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ARTICLE

Beneficial Effect of *Bifidobacterium longum* ATCC 15707 on Survival Rate of *Clostridium difficile* Infection in Mice

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Abstract

Clostridium difficile infection (CDI) is the main cause of hospital-acquired diarrhea that can cause colitis or even death. The medical-treatment cost and deaths caused by CDI are increasing annually worldwide. New approaches for prevention and treatment of these infections are needed, such as the use of probiotics. Probiotics, including *Bifidobacterium* spp. and *Lactobacillus*, are microorganisms that confer a health benefit to the host when administered in adequate amounts. The effect of *Bifidobacterium longum* ATCC 15707 on infectious disease caused by *C. difficile* 027 was investigated in a mouse model. The survival rates for mice given the pathogen alone, and with live cells, or dead cells of *B. longum* were 40, 70, and 60%, respectively. In addition, the intestinal tissues of the *B. longum*-treated group maintained structural integrity with some degree of damage. These findings suggested that *B. longum* ATCC 15707 has a function in repressing the infectious disease caused by *C. difficile* 027.

Keywords *Clostridium difficile*, *Bifidobacterium longum* ATCC 15707, infection model, probiotics

Introduction

Clostridium difficile is an anaerobic Gram-positive, endospore-forming bacteria that causes deadly intestinal infections (Liu *et al*., 2014). *C. difficile* infection begins with spore germination in the gut of infected persons and subsequent toxin production causing symptoms ranging from mild diarrhea to severe and life-threatening pseudomembranous colitis in infected patients (Surawicz *et al*., 2013). *Clostridium difficile*-associated disease (CDAD) is one of the main causes of healthcare-associated infections worldwide. The recent increase in CDAD has placed a rising clinical and economic burden on health systems (Yin *et al*., 2015). Current treatment for CDAD that typically includes metronidazole, vancomycin, and fidaxomicin, is usually effective; however, disease recurrence occurs in many patients (Cornely *et al*., 2012; Louie *et al*., 2011; Mullane *et al*., 2011). Antibiotics treatment is notorious for high causal rates of CDAD, because *C. difficile* have antibiotic resistance. To address this issue in the treatment of CDAD, new strategies such as probiotics are being explored.

Probiotics are nonpathogenic microorganisms that can resist digestion within the

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small intestine of the host and reach the colon alive (Gan *et al*., 2014); in addition, they confer beneficial health-effects when administered in adequate amount. Dairy-based foods containing intestinal species of lactobacilli, bifidobacteria, and others strains are the major source of probiotics for humans (Kim *et al*., 2012). Bifidobacteria are indigenous members of the human intestinal microbiota, comprising up to 90% of all bacteria in fecal samples of breast-fed infants (Salazar *et al*., 2012; Yuan *et al*., 2008). Bifidobacteria has the potential for use in enhancing digestive health and preventing disease. In particular, bifidobacteria are known to confer enteric protection against pathogenicity by inhibiting the growth of pathogens, including *Escherichia coli* O157:H7 (Asahara *et al*., 2004), *Helicobacter pylori* (Collado *et al*., 2005), *Listeria monocytogenes* (Touré *et al*., 2003), and *Clostridium difficile* (Kondepudi *et al*., 2012). In this study, we aimed at evaluating probiotics as a new treatment method for reducing the CDAD symptom caused by *Bifidobacterium* spp.

Materials and Methods

Bacterial cultures

Clostridium difficile PCR ribotype 027 was obtained from the Department of Pathobiology, University of Guelph (Canada). Prior to the experiments, stock culture was stored at -80°C in brain heart infusion (BHI) broth (Difco Laboratories, USA) containing 17% (v/v) glycerol as a cryoprotectant and supplemented with 0.5% yeast extract and 0.1% L-cysteine (BHIS). The strain was subcultured in BHIS broth and incubated overnight at 37°C under anaerobic conditions. *Bifidobacterium longum* ATCC 15707 was grown under anaerobic conditions at 37°C in blood-glucose-liver (BL) broth.

Co-culture of *C. difficile* and *B. longum* ATCC 15707

C. difficile growth in co-culture with *B. longum* ATCC 15707 was investigated. For each pair of species, three combinations of the initial cell concentrations were assessed. Viable counts and pH were determined at four time-intervals. All flasks contained 10^5 cells of *C. difficile*. Decimal dilution series (10^5 to 10^8 CFU/mL) of *B. longum* ATCC 15707 cultures were adjusted to equal volume. Experiments were conducted in triplicate and repeated three times. *C. difficile* cultures were grown in MRS broth and *B. longum* ATCC 15707 were grown in MRS broth supplemented with 0.1% L-cysteine at 37°C under anaerobic conditions. For enumeration of viable cells, samples were inoculated onto the CCFA agar (for *C. difficile*) and onto the BL-NPNL agar (for *B. longum* ATCC 15707), respectively, and incubated anaerobically at 37°C for 48 h (George *et al*., 1979; Teraguchi *et al*., 1978).

The experiment with mice

C57BL/6 female mice (5-7 wk old) were purchased from Jackson Laboratory (Bar Harbor, USA). Sixty female mice were housed in groups of 4 per cage under the same conditions, in sterile cages containing bedding, food pellets, and water. For each experiment, mice were randomly assigned to a given treatment group. Each experimental condition was tested in triplicate. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2012-45). The mouse model of *C. difficile* infection (CDI) was based on a previously reported method (Yun *et al*., 2014) with slight modification. An antibiotic mixture comprising 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 for 3 d, after which, the animals were provided with regular autoclaved drinking water. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally 1 d before the *C. difficile* challenge. Thereafter, the animals were infected by oral gavage with 7.0 Log CFU/mL of *C. difficile* PCR ribotype 027. We examined the effects of orally administered *B. longum* ATCC 15707 in the mouse model of CDI. The treatment groups received *B. longum* ATCC 15707 cells (~10^8 CFU/mL), or heat-killed *B. longum* ATCC (~10^8 CFU/mL) by gavage from 1 d to 4 d. The animals were monitored daily for symptoms such as diarrhea, hunched posture, and weight loss (Fig. 1). Body weight and survival data were collected daily on days 0 through 4. On day 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 *tcdA* gene (Yun *et al*., 2014).

Histopathological analysis was performed to evaluate mucosal damage and inflammation induced by the toxins. Some mice were euthanized on day 2, and the colon was excised to measure morphometric and histological indices of colitis. Cecal and colonic tissues were collected from

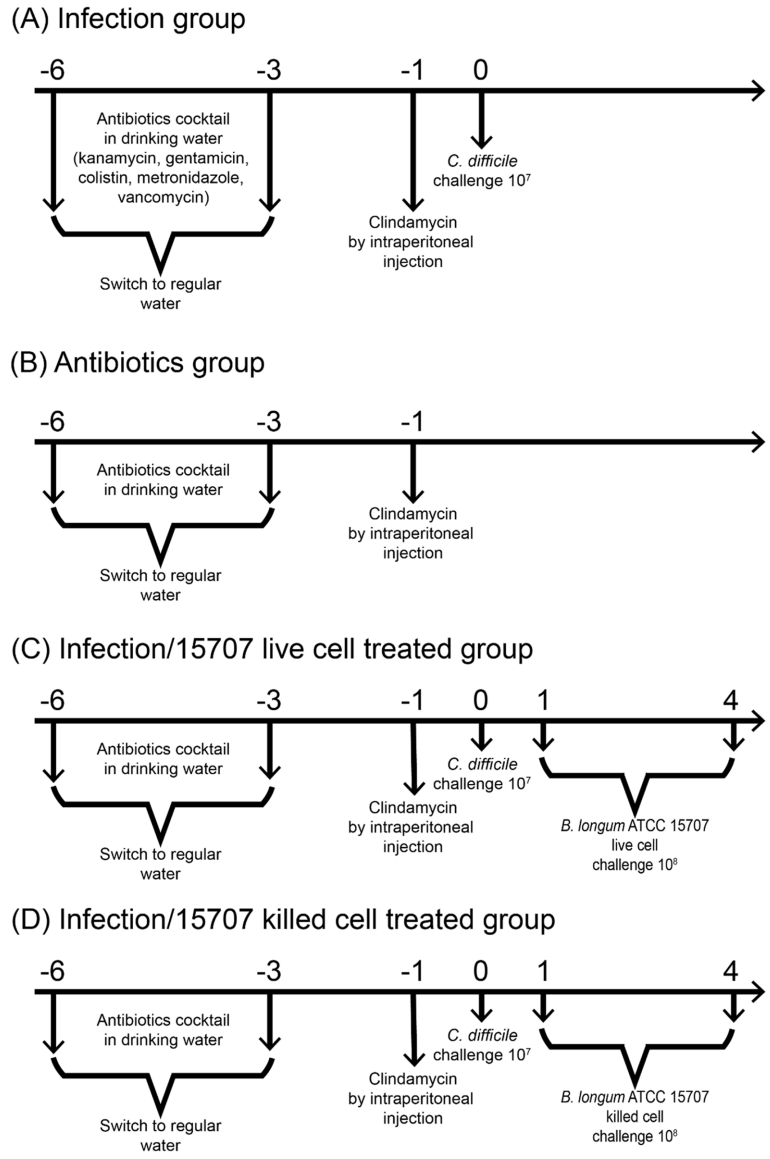

Fig. 1. Schematic overview of the CDI mouse model experimental design.

the 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 the fixation, the samples were washed twice with PBS and dehydrated prior to processing for embedding and sectioning. For histological analysis, deparaffinized 6-μm-thick sections were stained with hematoxylin and eosin (Yun *et al*., 2014).

Statistical analyses

The experiments were repeated thrice. The data are expressed as mean±standard error. The differences between means were evaluated by using one-way analysis of variance followed by the Bonferroni procedure for multiple-group comparison. Survival was evaluated using the Kaplan-Meier method. Differences with $p<0.05$ were considered as statistically significant.

Results and Discussion

Inactivation of *C. difficile* co-cultured with *Bifidobacterium longum* ATCC 15707

As shown in Fig. 2, *B. longum* ATCC 15707 exerted a growth-inhibitory effect on *C. difficile* when co-cultured. The growth inhibition of *C. difficile* was based on the growth curve of *C. difficile* per inoculum density of *B.*

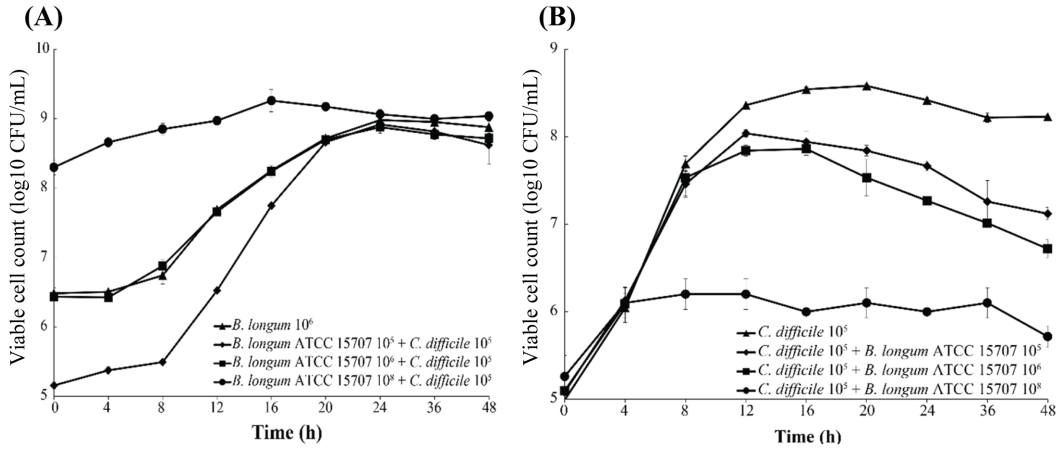

Fig. 2. Number of viable cell during incubation of co-cultured *Clostridium difficile* with *Bifidobacterium longum* ATCC 15707. (A) Effect of *C. difficile* on the growth of *B. longum* ATCC 15707, (B) Effect of *B. longum* ATCC 15707 on the growth of *C. difficile*.

longum ATCC 15707 (10^5 to 10^8 CFU/mL). The higher the inoculum density of *B. longum* ATCC 15707, growth of *C. difficile* has been suppressed. The efficacy was observed in all samples at the log phase of *B. longum* ATCC 15707. In addition, the growth inhibition of *C. difficile* occurred at pH values of <5.5 (Fig. 3), indicating that the inhibitory effect was possibly due to organic acid-production by bifidobacteria. *Bifidobacterium* species can produce different relative amounts of acetate, lactate and formate under the same conditions (Bezkorovainy *et al*., 1989). In this study, the main end product was usually lactic acid, which is in agreement with other reports of growth inhibition due to the ability of lactic acid bacteria to lower pH and produce organic acids (Annuk *et al*., 2003; Ridwan *et al*., 2008; Røssland *et al*., 2003; Tejero-Sariñena *et al*., 2012; Yun *et al*., 2014). Tejero-Sariñena *et al*. (2012) showed that the probiotic strains producing organic acid could inhibit the growth of pathogenic bacteria including *S. typhimurium*, *E. coli*, *E. faecalis*, *S. aureus* and *C. difficile*; and Yun *et al*. (2014) reported that the production of organic acids by *Lactobacillus acidophilus* could inhibit growth of *C. difficile*. The results of our study indicated that *B. longum* ATCC 15707 produced lactic acid that led to the lowering of pH and consequently, the inhibition of

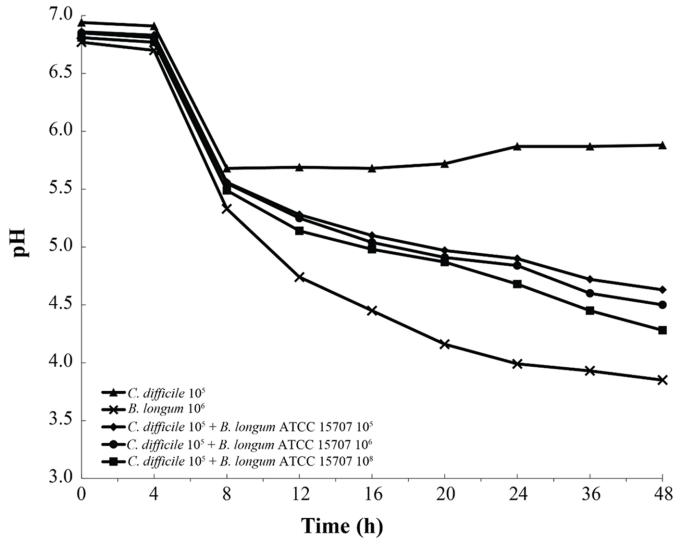

Fig. 3. Changes of pH during incubation of mixed *C. difficile* with *B. longum* ATCC 15707. All samples showed a decrease in pH. Inhibition of *C. difficile* growth was observed at < pH 5.5.

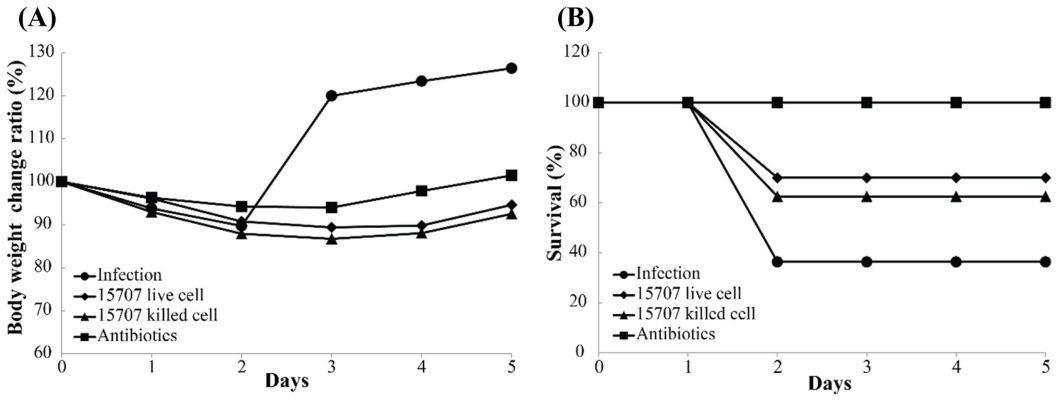

Fig. 4. Effect of *Bifidobacterium longum* ATCC 15707 on mice after the infection with *Clostridium difficile*. (A) Survival ratio by treatment with *B. longum* ATCC 15707 in *C. difficile*-infected (CDI) mice. (B) Change of BW ratio by treatment with *B. longum* ATCC 15707 in *C. difficile*-infected (CDI) mice. After two days, mortality was observed in every group.

C. difficile.

Effect of *B. longum* ATCC 15707 on CDAD Mice

We used a model of CDAD to determine the effects of *B. longum* ATCC 15707 on infection with *C. difficile*. This model has been previously used in studies on the effect of pre-and/or probiotics on CDI. Before infecting the mice with *C. difficile,* an antibiotic cocktail (kanamycin, gentamicin, colistin, metronidazole, and vancomycin) was used to disrupt the intestinal microbiota; and subsequently, *B. longum* ATCC 15707 was administered by gavage, in order to evaluate the effect of probiotics. All infected mice showed symptoms of CDAD, such as weight loss, hunching posture, and diarrhea. Consistent with some reports, most mice became moribund on days 2 or 3 post-infection. Nonetheless, the *B. longum* ATCC 15707-treated CDI mice showed a higher survival rate than that of the untreated CDI mice. As shown in Fig. 4, *B. longum* ATCC 15707 protected mice from death; whereas, untreated CDI mice showed 64% mortality; mice treated with live cells of *B. longum* ATCC 15707 showed 30% mortality; and mice treated with dead cells of *B. longum* ATCC 15707 showed 38% mortality. Probiotics have gained interest as a potential therapeutic modality for the prevention and treatment of CDAD. Probiotics, including *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG, have been most frequently evaluated in the prevention and treatment of CDAD in adults and children (Biller *et al*., 1995; Buts *et al*., 1993; Guslandi *et al*., 2000). Studies have shown that probiotic yogurt or mixed probiotics decrease the risk of CDCD (Hickson *et al*., 2007; Plummer *et al*., 2010; Szajewska *et al*., 2001). Moreover, various studies have indicated that probiotics induce improved gut health, via defending the gut against colonization by exogenous microorganisms (Wolvers *et al*., 2010), producing anti-microbial compounds including short chain fatty acids and bacteriocins that suppress the growth of pathogenic bacteria (De Vuyst *et al*., 2009), increasing the immunity of the host through their cell wall components (Matsuguchi *et al*., 2003). Our results suggested that organic acids produced by *B. longum* ATCC 15707 and cell membrane of *B. longum* ATCC 15707 could protect the CDAD mice. However, despite the reduced mortality in the *B. longum* ATCC 15707-treated CDI groups, body-weight profiles of the untreated CDI groups were more favorable, as compared to that of the treated groups because the measurements for that period could only be performed on surviving mice.

Histopathological analysis of gut tissues from mice infected with *C. difficile*

We investigated whether *B. longum* ATCC 15707 protects gut tissue from damage in the course of infection with *C. difficile*. In mice infected with *C. difficile*, colonic histopathology includes submucosal edema, epithelial necrosis, mucosal proliferation, and the presence of inflammatory cells (Chen *et al*., 2008). Histologic analysis of the colon and cecum of the infected mice revealed that *B. longum* ATCC 15707 alleviated *C. difficile* infection-induced damage to the lower intestinal tract tissues. The infection group showed inflammatory cell infiltration, edema, and epithelial cell loss by *C. difficile* toxins in the colon and cecum (Fig. 5); whereas, the intestinal tissues and epithelial cells in both live and dead cell *B. longum* ATCC 15707-treated mice maintained structural integrity. This

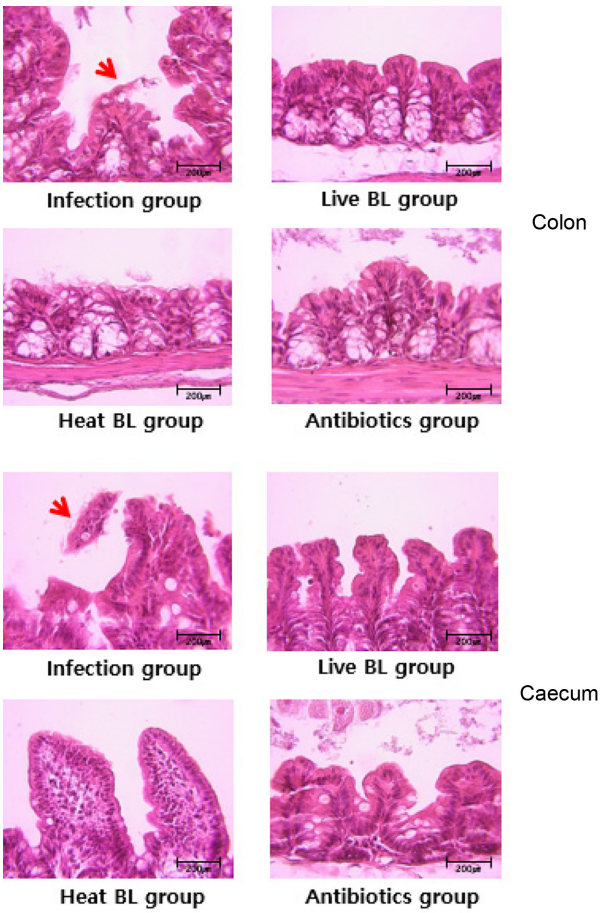

Fig. 5. Histopathologic analysis of the gut mucosa of C57BL/6 mice infected with *Clostridium difficile* and treated with *Bifidobacterium longum* ATCC 15707 (Live BL group) or heat-killed *B. longum* ATCC 15707 (Heat BL group). Histologic analysis of the lower intestinal tract of CDI mice revealed that *B. longum* ATCC 15707 alleviated the damage caused by *C. difficile* infection. Scale bar = 200 μm. The arrows indicate the loss of epithelial cells in the colon and cecum.

result suggested to have prevented the damage of intestinal tissue by preventing the growth of *C. difficile* due to organic acids produced by *B. longum* ATCC 15707. Also, a specific component of consists of the *B. longum* ATCC 15707 suggests that can prevent intestinal tissue damage caused by *C. difficile* infections. Similarly, some studies have shown that CDI symptom was alleviated after pre- and/or probiotics treatment (Yun *et al*., 2014; Yun *et al*., 2015). Thus, the finding that the epithelial cell loss in the lower intestinal tract was significantly reduced in CDAD mice following *B. longum* ATCC 15707 treatment, suggested that *B. longum* ATCC 15707 protects intestinal epithelial cells from apoptosis.

Conclusions

B. longum ATCC 15707 produced organic acid, which led to the lowering of pH and consequently, growth-inhibition of *C. difficile* when cocultured. *B. longum* ATCC 15707 also alleviated CDI symptoms in the CDI mouse model, which may be related to the reduced pH caused by organic acid-production by the probiotic bacterium. In addition, the cell wall components of *B. longum* ATCC 15707 were effective in alleviating CDI symptoms. Thus, this study supported that *B. longum* ATCC 15707 has potential for use in prevention as well as treatment of CDAD. Thus, *B. longum* ATCC 15707 may be utilized as an anti-pathogenic agent for CDAD.

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