al., 2012) and Campylobacter (Mundi et al., 2013). Epigallocatechin gallate, a major catechin compound found in tea extracts, attenuates the virulence phenotypes and the gene expression regulated by QS in E. coli O157:H7 (Lee et al., 2009).

**Effect of L. acidophilus GP1B on the Expression of Virulence Gene in C. difficile**

In this study, levels of expression of genes associated with AI-2 production (luxS) and virulence (tcdA, tcdB, and txeR) were determined by real-time PCR. Figure 5 shows the expression of virulence genes of C. difficile in the presence of CE of L. acidophilus GP1B. The amplification efficiency for each target (luxS, tcdA, tcdB, and txeR) and housekeeping gene (rpoA) was derived from a standard curve plotted as the cycle threshold (ΔCt) versus the copies. The results were expressed as ratios of luxS, tcdA, tcdB, and txeR mRNA to rpoA mRNA.

The transcription levels of the luxS and txeR genes were decreased to 31 and 43%, respectively. Also, the control levels in the presence of the CE of L. acidophilus GP1B decreased. These reduced levels of expression appeared to affect the transcription of tcdA and tcdB, which were reduced to 31 and 23% of the control values, respectively. The downregulation of luxS coupled with
the reduced level of AI-2 were observed in the presence of the GP1B extract. This provided more evidence that this quorum sensing system is involved in the control of virulence in C. difficile, as suggested by Lee and Song (2005). At the 10-μg/mL concentration, the CE of L. acidophilus GP1B decreased the pathogenicity of C. difficile by inhibiting QS, thereby lowering the expression of QS-regulated toxin genes (tcdA and tcdB) and sigma factor txeR (Figure 5). During infection, C. difficile produces 2 major virulence determinants, toxin A (tcdA) and toxin B (tcdB). Toxin A and toxin B both are large monoglucosyltransferases that catalyze the glucosylation, and hence inactivation, of Rho-GTPases (small regulatory proteins of the eukaryote actin cell cytoskeleton). This leads to disorganization of the cell cytoskeleton and cell death and causes extensive colonic inflammation and epithelial tissue damage in the infected host (Kuehne et al., 2010; Davies et al., 2011; Carter et al., 2012). The product of the txeR gene, positioned just upstream of the pathogenicity locus, was shown to be needed for toxin gene expression and to activate promoter-specific transcription of the toxin genes in conjunction with RNA polymerases from C. difficile (Mani and Dupuy, 2001). The results of real-time PCR assay verified that transcription of the genes encoding for virulence factors was decreased by the CE of L. acidophilus GP1B.

**Inactivation of C. difficile Cocultured with L. acidophilus GP1B**

The present in vitro study revealed that L. acidophilus GP1B exerts a growth inhibitory effect on C. difficile when they are cocultured (Figure 6A). Interestingly, such activity was observed at less than pH 4 (Figure 6B). These data suggest that the bactericidal effect of L. acidophilus GP1B on growth of C. difficile depends on its lactic acid production and pH reductive effect. Although the mechanism of action is poorly understood, probiotics are reported to exhibit an inhibitory effect against the growth of various pathogens, including Salmonella enterica serovar Typhimurium, Staph. aureus, C. perfringens, C. difficile, and pathogenic E. coli (Silva et al., 1987; Meurman et al., 1995; Carey et al., 2008). For instance, probiotics are believed to interfere with the growth of pathogens by production of antibacterial molecules, such as organic acids, hydrogen peroxide, and antimicrobial molecules (bacteriocins; McGroarty and Reid, 1988; Vandenbergh, 1993; Holzapfel et al., 1995; Fayol-Messaoudi et al., 2005; Dethlefsen et al., 2006; Mellefont et al., 2008). Probiotics, including Lactobacillus lactis, Lactobacillus casei Shirota, and L. acidophilus YIT 0070 strains reduced the growth of E. coli O157:H7 by lactic acid production and pH reductive effect (Brashears et al., 1998; Ogawa et al., 2001).
In particular, Asahara et al. (2004) determined that the antiinfectious activity of probiotic bifidobacteria against STEC O157:H7 was related to the combinatory effect of high acetic acid concentration and low pH in a fatal mouse STEC infection model.

**Effect of L. acidophilus GP1B on CDAD Mice**

Taken together, probiotics may be a valuable alternative therapy for CDAD by restoring intestinal microbial balance. Figure 1 provides a schematic representation of the *C. difficile*–infection (CDI) model used in this study. This study used an antibiotic mixture (kanamycin, gentamicin, colistin, metronidazole, and vancomycin), which has been used previously to disrupt the intestinal microbiota in mice. On study d 1 through 4, mice were given live *L. acidophilus* GP1B cells (~10⁸ cfu), heat-killed *L. acidophilus* GP1B (ca 10⁸ cfu), and *L. acidophilus* GP1B CE (10 mg/d) by gavage. Body weight and survival rate data were collected daily on d 0 through 4. On d 2, some of the mice were euthanized, and the colons were removed for measuring morphometric, biochemical, and histological indices of colitis. The disease status of the animals was also assessed by monitoring *C. difficile* in the mouse feces using a PCR assay targeting the *tdcA* gene (Figure 7A).

All mice showed symptoms of CDAD, such as weight loss, hunching posture, and diarrhea. Consistent with published reports for this mouse model (Chen et al., 2008), all mice became moribund on d 2 or 3 following infection. However, as shown in Figure 7A, *L. acidophilus* GP1B protected mice from death, with 20, 30, and 50% mortality observed in mice treated with live cells of *L. acidophilus* GP1B, heat-killed *L. acidophilus* GP1B, and *L. acidophilus* GP1B CE, respectively, as compared with 90% mortality in mice that received no treatment (control). The presence of *L. acidophilus* GP1B provides significant protection of CDAD mice from *C. difficile*–induced death (*P < 0.05*). However, no statistically significant difference existed in the BW profiles between any of the treatment groups. Mice in the control and 4 treatment groups lost approximately 10 to 15% of their weight between d 0 and 3 and then began to gain weight. Control-group mice increased weight profiles from d 2 onward because the measurements for that period were obviously performed only on surviving mice (Figure 7B). *Clostridium difficile* is the most common clinical problem occurring in acute care settings; approximately 25% of patients treated for CDAD with metronidazole or vancomycin experience recurrent symptoms, and the incidence and severity of *C. difficile* appear to be increasing (Barbut et al., 1996; Bartlett, 2002; Katz, 2006; Zar et al., 2007). The prevention and therapy of CDAD are significant objectives, and better treatments are needed. Several different probiotics, including *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG, have been evaluated in the prevention and treatment of CDAD in adults and children (Floch et al., 2011; Na and Kelly, 2011; Hell et al., 2013).
Inhibitory Effects of L. acidophilus GP1B on Inflammation in Intestines

This study investigated whether L. acidophilus GP1B protects both the cecal and colonic tissue from damage during C. difficile infection. A mouse model of CDAD is associated with a colonic histopathology that includes submucosal edema, epithelial necrosis, mucosal proliferation, and the presence of inflammatory cells (Chen et al., 2008). Histologic analysis of colon and cecum from these mice revealed that L. acidophilus GP1B protects tissues of the lower intestine from damage during C. difficile infection.

Inflammatory cell infiltration, edema, and epithelial cell loss in the colon and cecum are evident in the mice, which were only administered PBS (Figure 8, CDI group, arrows). In contrast, intestinal tissues and epithelial cells in L. acidophilus GP1B–treated mice maintain structural integrity. In addition, intestinal tissues and intestinal epithelial cells of the other treatment group maintained structural integrity but some damage was present. Thus, epithelial cell loss in the colon and cecum was significantly reduced in CDAD mice following treatment, suggesting that L. acidophilus GP1B protects intestinal epithelial cells from apoptosis (Figure 8).
Effect of L. acidophilus GP1B on Colon Microbiota

The effect of *L. acidophilus* GP1B on the changes of *C. difficile* in the gut is shown in Figure 9. When live-cell (~$10^8$ cfu) and CE (10 mg/d) of *L. acidophilus* GP1B were administrated to CDAD mice, a decrease in the viable cells of *C. difficile* was observed. From an initial number of log 8.2 cfu/mL in colon, *L. acidophilus* GP1B or CE allowed the decrease to log 1.5 (live-cell) and 0.8 (CE) cfu/mL, respectively ($P < 0.05$). Interestingly, viable cells of *Lactobacillus* in colon increased with decreasing *C. difficile*, whereas the total number of aerobes did not changed. This result indicates that *L. acidophilus* GP1B may be effective probiotics in alleviating viable *C. difficile* cells in colon. In addition, *L. acidophilus* GP1B and GP1B CE are believed to positively affect the growth of *Lactobacillus* sp.

Probiotics are known to have a beneficial effect on the growth of gut microbiota. Although individual variation in the gut microbiota is possible, most intestinal microbes belong to the *Firmicutes* and *Bacteroidetes* families (Dethlefsen et al., 2006; Ley et al., 2006). Subsequent environmental influences, such as diet, host genetics, medication use, and exposure to infectious agents, affect the resultant gut microbial community (Dethlefsen et al., 2006).

It has been reported that administration of antibiotics could result in change in the gut microbial community (Löfmark et al., 2006; Jernberg et al., 2007; Dethlefsen et al., 2008). The CDI model may result from a loss of the intrinsic colonization resistance of the
Figure 8. Representative colon and cecum histology pictures about effects of *Lactobacillus acidophilus* GP1B on *Clostridium difficile* in *C. difficile* infection (CDI) mouse model. Scale bar = 200 μm. The arrows indicate the loss of epithelial cells in colon and cecum. Color version available in the online PDF.
gut microbiota (Mylonakis et al., 2001; Bartlett, 2008). Reeves et al. (2011) reported that the loss of colonization resistance against \textit{C. difficile} following antibiotic administration was not directly related to changes in the overall density of the gut microbiota. In particular, studies have recently determined that the antinfectious activity of probiotics against \textit{E. coli} O157:H7 was related to the combinatory effect of high acetic acid concentration and low pH (Asahara et al., 2004). \textit{Clostridium difficile} is an inhabitant of the human intestinal tract, and it has the opportunity to interact with a variety of intestinal organisms. It is likely that normal commensal bacteria including \textit{L. acidophilus} GP1B suppress the overgrowth of \textit{C. difficile} and may protect the intestinal tract from pathogens.

\textbf{CONCLUSIONS}

The CE of \textit{L. acidophilus} GP1B was capable of interfering with QS in \textit{C. difficile} by decreasing AI-2 production. Furthermore, the addition of a CE of \textit{L. acidophilus} GP1B resulted in downregulation of virulence genes in \textit{C. difficile} at the level of mRNA. \textit{Lactobacillus acidophilus} GP1B also exhibits an inhibitory effect against the growth of \textit{C. difficile} in the CDI mouse model, which may be related to a reduced pH as a result of organic acids produced by the probiotic bacterium. Results indicated that the \textit{L. acidophilus} GP1B used in this study did elicit a bactericidal effect on \textit{C. difficile}. Overall, the results obtained in this study support that probiotic preparations have potential for both prevention and treatment of \textit{C. difficile}–associated diarrhea.

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