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ARTICLE

Evaluation of Two *Bacillus subtilis* Strains Isolated from Korean Fermented Food as Probiotics against Loperamide-induced Constipation in Mice

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Abstract Probiotics are live microbes that confer health benefits on the host when administered in adequate amounts. To evaluate the probiotic potential of *Bacillus subtilis* isolated from Korean fermented foods, we investigated the resistance to biological barriers and improvement of loperamide-induced constipation. The values of resistance to gastric acidity of *B. subtilis* CBD2 and KMKW4 strains were 55.34±2.12 and 64.58±2.95%, respectively, whereas the survival rate of *B. subtilis* KMKW4 (31.17±5.78%) in bile acids was superior to that of CBD2 (8.62±2.09%). These strains also demonstrated adhesiveness to intestinal epithelial HT-29 cells and an inhibitory activity against pathogenic microflora. Furthermore, *B. subtilis* CBD2 and KMKW4 strains improved gastrointestinal activity when tested in a loperamide-induced mouse model of constipation. Pre-treatment with CBD2 and KMKW4 strains before the onset of constipation improved fecal output and gastrointestinal transit in loperamide-treated mice. These strains also showed inhibitory effects on the activity of β-glucosidase and tryptophanase, harmful enzymes of intestinal microflora. Taken together, these finding show that *B. subtilis* CBD2 and KMKW4 have high adaptability to gastrointestinal environment, and the ability to inhibit pathogenic microflora and prevent constipation, suggesting their activity as potential probiotics.

Keywords *Bacillus* spp. · *Bacillus subtilis* · constipation · Korean traditional food · probiotic

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Introduction

Probiotics were first described by Lilly and Stillwell (1965) as bacterial factors that stimulated the growth of other microbial species. Currently, the term is applied to microorganisms that, if ingested in adequate numbers, can provide health benefits to the host (Schrezenmeir and de Vrese, 2001). The market for probiotics has grown concurrently with their recognition as healthy food supplements (Stanton et al., 2001). Probiotic bacteria are considered to be resistant to biological barriers such as gastric juice and bile salts and possess the ability to adhere to enterocytes and inhibit the growth of enteropathogenic microbes (Ouwehand et al., 2001; Vinderola and Reinheimer, 2003; Servin and Coconnier, 2003). *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, and *Bacillus* spp. are well-known probiotics that colonize the human gastrointestinal tract and exert immunomodulatory effects (Ouwehand et al., 2002). Probiotic bacteria can also affect food quality. Probiotics participate in the fermentation of food products and release saccharolytic, proteolytic, and lipolytic enzymes, which contribute to nutrient assimilation and significantly influence the food matrix (Heller, 2001; Crittenden et al., 2002).

Korean traditional fermented food such as *kimchi* (fermented vegetable product), *Doenjang,* Korean fermented soybean paste, and *Gochujang*, Korean hot pepper paste contain dynamic microbial communities composed of bacteria, fungi, and yeast (Lee et al., 2005; Kim et al., 2009; Jang et al., 2011). Lim and Im (2009) isolated probiotic lactic acid bacteria (LAB) from Korean fermented food and described *Lactobacillus plantarum* K21 as a novel probiotic LAB strain. LAB isolated from traditional dairy products have been extensively studied as probiotic bacteria; *Lactobacillus* spp. added to Spanish fermented sausages have shown health-promoting effects (Rubio et al., 2013). Although *Bacillus* spp. are also frequently observed in Korean fermented food research on *Bacillus* probiotic activity has started much later, because bacilli have often been considered as soil organisms.

Bacillus spores are able to germinate in the gastrointestinal tract and exert probiotic activity (Casula and Cutting, 2002). The administration of *Bacillus* spp. in shrimp increased the content of digestive enzymes such as amylase, protease, and lipase (Ziaei-Nejadn et al., 2006). *Bacillus*-based probiotics have proved beneficial to human health through the expression of antimicrobial and immunomodulatory factors and reduction of blood lipids without any adverse effects (Sanders et al., 2003). A number of *Bacillus* species are currently used as probiotic dietary and medicinal supplement in several countries (Cutting, 2011).

Constipation, defined as infrequent or difficult evacuation of feces, is prevalent in modern societies and is a common functional gastrointestinal disorder (Bharucha, 2007). Constipation is often caused by insufficient dietary fiber intake, inadequate fluid intake, decreased physical activity, hypothyroidism, and obstruction by colorectal cancer (Leung, 2007). Various chemical drugs such as magnesium oxide, senna, senokot, and gaviscon are commonly used to treat constipation, but they are expensive and have undesirable side effects (Erasto et al., 2005; Wintola et al., 2010). Mitsui et al. (2006) demonstrated that ingestion of Natto containing *Bacillus subtilis* spores improved the intestinal condition in volunteers with constipation. *B. subtilis* have been used in fermented foods for several centuries without harmful effects and therefore is generally recognized as safe. A study on *B. subtilis* health beneficiary effects has confirmed that *B. subtilis* can act as probiotic bacteria (Wang et al., 2006).

The aim of the present study was to screen *B. subtilis* isolated from fermented Korean food for the probiotic activity and potential application in food industry. We report that *B. subtilis* isolates exhibited tolerance to gastric fluid *in vitro* and retained their probiotic properties, including inhibitory activity against pathogenic bacteria. Most importantly, the administration of the isolated strains in mice could prevent constipation and improve gastrointestinal functions.

Materials and Methods

Isolation and identification of *Bacillus* spp. One gram each of *kimchi*, *doenjang*, and *soy sauce* was suspended in 9 mL of sterilized distilled water, and 100 μL of the suspension was spread on nutrient agar medium and cultured for 24 h at 30°C. The morphology of isolated bacteria was examined microscopically after Gram staining, and Gram-positive rod-shaped microorganisms were selected for further identification based on the sequences of 16S rRNA genes. Chromosomal DNA was purified using the Wizard Genomic DNA Kit (Promega Co., USA), and 16S rRNA genes were amplified using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACC TTG TTA CGA CTT-3'). Partial DNA sequencing of 16S rRNA was performed by Solgent Co. Ltd (Korea), and sequence homology was analyzed using the BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences of isolated bacteria were aligned with those of closely related neighbors using the Clustal X (http://www.clustal.org/) and BioEdit programs (http://www.mbio.ncsu.edu/bioedit/bioedit.htm).

Antimicrobial activity. Pathogenic microorganisms were obtained from the Korean Collection for Type Cultures (KCTC, Korea), the Korean Culture Center of Microorganisms (KCCM, Korea), and Yeungnam University (Korea). The standard culture conditions for the strains are listed in Table 1. Each pathogenic bacteria culture broth was spread on agar medium according to the species of pathogenic bacteria. Isolated bacteria were inoculated with a toothpick, and incubated for 1 day at 30 or 37°C. The diameter of the clear zone resulting from suppression of pathogenic bacterial growth was measured.

Resistance to gastric juice and bile acids. The isolated bacteria were incubated for 18 h in 10 mL of Nutrient broth (Difco, USA) at 37°C, centrifuged for 15 min at 6,000 rpm, and resuspended in 10 mL of 0.5% NaCl (w/v). Gastric acid tolerance was examined by the incubation of 1 mL bacterial suspension in 10 mL of gastric juice consisting of 0.3% pepsin (w/v) and 0.5% NaCl (w/v) in Nutrient broth adjusted to pH 3.0 for 3 h at 37°C (Vinderola and Reinheimer, 2003). Cell viability was determined by culturing on Nutrient agar for 24 h at 37°C and calculated as the percentage of viable cells before (control) and after (sample) incubation in gastric juice: % survival = (control – sample)/control ×100%.

To examine bile tolerance, 0.5% of bacterial suspension was inoculated in Nutrient broth containing 0.3% oxgall (w/v) for 24 h at 37°C. Bacterial growth was determined by measuring the optical density at 600 nm (Hyronimus et al., 2000) using a spectrophotometer (Ultraspec 2100, Amersham Biosciences Co., UK). The survival rate was expressed as the percentage of control cultures grown without bile acids.

***In vitro* adherence assay.** The human colonic adenocarcinoma cell line HT-29 (Korea cell line bank, Korea) was cultured in RPMI 1640 (WELGENE Co., Korea) supplemented with 10% fetal bovine serum (FBS, WELGENE), penicillin (100 U/mL), streptomycin (100 μg/mL) (GIBCO, USA), 25 mM $NaHCO_3$, and 25 mM HEPES (both from WELGENE) at 37°C in a 5% CO_2 humidified environment. For the adherence assay, 1×10^5 cells/well were seeded in 24-well plate and incubated for 24 h at 37°C. Subsequently, the medium was changed to antibiotic-free RPMI 1640 for additional 3 h, and the monolayers were rinsed twice with sterile phosphate buffer saline (PBS). Bacteria were washed in 0.5 mL of antibiotic-free RPMI 1640 to remove culture broth, centrifuged, and added to HT-29 monolayers in 1 mL (1×10^7 cells) for 2 h at 37°C. HT-29 monolayers were then washed twice with PBS, and the adhered bacteria were recovered by the incubation with 1 mL 0.1% triton X-100 in PBS for 30 min at 37°C (Wang et al., 2008). Viable bacteria were counted as described above, and the adhesion rate was determined as the percentage of live bacteria recovered from HT-29 monolayers to total added bacteria (Rowan et al., 2001).

Animals. Four-week-old male BALB/c mice were purchased from Central Lab Animal Inc. (Korea) and allowed to adapt for 1

Table 1 Pathogenic microorganisms and culture conditions

Group	Microorganism	Medium	Incubation temperature (°C)
Gram-positive bacteria	*Staphylococcus aureus* (KCTC 1621)	Trypticase soy agar Trypticase soy broth	37
	Bacillus cereus (KCTC 1012)	Nutrient agar Nutrient broth	30
	Listeria monocytogenes (KCTC 3569)	Brain-heart infusion agar brain-heart infusion broth	37
Gram-negative bacteria	*Vibrio parahaemolyticus* (KCTC 2471)	Marine agar marine broth	37
	Salmonella typhimurium (KCTC 2514)	Nutrient agar Nutrient broth	37
	Escherichia coli (KCTC 2593)	Nutrient agar Nutrient broth	37
	Shigella sonnei (KCCM 41282)	Nutrient agar Nutrient broth	37
	Escherichia coli O157 (Yeungnam University, Kyungsan, Korea)	Nutrient agar Nutrient broth	37
	Pseudomonas aeruginosa (KCCM 12535)	Nutrient agar Nutrient broth	37
Fungi	*Candida albicans* (KCTC 7965)	Yeast mold agar	25

week. Mice were housed in a room maintained under controlled temperature of 22±2°C, humidity of 55±15%, and 12/12 h light/dark cycle. Food pellets and tap water were provided *ad libitum*. All procedures were performed in accordance with the animal protocol approved by the Daegu Technopark Bio-Health Convergence Center Institutional Animal Care and Use Committee (Korea).

***In vivo* experimental design and treatment.** At 5 weeks of age, mice were randomly divided into 4 groups (n =8). Normal (NOR) group and Constipated control (CON) group received 200 μL PBS, whereas bacteria-treated groups were orally administered *B. subtilis* CBD2 or KMKW4 strains at a daily dose of 1×10^7 CFU in 200 μL PBS for 7 days.

One day after the last dose, constipation was induced in CON, CBD2, and KMKW4 groups by subcutaneous injection of loperamide hydrochloride (4 mg/kg body weight; Sigma-Aldrich, USA) in 0.5% Tween 20 (Sigma-Aldrich) twice a day for 5 days. The non-constipated NOR group was subcutaneously injected with 0.5% Tween 20 alone (Shimotoyodome et al., 2000).

Analysis of body weight and food intake. Alterations in mouse body weight and food intake were determined at day 1 (before the experiment), day 8 (1 day after the last administration of *B. subtilis*) and day 13 (1 day after the last injection of loperamide hydrochloride) using an electric balance (CP423S, Sartorius AG, Germany). All measurements were performed in triplicate to ensure accuracy.

Fecal parameters. Fecal output was assayed by counting fecal pellets produced in 1 h at days 8 and 13 and assessing fecal wet and dry weights (Devries et al., 2010). Dry weight was measured after the pellets have been treated for 24 h in a laboratory dry oven at 70°C. The percentage of water content was calculated as: (wet weight – dry weight)/wet weight of fecal pellets ×100%. At day 13, 5 mice in each group were sacrificed, and the distal colon was removed to assess the number of fecal pellets. Other mice (n =3 per group) were used to measure gastrointestinal transit ratio.

Measurement of gastrointestinal transit (GIT) ratio. Gastrointestinal motility was evaluated as previously described (Miki et al., 2005). The mice were fasted with free access to drinking tap water for 24 h. On the day the experiment was initiated, the animals received orally 20 μL/g weight of 25% barium sulfate (Sigma-Aldrich) suspended in water. The mice were sacrificed 15 min later, and the gastrointestinal tract was quickly removed. The lengths from the pylorus to the most distal point of barium sulfate migration (A) and from the pylorus to terminal ileum (B) were measured. GIT ratio was expressed as the percentage of A to B.

β-Glucosidase and tryptophanase activity. The caecum of the sacrificed mice was removed and suspended 1:10 in cold 0.1 M phosphate buffer (pH 7.0). Non-bacterial debris was removed by centrifugation at 6,000 rpm for 10 min (Gudiel-Urbano and Goñi, 2002) and the supernatant was assayed for the activity of β-glucosidase and tryptophanase.

β-Glucosidase activity was measured in a 2-mL reaction mixture containing 800 μL of 2 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) and 200 μL of enzyme solution (caecum supernatant). The mixture was incubated for 30 min at 37°C and stopped by adding 1 mL of 0.5 N NaOH. The reaction mixture was centrifuged at 3,000 rpm for 10 min, and the enzymatic activity was measured by absorbance at 405 nm (An et al., 2011).

Table 2 BLAST search for the 16S rRNA gene sequences of isolated bacteria

Isolated strain	Putative species	Related GenBank sequence	Identity (%)
CBD2	*Bacillus subtilis*	JF932296.1	98
CBKW3	*Bacillus subtilis*	HM055602.1	99
KGC1	*Bacillus subtilis*	JN400257.1	99
KKD1	*Bacillus amyloliquefaciens*	KC692163.1	99
KKD2	*Bacillus amyloliquefaciens*	KC692179.1	99
KKD4	*Bacillus amyloliquefaciens*	KF112077.1	99
KKD6	*Bacillus methylotrophicus*	KC790268.1	99
KMKW2	*Bacillus amyloliquefaciens*	KC692163.1	99
KMKW4	*Bacillus subtilis*	KC441757.1	98

Tryptophanase activity was assayed in a 2.5-mL reaction mixture containing 200 μL of complete reagent solution (2.75 mg pyridoxal phosphate, 19.6 mg disodium EDTA dihydrate, and 10 mg bovine serum albumin in 100 mL of 0.05 M potassium phosphate buffer, pH 7.5), 200 μL of 20 mM tryptophan (Sigma-Aldrich), and 100 μL of the enzyme solution. The reaction mixture was incubated for 1 h at 37°C and stopped by adding 2 mL of color reagent (14.7 g p-dimethylaminobenzaldehyde in 52 mL H_2SO_4 and 948 mL of 95% ethanol). The reaction mixture was centrifuged at 3,000 rpm for 10 min, and the enzymatic activity was measured by reading absorbance at 550 nm (An et al., 2011).

Statistical analysis. Data were expressed as the means ± SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's *t*-test. Differences between means were considered statistically significant at $p<0.05$. All statistical analyses were performed using the SPSS/Windows software (Version 12.0, SPSS Inc., USA).

Results and Discussion

Isolation and identification of bacteria from Korean traditional food. Among the 162 isolates, 11 were identified as Gram-positive rod-shaped bacteria. Phylogenetic analysis based on 16S rRNA gene sequences of 9 isolates revealed over 99% similarity to *B. subtilis*, *B. tequilensis*, *B. amyloliquefaciens*, and *B. methylotrophicus* (Table 2). The strains were named as *B. subtilis* CBD2, *B. subtilis* CBKW3, *B. subtilis* KGC1, *B. amyloliquefaciens* KKD1, *B. amyloliquefaciens* KKD2, *B. amyloliquefaciens* KKD4, *B. methylotrophicus* KKD6, *B. amyloliquefaciens* KMKW2, and *B. subtilis* KMKW4. *Bacillus* spp. represented a significant portion of microbial community in Korean fermented food such as Korean fermented soybean paste (*Doenjang*), salted seafood (*Jeotgal*), and Korean hot pepper paste (*Gochujang*) (Guan et al., 2011; Jang et al., 2011; Nam et al., 2012a; Nam et al., 2012b). Among the *Bacillus* spp., *B. cereus* that was transferred from soil to food was identified as foodborne pathogen (Stenfors Arnesen et al., 2008), whereas *B. coagulans*, *B. natto*, *B. polyfermenticus*, and *B. subtilis* were considered by the Ministry of Food and Drug Safety as safe bacilli species. Hong et al. (2008) reported that upon feeding *B. subtilis* to rabbits and guinea pigs, it was not toxic, according to hematology indexes, weight gain, and histological evaluation of visceral organ. Another study confirmed that *B. subtilis* showed neither toxicity nor histological changes in mice, rabbits, and piglets (Sorokulova et al., 2008). Considering the safety to human consumption, we selected *B. subtilis* CBD2, *B. subtilis* CBKW3, *B. subtilis* KGC1, and *B. subtilis* KMKW4 strains as candidate probiotic bacilli.

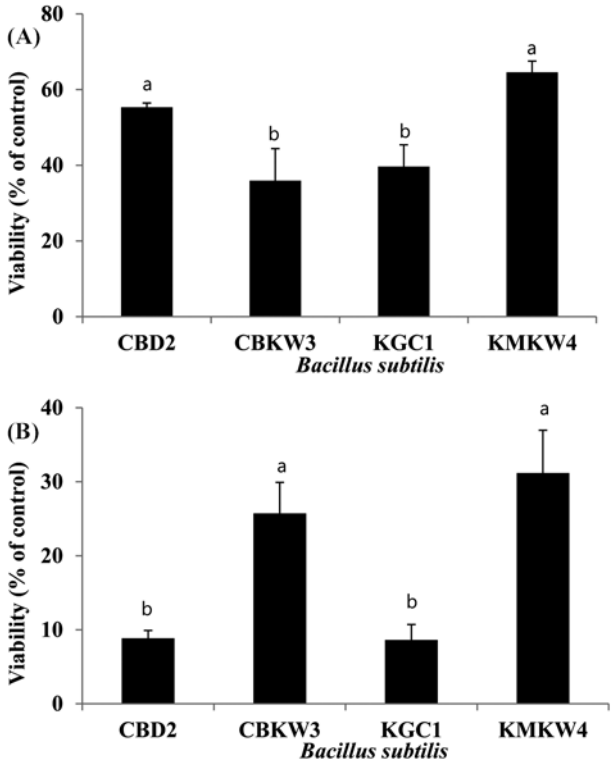

Fig. 1 Effect of artificial gastrointestinal juice (A) and 0.3% bile salts (B) on the growth of *Bacillus subtilis* strains isolated from Korean traditional fermented food. The data are expressed as the means ± SD ($n=3$). The letters with bars indicate significant differences at $p<0.05$.

Resistance to gastric and bile acids. It is essential that the ingested probiotics should survive the acidic environment in the

stomach and small intestine to exert their health beneficial effects (Bezkorovainy, 2001). Erkkilä and Petäjä (2000) screened potential probiotics for survival at low pH and in the presence of bile salts and found that *Lactobacillus sakei* and *Pediococcus acidilactici* could be used as commercial meat starter cultures. Cell viability of *B. subtilis* CBD2, CBKW3, KGC1, and KMKW4 strains declined in artificial gastric juice (Fig. 1A): the survival counts of CBKW3 and KGC1 were, respectively, 35.93±8.47% and 39.66±5.74% of the initial viable cell count. However, the CBD2 and KMKW4 strains showed significantly higher resistance to low pH and digestive enzymes in gastric juice than CBKW3 and KGC1 strains (p <0.05). A relatively high tolerance of *B. subtilis* KMKW4 (64.58±2.95% of initial viable cells) was similar to that of *Bifidobacterium infantis* microencapsulated with skim milk (Lian et al., 2003). Bile salt oxgall significantly decreased the viability of *B. subtilis* CBD2 (8.62±2.09% of control cell growth) and KGC1 (8.84±1.07%), which showed high tolerance to gastric juice, whereas was sensitive to bile salts (Fig. 1B). However, *B. subtilis* KMKW4 showed relatively high resistance to both low acidity and bile salts. Bile consists of bile acids, cholesterol, and phospholipids, and functions as a biological fat emulsifier. In addition, bile exhibits bactericidal activity by damaging cell wall of Gram-positive microorganisms, which seem to be more sensitive to the deleterious effects of bile than Gram-negative bacteria (Begley et al., 2005). Hyronimus et al. (2000) showed that *B. laevolacticus* was resistant to acidic conditions but not to oxgall. Consistent with these data, the *B. subtilis* strains isolated in the present study showed low tolerance to bile acids.

Adhesion to intestinal epithelial cells. The adhesion to intestinal mucosa is essential for probiotic colonization of the gastrointestinal tract and for subsequent beneficial functional effects (Blum et al., 1999). The adhesion values of *B. subtilis* CBD2 and KMKW4 to epithelial HT-29 cells were 4.01±0.55% and 3.68±0.30%, respectively, of initial cell counts, whereas KGC1 and CBKW3 showed lower adhesive abilities (1.67±0.12% and 2.97±0.37%, respectively; Fig. 2). The adhesion rates of *B. subtilis* CBD2 and KMKW4 were lower than that of *Lactobacillus reuteri* (21.30±1.56%), which is a well known probiotic bacterium (Wang et al., 2008), but higher than that of *B. subtilis* H7 (0.35±1.56%) reported by Rowan et al. (2001).

Antimicrobial activity of the isolated strains. *B. subtilis* was reported to produce antibiotics active against fungi, yeast, and bacteria, such as inturin A and subtillin (Stein, 2005; Peypoux et al., 1978). The consumption of probiotics producing bacteriocins and other antimicrobials inhibits the growth of enteric microbial pathogens (Klaenhammer and Kullen, 1999) and is beneficial for gastrointestinal activity and overall health status. The antagonistic activities of *B. subtilis* strains CBD2, CBKW3, KGC1, and KMKW4 were tested against a variety of microorganisms including foodborne pathogens (Table 3). All of the tested strains showed highest inhibitory activities against *Candida albicans*. *B. subtilis* CBD2, and KMKW4 strains also inhibited the growth of *B. cereus*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Salmonella typhimurium*, *Escherichia coli*, and *Shigella sonnei*, demonstrating broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative species. Given that *B. subtilis* CBD2 and KMKW4 strains also showed high adaptability to gastrointestinal environment, they were selected as candidate probiotics for *in vivo* experiments.

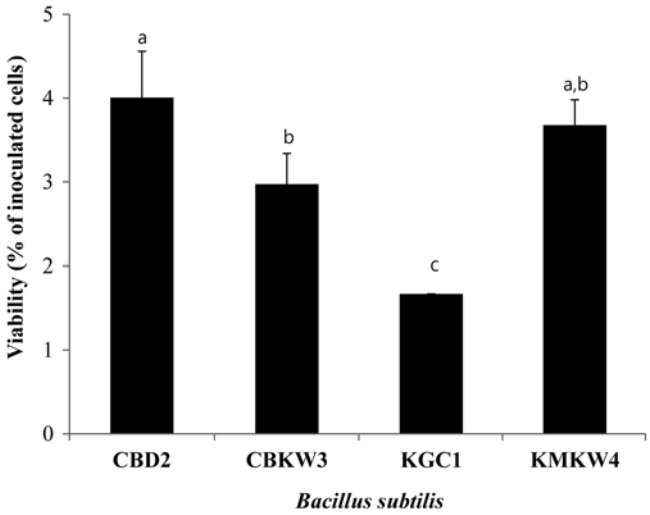

Fig. 2 *Bacillus subtilis* adhesion on intestinal epithelial HT-29 cells. Vegetative cells of *B. subtilis* CBD2, CBKW3, KGC1, and KMKW4 were added to HT-29 cell monolayers and incubated for 2 h at 37°C. Adherent bacteria were assessed as described in Materials and Methods and expressed as the percentage to initial bacterial load (means ± SD; n =3). The letters with bars indicate significant differences at p <0.05.

Body weight and feeding. To determine whether *B. subtilis* CBD2 and KMKW4 strains ameliorated the constipating effect of loperamide, we examined body weight and food intake of mice administered *B. subtilis* CBD2 and KMKW4 prior to loperamide treatment. The experimental groups did not demonstrate significant differences in body weight or food intake before the experiment (day 1), after the last *B. subtilis* administration (day 8) or after the last injection of loperamide hydrochloride (day 13; p >0.05) (Table 4).

Body weight and food intake are considered as important parameters for the evaluation of constipation symptoms and preventive treatment. However, animal models of loperamide-induced constipation have demonstrated inconsistent effects with regard to body weight and food intake. Wintola et al. (2010) indicated that loperamide treatment decreased body weight and food intake, whereas Lee et al. (2012) did not observe any changes in these parameters following loperamide administration. In our model, we did not detect loperamide effects on either body weight or food intake.

Fecal output. To examine the constipation-preventive effects of *B. subtilis* CBD2 and KMKW4, the frequency of excretion and fecal weight, and water content were analyzed in mice pre-treated with *B. subtilis* CBD2 and KMKW4 for 7 days prior to the induction of constipation with loperamide. Administration of

Table 3 Antimicrobial activity of *Bacillus subtilis* strains against pathogenic intestinal microflora

Group	Microorganism	Inhibition zone diameter(mm)			
		CBD2	CBKW3	KGC1	KMKW4
Gram-positive bacteria	*Staphylococcus aureus* (KCTC 1621)	7.67±0.58[a]	3.33±1.00[b]	3.33±1.6[b]	3.33±0.58[b]
	Bacillus cereus (KCTC 1012)	3.00±0.00	-	-	-
	Listeria monocytogenes (KCTC 3569)	7.33±1.15[a]	6.33±0.58[a]	-	6.33±0.58[a]
Gram-negative bacteria	*Vibrio parahaemolyticus* (KCTC 2471)	3.67±0.58	2.33±0.58*	-	-
	Salmonella typhimurium (KCTC 2514)	3.37±0.58[a]	2.33±0.58[a]	2.33±0.58[a]	3.33±0.58[a]
	Escherichia coli (KCTC 2593)	4.00±1.00[a]	2.33±0.58[b]	2.33±0.58[b]	-
	Shigella sonnei (KCCM 41282)	3.67±0.58[a]	2.33±0.58[b]	3.33±0.58[a,b]	3.67±0.58[a]
	Escherichia coli O157 (Yeungnam University, Kyungsan, Korea)	3.67±0.58	-	-	-
	Pseudomonas aeruginosa (KCCM 12535)	-	2.33±0.58	-	-
Fungi	*Candida albicans* (KCTC 7965)	18.33±0.58[a]	11.38±0.58[b]	16.67±1.15[a]	17.67±1.15[a]

-; no inhibition. The data are expressed as the mean ± SD (n =3). The mean values with different superscript letters in the same row represent significantly different antimicrobial activity among the isolated bacteria (n =3) at p <0.05. * represents significant difference (p <0.05) compared to antibacterial activity of CBD using Student's t-test.

Table 4 Effects of *B. subtilis* strains CBD2 and KMKW4 on mouse body weight and food intake

Group	Body weight (g)			Food intake (g/day)		
	Day 1	Day 8	Day 13	Day 1	Day 8	Day 13
NOR	21.80±0.90	22.08±0.83	22.49±1.23	4.38±0.23	4.94±0.11	5.29±0.02
CON	21.19±0.84	22.89±0.75	24.12±0.67	4.29±0.20	4.95±0.03	4.76±0.25
CBD2	20.94±0.97	21.89±0.96	22.72±1.04	4.26±0.13	5.06±0.18	5.05±0.12
KMKW4	21.62±0.94	22.24±0.80	22.98±0.78	4.16±0.10	4.77±0.22	5.18±0.11

NOR, normal mice; CON; loperamide-treated mice; CBD2, mice treated with *B. subtilis* CBD2 and loperamide; KMKW4, mice treated with *B. subtilis* KMKW4 and loperamide.

loperamide hydrochloride (4 mg/kg) significantly reduced the excretion frequency and fecal weight and water content to 42.5±20.1, 40.4±18.5, and 33.0±11.6% of those in the NOR group, respectively (p <0.05; Fig. 3A, B, and C). Administration of *B. subtilis* CBD2 ameliorated loperamide-induced constipation, because mice in the CBD2 group showed increased fecal frequency, wet weight, and water content (73.8±32.9, 26.7±14.1, and 28.6±22.4%, respectively) compared to the control group treated with loperamide alone; however, the wet fecal weight and water content did not differ significantly (p >0.05). Similarly, *B. subtilis* KMKW4 also demonstrated preventive activity. Although the wet fecal weight was reduced to 22.2±25.9% of that in the control group, the fecal frequency and water content increased to 57.1±60.7 and 24.1±31.3%, respectively, of that in the control group. However, these results did not differ significantly (p >0.05). At the end of the experiment, the mice were sacrificed, and the distal colons were analyzed for the presence of fecal pellets. In the constipated control group, the number of fecal pellets in distal colon increased to 38.1±31.0% of that in normal mice. However, the mice pre-treated with *B. subtilis* CBD2 and KMKW4 showed lower constipation and less fecal pellets in distal colon: 31.0±21.1 and 31.0±17.2%, respectively, compared to the control group. However, no significant differences were observed in all experimental groups (p >0.05; Fig. 3D).

Constipation is a chronic gastrointestinal disorder characterized by infrequent bowel movements, difficulty during defecation, and sensation of incomplete bowel evacuation (Emmanuel et al., 2009; Walia et al., 2009). A mechanism of slow transit constipation is a failure of peristalsis to move luminal contents through the colon, which increases the time for the bacterial degradation of fecal

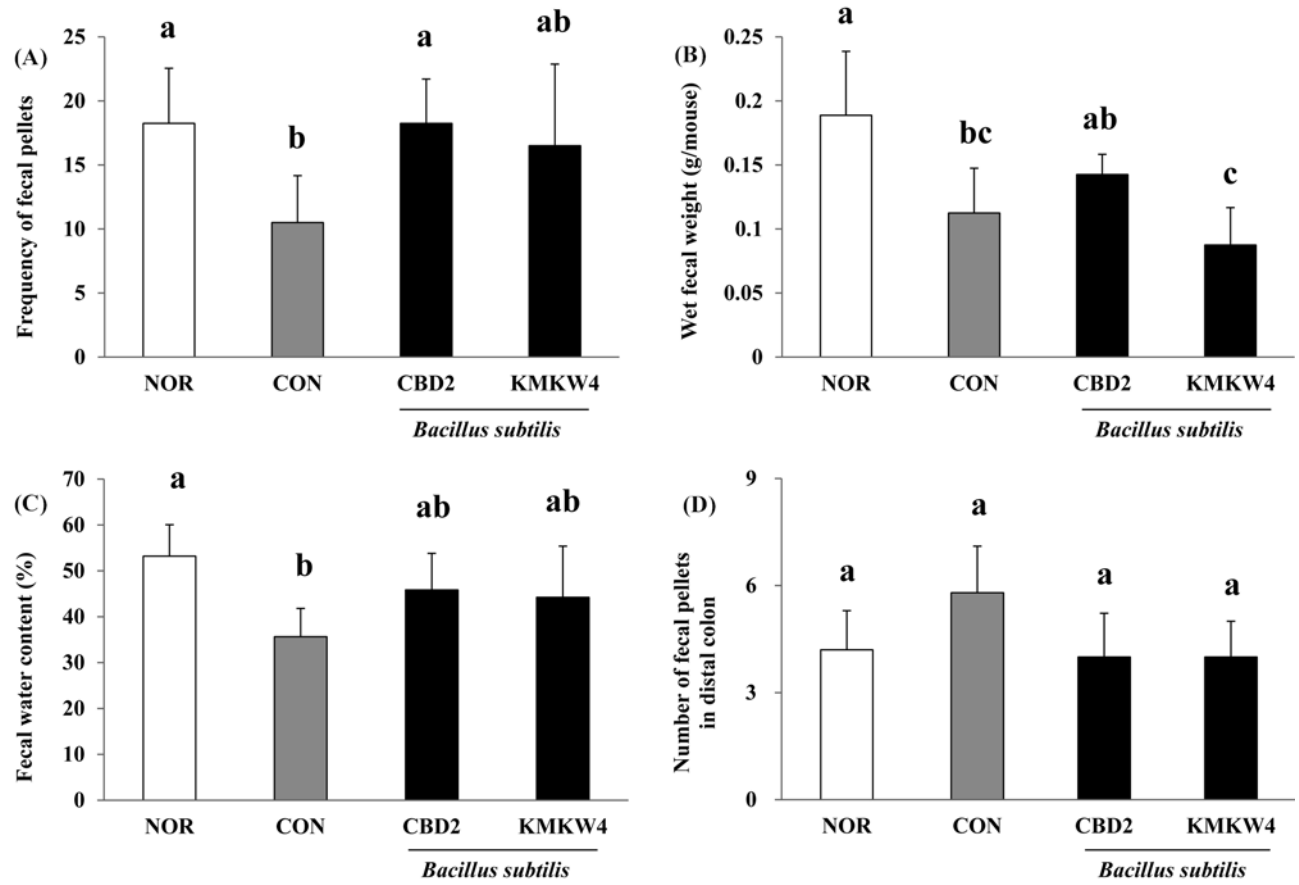

Fig. 3 *B. subtilis* CBD2 and KMKW4 prevented negative effects on fecal output in loperamide-treated constipated mice. Excretion frequency (A), fecal wet weight (B), and water content (C) were assayed by counting fecal pellets produced in 1 h at day 13 (1 day after the last injection of loperamide; n =8 per group). At the end of the analysis, the mice were sacrificed (n =5 per each group and fecal pellets were counted in the distal colon (D). The data are expressed as the means ± SD. Superscript lower case letters indicate significant differences at p <0.05.

solids and water absorption and reduces the frequency and weight of feces (Schiller, 2001). Loperamide is an opioid-receptor agonist that acts on the μ-opioid receptors in the intestinal myenteric plexus, decreasing its activity, which in turn inhibits contractility of circular and longitudinal smooth muscles in the intestinal wall (Tan-No et al., 2003; di Bosco et al., 2008). Loperamide induces constipation by slowing intestinal transit and increasing contact time, inhibiting fluid and electrolyte secretion, and stimulating salt and water absorption (Awouters et al., 1983; Schiller et al., 1984); it also decreases colonic mass movement and suppresses the gastrocolic reflex (Katzung, 2004). In the present study, loperamide decreased excretion frequency and fecal wet weight and water content, which was consistent with previous findings (Kakino et al., 2012). These symptoms of constipation were ameliorated by the pre-treatment with *B. subtilis* CBD2 and KMKW4 strains. Although some effects were not statistically significant, these results demonstrate that *B. subtilis* strains can prevent loperamide-induced constipation in mice.

Gastrointestinal transit. To determine the effects of *B. subtilis* CBD2 and KMKW4 on the gastrointestinal function in mice, we used the barium sulfate method (Fig. 4). GIT was assessed by measuring barium sulfate migration from the pylorus to the most distal point and from the pylorus to terminal ileum. Loperamide reduced the GIT to 31.8±9.1% of that in normal group, whereas *B. subtilis* CBD2 and KMKW4 significantly incteased the GIT to 45.8±27.6 and 40.6±23.5%, respectively, compared to the constipated control group (p <0.05).

The measurement of GIT time is a direct method to evaluate gastrointestinal motility disorders, including constipation (Padmanabhan et al., 2013) and is very useful for diagnostic screening (Lin et al., 2005). Loperamide inhibits intestinal fluid secretion and intestinal wall movement, delaying fecal evacuation and GIT (Holzer, 2009). In our loperamide-induced constipation model, *B. subtilis* CBD2 and KMKW4 strains increased the GIT to about 20% compared to the control group, which may explain the increase of fecal frequency described above (Fig. 3).

Harmful enzymatic activity in the gastrointestinal tract. We then examined the effects of *B. subtilis* CBD2 and KMKW4 on the activity of harmful enzymes in the cecum of loperamide-constipated mice. The cecum extract of mice sacrificed after the

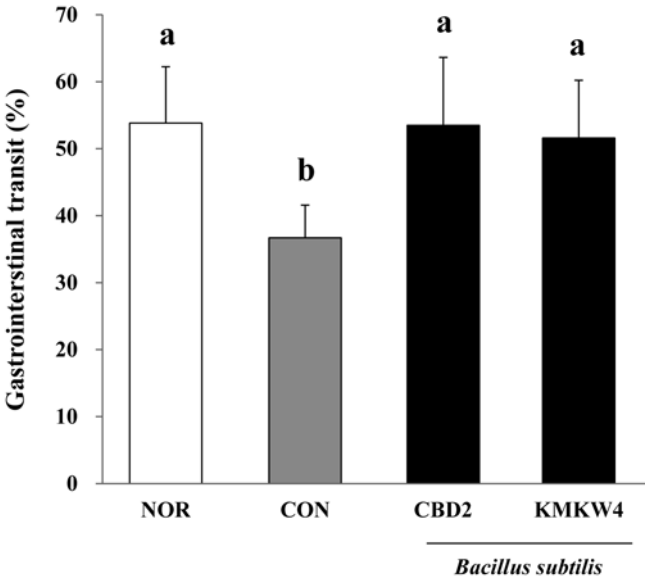

Fig. 4 *B. subtilis* CBD2 and KMKW4 improved gastrointestinal transit (GIT) in loperamide-treated constipated mice. Mice were fasted for 24 h prior to barium sulfate administration for 15 min. Barium migration was measured from the pylorus to the most distal point and from the pylorus to terminal ileum (n =3 per group). The data are expressed as the means ± SD (n =3). Superscript lower case letters indicate significant difference (p <0.05).

last injection of loperamide was used to analyze the activity of β-glucosidase and tryptophanase (Fig. 5). β-Glucosidase was significantly activated by loperamide treatment (176.0±35.2% of the normal untreated group; p <0.05), whereas *B. subtilis* CBD2 inhibited the upregulation of β-glucosidase (109.7±21.7% of the normal group; p <0.05). The *B. subtilis* KMKW4 strain also exhibited positive effects, reducing the upregulation of β-glucosidase in loperamide-treated mice (141.4±30.5% of the normal group). However, no significant difference was observed (p >0.05; Fig. 5A). Similarly, tryptophanase was activated by loperamide treatment (123.7±18.0% of the normal group), whereas *B. subtilis* CBD2 and KMKW4 inhibited the upregulation of tryptophanase in loperamide-treated mice (102.4±12.3 and 98.6±12.8%, respectively, of that in the normal group). However, these results were not significantly different (p >0.05; Fig. 5B).

The enzymatic activity of intestinal microflora is implicated in the enterohepatic circulation of toxic and carcinogenic substances (Guerin-Danan et al., 1998). In particular, colonic β-glucosidase and tryptophanase have been associated with colorectal carcinogenesis (Chung et al., 1975; Chadwick et al., 1992). Several studies have shown that specific bacteria can reduce the intestinal endotoxin levels and improve intestinal function (Griffiths et al., 2004; Wang et al., 2004; Wang et al., 2006). Furthermore, previous studies have demonstrated that the decrease in the activity of harmful enzymes improves the GIT and relieves constipation (Lee et al., 2009; An et al., 2010). The results of the present study show that *B. subtilis* CBD2 and KMKW4 strains inhibit the activity of β-glucosidase and tryptophanase, identified as harmful enzymes of the intestinal microflora, confirming beneficial probiotic-like effects of these strains on the gastrointestinal tract.

In conclusion, our data indicate that *B. subtilis* strains CBD2 and KMKW4 isolated from Korean traditional fermented food positively affect the intestinal function by suppressing the growth of pathogenic microflora and the activity of harmful enzymes as well as ameliorating constipation. The results of this study suggest that these *B. subtilis* strains can be used as probiotics to improve gastrointestinal health.

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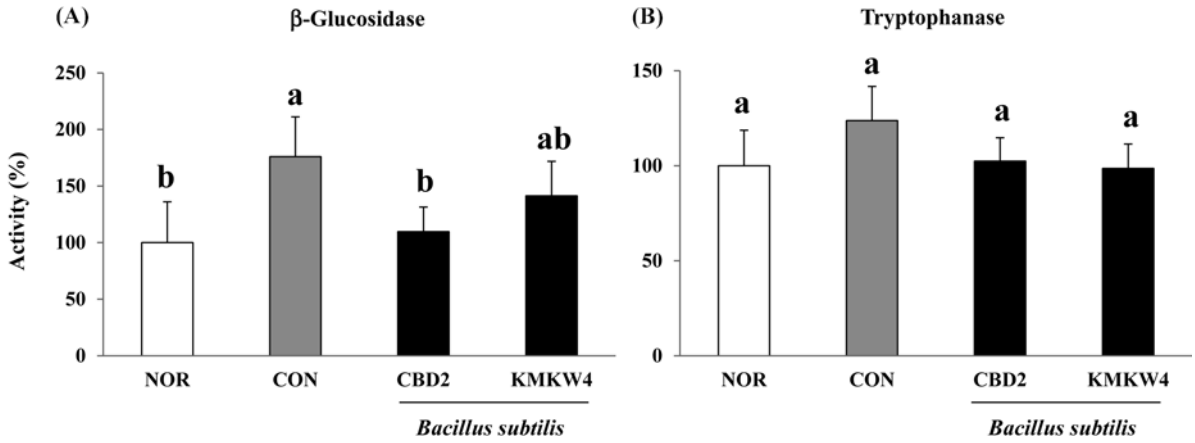

Fig. 5 *B. subtilis* CBD2 and KMKW4 inhibited the activity of β-glucosidase and tryptophanase in loperamide-treated constipated mice. After last injection of loperamide, the mice were sacrificed (n =5 per group), the caecum extract was prepared and used to assess the enzymatic activity of β-glucosidase (A) and tryptophanase (B). The data are expressed as the means ± SD. Superscript lower case letters indicate significant difference (p <0.05)

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