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Synergistic Antimicrobial Effect of *Achyranthes japonica* Nakai Extracts and *Bifidobacterium* Supernatants Against *Clostridium difficile*

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Abstract The synergistic antimicrobial effect of *Achyranthes japonica* Nakai (AJN) and *Bifidobacterium* extracellular factors against *Clostridium difficile* were measured using a turbidity method. Each broth supernatant of *Bifidobacterium infantis* (68.8±0.02%) and *Bifidobacterium adolescentis* (33.2±0.2%) obtained by adding ethyl acetate soluble fractionate from *A. japonica* Nakai ethanolic extracts (AJNEA, 100 ppm, no inhibition) showed high synergistic antimicrobial activity against *C. difficile*. In addition, the antimicrobial activity in a laboratory medium and yogurt products against *C. difficile* were evaluated. In yogurt prepared with a starter 5 (*Lactobacillus acidophilus*: *Streptococcus thermophilus*: *B. adolescentis*=1 : 1 : 1) and a starter 4 (*L. acidophilus*: *S. thermophilus*: *B. infantis*=1 : 1 : 1) and 0.5% AJNEA powder, high antimicrobial effects were recorded that measured 79.0 and 65.2%, respectively. The results indicated the potential of AJN extract for use as an antimicrobial agent. In addition, the efficiency of the antimicrobial activity of the extracts was further improved in combination with lactic acid bacteria, which suggests that they have the potential to be used as a highly effective antibiotic-tolerant microorganism prevention system. Such a strategy can be used for alternative drugs or functional food additives for treatment of antibiotic-associated diarrhea.

Keywords: *Achyranthes japonica* Nakai, *Clostridium difficile*, lactic acid bacteria, synergistic antimicrobial effect, yogurt

Introduction

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that is an important nosocomial pathogen (1,2). It is associated with outbreaks of Pseudomembranous colitis, antibiotic-associated diarrhea, and other intestinal disorders such as diarrhea in children and adults (3,4). Although most antibiotics have been implicated with *C. difficile*-associated diarrhea, broad-spectrum antibiotics are among the antibiotics most commonly associated with this ailment (5-7). The most common treatment for antibiotic-associated diarrhea and *C. difficile* colitis is oral administration of bacitracin, vancomycin, metronidazole, teicoplanin, and fusidic acid.

Recent reports describe that an intake of probiotics for the treatment of *C. difficile* diarrhea can restore or optimize the microbial balance in the gut and promote good health. Probiotics are associated with a wide range of health benefits, including modulation of the immune function, protection against enteric infections and immunoinflammatory disorders including inflammatory bowel disease, the alleviation of lactose intolerance, the lowering of blood cholesterol and the prevention of cancer (8-13). Several investigations have shown the positive effects of probiotics supplements in the treatment of diarrhea. For example, the intake of lactic acid bacteria (LAB) has been shown to reduce the duration and severity of diarrhea in infants and children hospitalized for acute rotavirus diarrhea (9,14-18). Some probiotics including *Saccharomyces boulardii*, *Lactobacillus GG*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *B. longum* with *Lactobacillus acidophilus* given with antibiotics showed a significant reduction in the incidence rate of antibiotic-associated diarrhea (19-22).

In previous screening tests, it was shown by the authors that *Achyranthes japonica* Nakai (AJN) showed the highest inhibitory effect against *C. difficile* among various herb extracts. AJN is a perennial herb that has a wide distribution in Asian countries including South Korea, China, and Japan. AJN has various physiological effects including the control of blood circulation, the removal of extravasated blood, and the inteneration of joint actions. The root of *Achyranthes fauriei* or *Achyranthes bidentate* (Amaranthaceae) has also been used as a diuretic or an analgesic (23,24). It has been established that AJN contains various active components, including phytoecdysteroid, saponin, polysaccharide, 20-hydroxyecdysone, and inokosterone (25). However, thus far, both its effect on intestinal positive microflora including *Bifidobacterium* spp. as well as its antimicrobial activity against *C. difficile* has not been established.

The aim of this study was to determine the potential of using *A. japonica* Nakai ethyl acetate soluble fractionates (AJNEA) combined with *Bifidobacterium* strains to inhibit the growth of *C. difficile* in laboratory media and milk products.

Materials and Methods

Strains and culture conditions *Clostridium difficile* ATCC 9689 as an indicator strain was purchased from the National Institute of Health (Seoul, Korea). *C. difficile* was incubated in a reinforced clostridial medium (RCM) semi-broth (Difco, Detroit, MI, USA) at 37°C for 18 hr. Five types of *Bifidobacterium* strains from human origin, *B. longum* ATCC 15707, *B. breve* ATCC 15700, *B. bifidum* ATCC 29521, *B. infantis* ATCC 15697, and *B. adolescentis*

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ATCC 15703 were purchased from the National Institute of Health (NIH, Seoul, Korea). *L. bevies* ATCC 14869, *Lactobacillus helveticus* ATCC 15009, *Streptococcus thermophilus* KCTC 21185. *Lactobacillus bulgaricus* CH-2, ABT YC-180, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus rhamnosus* GG IFO 3863 were purchased from Hansen's Laboratory of Denmark and the NIH (Seoul, Korea). Lactic acid bacteria were cultured in a trypticase-proteose peptone-yeast extract (TPY) broth supplemented with 0.5% glucose and 0.05% L-cysteine HCl or Lactobacilli MRS broth (Difco) and were incubated for 18 hr at 37°C. Stock cultures of Bifidobacteria and *C. difficile* were maintained at −80°C using sucrose 0.3 M as a cryoprotectant and were reactivated twice before the experiments commenced.

Preparation of extracellular factors from LAB LAB was harvested by centrifugation at 1,500×g for 10 min, after an incubation period of 18 hr at 37°C. Each supernatant was then collected in a new tube. Each culture supernatant was lyophilized by using a freeze dryer (Eyela Ltd., Tokyo, Japan). Each powdered supernatant of LAB was prepared by adding the appropriate volume of a RCM media solution and was filtered using a syringe filter (0.45 μm) to test for antimicrobial activity.

Preparation of AJN ethanolic extract AJN was purchased from Kyungdong herbal market (Seoul, Korea) in 2005. The ethanolic extract of AJN was prepared as follows (26): 2 kg of dried and ground material was extracted with 95% ethanol at room temperature. The supernatant was then filtered and evaporated in a vacuum at a temperature below 50°C using a rotary evaporator (Eyela Ltd.). The ethanol extracts (44.67 g) were then fractionated successively with *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous. These fractionates were concentrated by evaporation or dryness, yielding *n*-hexane (6.36 g), chloroform residue (0.86 g), ethyl acetate residue (AJNEA, 1.16 g), and *n*-butanol residue (30.32 g), and aqueous residue (11.50 g), respectively. Each fractionate was then dissolved in RCM media, and filtered using a syringe filter (0.45 μm) and used as a sample to test for antimicrobial activity.

Antimicrobial activity Antimicrobial activity was measured using a turbidity assay (27). *C. difficile* (0.3, OD 600 nm) was inoculated into 10 mL of RCM broth along with a test sample and was incubated at 37°C for 12 hr. The control was inoculated only with *C. difficile* without AJNEA. After incubation at 37°C for 12 hr, the optimal density (OD) was measured at 600 nm. To test for synergistic effect, *Bifidobacterium* spp. and *Lactobacillus* spp. were cultured in a TPY broth containing the AJNEA for 12 hr at 37°C. After incubation, each supernatant was then collected into a new tube and made into a powder using a freeze dryer. *C. difficile* (0.3, $OD_{600\,nm}$) was inoculated into 10 mL of RCM broth dissolving each lyophilized supernatant. After incubation at 37°C for 12 hr, the OD was measured at 600 nm. The control was inoculated with only *C. difficile* without AJNEA and each culture supernatant of LAB. The sample control was inoculated with each extract or each culture supernatant of *Bifidobacterium* spp. and *Lactobacillus* spp. To determine minimum inhibition concentration (MIC), each culture broth (50 μL) was taken from each tube spread over sterile nutrient agar plates. They were then incubated at 37°C for 12 hr and the development of the microorganisms was checked.

Table 1. Composition of starter used for preparation of yogurt

Starter group	Yogurt starter composition
1	ABT (*L. acidophilus*: *L. bulgaricus*: *S. thermophilus*)
2	ABT: *B. adolescentis*=1 : 1
3	ABT: *B. infantis*=1 : 1
4	*L. acidophilus*: *S. thermophilus*: *B. infantis*=1 : 1 : 1
5	*L. acidophilus*: *S. thermophilus*: *B. adolescentis*=1 : 1 : 1

C. *difficile* toxin A assay *C. difficile* toxin A was detected using a toxin A kit (Oxoid, Basingstoke, UK) and tested according to the instructions of the manufacture. Activated *C. difficile* culture broth was centrifuged at 18,000×g for 10 min, and the supernatant was used as the test sample. The separated toxin was mixed with each AJNEA at different concentration and was then reacted for 30 min. *C. difficile* toxin A appeared as a blue line in the control and the resulting window of the kits.

Preparation of yogurt Yogurt was prepared with various starter groups (Table 1) containing 0.5% AJNEA powder. LAB was subcultured in TPY broth at 37°C for 12 hr. Yogurt starter was incubated in 10% skim milk at 37°C for 8 hr. Yogurts were prepared in fresh milk together with 5% skim milk, 1.75% sucrose, and 0.5% of AJNEA powder fermented with LAB at 37°C for 24 hr. The yogurt samples were harvested by centrifugation at 1,500×g for 10 min. Each yogurt supernatant was then lyophilized by using a freeze dryer and was used as test sample. Each powdered supernatant of yogurt was prepared by adding the appropriate volume of RCM media solution and was then filtered using a syringe filter (0.45 μm) to test for antimicrobial activity.

Results and Discussion

Inhibition of *C. difficile* growth by LAB supernatants The antimicrobial effect of LAB against *C. difficile* was investigated. As shown in Fig. 1, although the supernatant of *B. adolescentis, B. bifidum, and B. longum* did not show growth inhibitory activity, only the supernatant of strain *B. breve* displayed slight activity of 29.0%. In addition, among the tested strains, *L. rhamnosus* GG (positive control), *L. bulgaricus*, and *L. acidophilus* showed an inhibitory growth effect of more than 40%. Pharmaceutical probiotics have been used as alternative treatments or preventive therapies for a variety of clinical diseases (28-30). In particular, these strains have been used in the treatment or prophylaxis of antibiotic-associated diarrhea (22). The study of their use as probiotics to prevent clindamycin diarrhea or erythromycin diarrhea has shown the same benefit (31). Recently, accumulated research evidence suggests that a supplement of probiotics given either as a

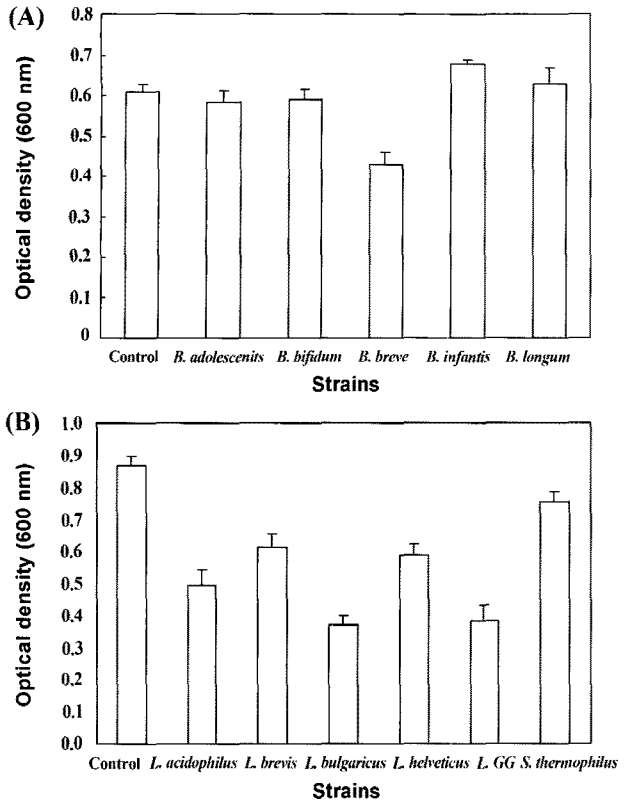

Fig. 1. Antimicrobial effect of *Bifidobacterium* spp. (A) and *Lactobacillus* spp. (B) supernatants against *C. difficile*. The control was inoculated with only *C. difficile* (0.3, $OD_{600\,nm}$) without any treatment and incubated at 37°C for 12 hr. Tested concentration of each lyophilized supernatant powder from LAB strains was 500 ppm at the final concentration. The results are means of triplicate±SE.

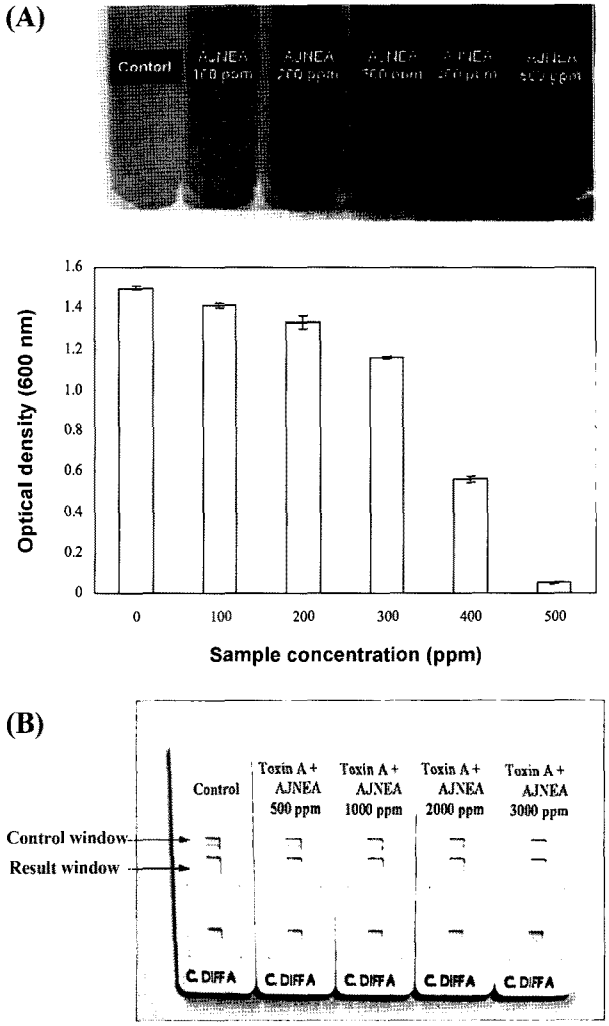

Fig. 2. Inhibition of *C. difficile* growth (A) and *C. difficile* toxin A (B) by AJNEA with different concentration. Toxin separated from *C. difficile* was mixed with each AJNEA with different concentrations, and reacted for 30 min. *C. difficile* toxin A appeared as a blue line in the control and resulting window of kits, respectively. AJNEA is defined as the ethyl acetate soluble fractionates from ethanolic extract of AJN.

single or a mixture of living microorganisms may play a role in the treatment or prevention of human disease including intestinal infections. In previous tests by the authors, antimicrobial activity was screened against *C. difficile* of 10^9 LAB isolated from 32 healthy Korean infants and identified *B. infantis* and *L. salivaricus* were identified as the active strains (32). *B. infantis* was the predominant microorganism in the intestines of normal breast-fed infants, as reported previously. However, probiotics therapy remains largely unproven for *C. difficile* infection. Therefore, more detailed studies are necessary to determine the potential application of probiotics for the prevention or treatment of antibiotic-associated diarrhea or Pseudomembranous colitis.

Inhibition of *C. difficile* growth by AJNEA In a previous study by the authors, the antimicrobial activities against *C. difficile* of ethanolic extracts of 40 species of traditional herbal medicines were screened. The results indicated that AJNEA exhibited the highest growth inhibitory activity against *C. difficile*. In turbidity assays, as shown in Fig. 2A, the inhibitory effect against *C. difficile* growth corresponded to the treated concentration of AJNEA. A concentration as low as 500 μg/mL of AJNEA inhibited 96.8% of the growth *C. difficile* and the MIC value of AJNEA against *C. difficile* was 625 μg/mL. These results indicated AJNEA can be utilized as a potential antimicrobial agent against disease related to *C. difficile*. In our knowledge, this is the first report in which AJN shows antimicrobial activity against *C. difficile*.

Inhibition of AJNEA on C. *difficile* toxin A The inhibitory activity of AJNEA was determined on *C. difficile* toxin A, which is well known to be connected with *C. difficile* associated disease (33). When the epithelial cell intestine is stimulated, *C. difficile* toxin A expresses inflammatory mediators such as interleukin-8 and chemokine and then induces mitochondrial injury and tissue damage as a consequence. For the determination as to whether AJNEA directly affected toxin A production from *C. difficile*, the effect was tested using a toxin A immunoassay. The results indicated, at 3,000 ppm of the AJNEA treated group, that the toxin A band was not detected (Fig. 2B). Further study is necessary to isolate and identify the active component from AJN that possesses

antimicrobial activity against *C. difficile*. This study is currently ongoing.

Synergistic inhibition of a mixture of LAB supernatants and AJNEA against *C. difficile* Figure 3 shows the inhibitory effect of LAB strains and AJNEA against *C. difficile* according to the culture time along with their effect compared to vancomycin (12.5 μg/mL) and *L. GG* supernatant as a positive control. In particular, *L. acidophilus* and AJNEA (500 ppm) displayed the same inhibition activity as that of *L. GG* and vancomycin, respectively. In our previous study, the growth effect of AJNEA was confirmed on the LAB strains. AJNEA did not inhibit the growth of tested *Bifidobacterium* spp. and *Lactobacillus* spp., with the exception of *B. longum*, *S. thermophilus*, and *L. helveticus* (data not shown). Considering these results, the synergistic antimicrobial effects by a co-treatment of AJNEA and each culture supernatants of *Bifidobacterium* spp. and *Lactobacillus* spp. were investigated against *C. difficile*. The results (Fig. 4A) showed that although AJNEA (100 ppm) did not have the inhibitory activity against *C. difficile* growth, among the tested *Bifidobacterium* spp., an extracellular factor (in culture media) of *B. infantis* ATCC 15697 and *B. adolescentis* ATCC 15703 showed a synergistic effect with AJNEA on *C. difficile* growth inhibition. The individual treatment of *L. bulgaricus* and *L. GG* (positive control) showed an inhibitory effect on *C. difficile* growth, which yielded the same results as previously reported from various studies (20,22,28,29). An extracellular factor of the tested *Lactobacillus* species, except for *L. helveticus*, with AJNEA did not show any synergistic effect against *C. difficile* (Fig. 4B). Although *L. helveticus* was shown to have a synergistic effect to AJNEA, however, in a previous study, growth of the strain was inhibited by the addition of AJNEA.

From the above results, the synergistic antimicrobial effect of *B. infantis* and *B. adolescentis* was assessed according to the concentration of AJNEA. As shown in Fig. 5, the culture supernatant of *B. infantis* ATCC 15697 containing AJNEA (50 ppm) presented an especially clear synergistic effect (73.4±0.06%) compared to the control treated with only the culture media supernatant of *B. infantis* (no inhibition) or AJNEA (50 ppm, 1.5±0.1%). This activity increased in a concentration-dependent manner. Moreover, the media supernatant of *B. adolescentis* ATCC 15703 with AJNEA (200 ppm) showed a synergistic effect (45.4±0.01%). The results of this study indicated that AJNEA had synergistic effects with *B. infantis* ATCC 15697 and *B. adolescentis* ATCC 15703. As the AJNEA treatment resulted in no significant inhibitory effect on the

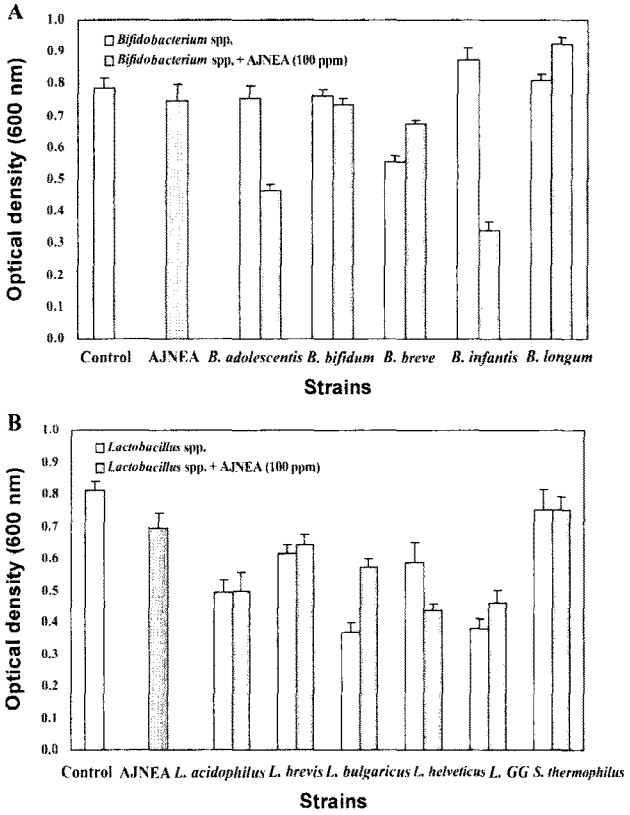

Fig. 4. Synergistic antimicrobial effect of each lyophilized supernatants of *Bifidobacterium* spp. (A) or *Lactobacillus* spp. (B) containing AJNEA against *C. difficile*. Tested concentration of AJNEA was 100 ppm at the final concentration. The results are the means of triplicate±SE.

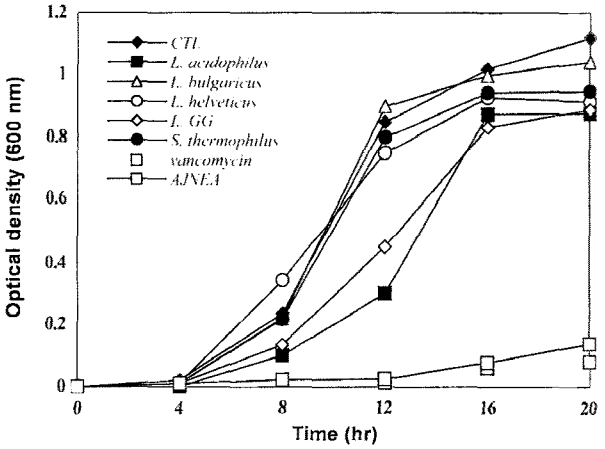

Fig. 3. Inhibition of *C. difficile* growth by LAB strains and AJNEA according to the culture period. *L. GG* and vancomycin (12.5 μg/mL) were used as positive controls and the tested concentration of AJNEA was 500 ppm at the final concentration.

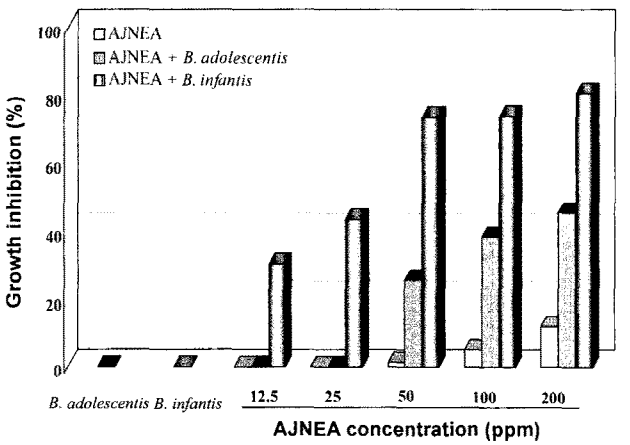

Fig. 5. Synergistic antimicrobial effect of each culture media supernatant powder of *B. adolescentis* and *B. infantis* containing AJNEA with different concentrations against *C. difficile*.

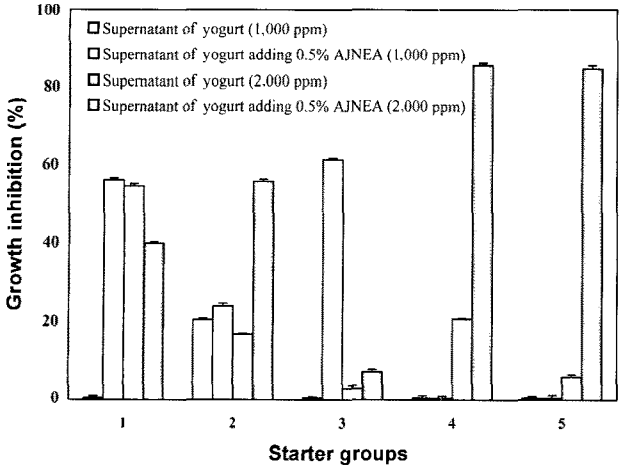

Fig. 6. Inhibition of *C. difficile* growth by lyophilized supernatants of yogurt prepared with AJNEA and different starter species. The results are the means of triplicate±SE.

growth of the *Bifidobacterium* strains, it is believed that a mixture of *Bifidobacterium* strain combined with AJNEA is expected to be a useful source for the prevention of *C. difficile*-associated diarrhea. However, further investigations of the safety issues relating to AJNEA in addition to the determination of the mechanism leading to the inhibitory actions of these mixtures are needed.

Inhibition of *C. difficile* growth by yogurt supernatants

To assess the effect of yogurt containing AJNEA against *C. difficile* growth, yogurt was prepared by adding each different starter species (Table 1) and AJNEA. The results indicated that in yogurt prepared with starter group 4 (*L. acidophilus*: *S. thermophilus*: *B. adolescentis*=1 : 1 : 1) or starter group 5 (*L. acidophilus*: *S. thermophilus*: *B. infantis* =1 : 1 : 1) as the starter and 0.5% AJNEA powder, high synergistic antimicrobial effects against *C. difficile* resulted, at 79 and 65.2%, respectively (Fig. 6). Relapsing *C. difficile* diarrhea occurs in 15-35% of patients, and in most cases, the diarrhoeal management problem remains unsolved (20, 22,32). The results of several other related studies for the treatment and management of relapses against *C. difficile* diarrhea prescribed oral yogurt, and *L. GG* fermented yogurt was shown to be effective in lessening diarrhea more than in the control group (28,29). *L. casei GG* was administered in the form of yogurt (31). However, the patients given a mixture of *L. acidophilus* and *L. bulgaricus* experienced no preventive effect on diarrhea (34).

In conclusion, *C. difficile*-associated diarrhea accounts for as much as 15% of diarrhoeal disease associated with antibiotics, and 60% of those undergoing relapse. Therefore, the development of new drugs or sources in which non-absorbed antibiotics, exhibiting potent activity against *C. difficile* is necessary. In this study, it was determined that AJNEA may represent an important therapeutic alternative for the treatment of *C. difficile*-associated diarrhea as a synergist with *Bifidobacterium* spp. Further investigations to identify the active component from AJN and to elucidate the underlying mechanism responsible for the synergistic antimicrobial properties are in progress.

Acknowledgments

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