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ARTICLE

Effect of microencapsulated *Bacillus subtilis* strain CBD2-fermented grain on loperamide-induced constipation in mice

Bae Jin Kim[1] · Hee Kyoung Jung[1] · Yoo Seok Jeong[1] · Su Jin Yang[2] · Joo-Heon Hong[2]

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Abstract Constipation is a common cause of discomfort that affects the quality of life. The aim of the present study was to evaluate the ameliorative effects of microencapsulated food products using *Bacillus subtilis* CBD2 strain-fermented grain in mice with loperamide-induced constipation. Microcapsules prepared by spray-drying with 2 % pectin and 2 % alginic acid (SD-P2A2) showed higher adhesion and dissolution of amylase than those prepared by freeze-drying. Experimental mice were fed a diet containing SD-P2A2 for 7 days prior to loperamide treatment. The ameliorative effects of an experimental diet containing SD-P2A2 on constipation were compared with those observed in mice fed a control diet containing non-fermented grain. Following the induction of constipation in mice, a marked alteration was observed in fecal parameters (frequency of defecation, fecal weights, fecal water content, and fecal pellet numbers in the distal colon), gastrointestinal transit ratios, harmful enzyme activities, and gene expression differences related to intestinal muscle contraction. These symptoms were significantly ameliorated in mice fed the experimental diet with SD-P2A2 versus constipated control mice. Thus, the intake of microencapsulated food products using *B. subtilis* CBD2 strain-fermented grain exhibited probiotic effects; these products may be used as a functional material for reducing constipation.

Keywords *Bacillus subtilis* · Constipation · Fermentation · Microencapsulation · Spray-drying

Introduction

Constipation is characterized by a constellation of symptoms and complaints: the most common of which are low defecation frequency, irregular fecal expulsion, painful and strained defecation, hard and dry fecal pellets, a feeling of incomplete rectal defecation, and the passing of abnormally small fecal pellets (Bosaeus 2004). Loperamide acts on the mu-receptors in the intestinal mucosa, which leads to decreased gastrointestinal motility by reducing circular and longitudinal smooth muscle activity of the intestinal wall. Loperamide is approved for the control of diarrhea symptoms and is available without a prescription (Arafat et al. 2014). The major mechanism underlying constipation is the failure of peristalsis to move the luminal content through the colon, resulting in increased time required for bacterial feces degradation and more time for salt and water absorption, which manifests in reduced defecation frequencies and fecal weights (Schiller 2001). Various laxative drugs that contain magnesium oxide, senna, senokot, or gaviscon are commonly used to treat constipation, but these drugs are expensive and induce side effects, such as severe diarrhea. In addition, the repeated use of some drugs can induce colic melanosis, a risk factor for developing colorectal neoplasms (Siegers et al. 1993; Wintola et al. 2010). Thus, substantial efforts have been made to develop new laxative drugs with reduced side effects and increased beneficial effects.

Probiotic bacteria can confer health benefits to the host when administered in adequate amounts (Schrezenmeir and

✉ Joo-Heon Hong
jhhong@cu.ac.kr

[1] Biohealth Convergence Center, Daegu Technopark, 46-17, Seongseogongdan-ro, Dalseo-gu, Daegu, Republic of Korea

[2] Department of Food Science and Technology, Catholic University of Daegu, 13-13, Hayang-ro, Hayang-Eup, Gyeongsan-Si, Gyeongbuk, Republic of Korea

de Vrese 2001). Probiotic bacteria can be effective in treating chronic constipation, and their laxative effects are similar to those of magnesium oxide (Bu et al. 2007). Moreover, probiotic bacteria ameliorate colic, one of the symptoms of constipation (Szajewska et al. 2013). 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). *Bacillus* spores are able to germinate in the gastrointestinal tract and exert probiotic activity (Casula and Cutting 2002). The most abundantly used bacterial α-amylases for industrial purpose are derived from members of the *Bacillus* species.

Biopolymers such as pectin and alginic acid have attracted interest as matrices for the immobilization or controlled release of several enzymes and have been applied in the pharmaceutical, food, biomedical, chemical, and waste-treatment industries (Ghanem and Skonberg 2002). Alginic acids have been used in several applications related to microencapsulation and controlled release delivery systems, namely, for proteins and enzymes (George and Abraham 2006; Möbus et al. 2012). The alginic acid gel structure is relatively stable at acidic pH, but it is easily swollen and disintegrated under mild alkali conditions (Yoo et al. 2006). Spray-drying is the most common microencapsulation method used in the food industry. The benefits of encapsulation to protect probiotics against low gastric pH have been shown in numerous reports (Anal and Singh 2007).

We previously described the potential of the *Bacillus subtilis* CBD2 strain probiotic isolated from Korean fermented food in treating mice with loperamide-induced constipation (Kim et al. 2013). The probiotic potential of *B. subtilis* may be enhanced via modification, such as with fermentation and microencapsulation. Therefore, we investigated whether food products containing microencapsulated *B. subtilis* CBD2-fermented grain could exert probiotic effects in mice with loperamide-induced constipation. In the present study, changes in fecal parameters, the gastrointestinal transit (GIT) ratio, harmful enzyme activities, and mRNA expression levels of intestinal muscle contraction-related genes were observed in mice with loperamide-induced constipation to evaluate the probiotic effects of microencapsulated food products containing *B. subtilis* CBD2-fermented grain. Our results suggested that microencapsulated food product containing *B. subtilis* CBD2-fermented grain may be used as a functional material for ameliorating constipation.

Materials and methods

Preparation of samples and microbiological cultures

Rice and amaranth were purchased from an online market, ground in a blender, and passed through a 200-mesh screen (rice) or a 40-mesh screen (amaranth). The *B. subtilis* CBD2 strain was isolated from *Doenjang*, Korean fermented soybean paste, as described in a previous study (Yang et al. 2014). *B. subtilis* CBD2 was used for the production of fermented rice and amaranth. The strain was cultured in nutrient broth (Difco, Detroit, MI, USA) at 37 °C for 18 h. Activated cultures were inoculated into 10 mL of nutrient broth and then incubated at 37 °C for an additional 24 h.

Preparation of fermented grain cultures

Fermented grain (FG) was prepared by mixing amaranth powder and rice powder at a 1:4 (w/w) ratio in an Erlenmeyer flask. Subsequently, sterile distilled water (1:2, w/v) and 2 % (v/v) *B. subtilis* CBD2 was added to the flask, and the fermentation proceeded in a shaking incubator set at 37 °C for 72 h. The FG was subjected to freeze-drying (FD) or spray-drying (SD) to form a powder.

Spray-drying procedure

Microencapsulation was performed with fermented grain medium using different agents (2 % pectin and either 0.5, 1, or 2 % alginic acid), using a spray drier. The mixture was stirred at 4,000 rpm for 5 min using a WiseTis homogenizer (DAIHAN Scientific Co., Ltd, Seoul, Korea). The grain flour liquid culture was processed using an atomizer-type Pilot KL-8 spray drier (Seogang Engineering Co., Ltd, Cheonan, Korea) with a flow rate of 12 mL/min. The inlet (controlled) and outlet (measured) air temperatures were 160 and 110 °C, respectively. The spray-dried microcapsules (2 % pectin, SD-P2; 2 % pectin with 0.5 % alginic acid, SD-P2A0.5; 2 % pectin with 1 % alginic acid, SD-P2A1; and 2 % pectin with 2 % alginic acid, SD-P2A2) were stored at −70 °C until further analysis.

Yield

The yield was calculated as the ratio between the total weight of freeze-dried or spray-dried powders and the total weight of fermented grain measured after adding the forming agent.

Particle size

The particle size of the powders was measured by laser diffraction (LS-320, Beckman Coulter, Fullerton, CA, USA). Approximately 0.1 g of each powder was used to achieve the required obscuration percentage of 8–10 %, and each sample was measured in triplicate.

Microbiological analysis (total viable bacterial counts)

FG (10 mL) was homogenized with 90 mL of sterile physiological saline (0.85 % NaCl). Serial dilutions of FG were prepared in sterilized physiological saline. Then, 0.1 mL of each dilution was transferred to the surfaces of nutrient agar plates and was spread over the entire surface of the agar by rotary motion. The cultures were incubated at 37 °C for 24 h. The colonies were then counted and expressed as logarithmic colony forming units per milliliter or gram of sample.

Amylase assays

Amylase activities were determined by measuring the amount of reducing sugars released during starch hydrolysis using the dinitrosalicylic acid (DNS) method (Miller 1959). The reaction mixture included 0.5 mL of sample and 0.5 mL of 1 % of soluble starch dissolved in 50 mM sodium phosphate buffer at pH 7.0. The mixture, after incubation for 30 min at 37 °C, was boiled for 10 min in the presence of 1.5 mL of DNS. After cooling to 15 °C, the absorbance at 550 nm was measured.

In vitro dissolution studies

For in vitro dissolution in the Erlenmeyer flask, 0.8 g of sample was mixed with 12 mL of 0.1 N hydrochloric acid (pH 1.2) and shaken for 2 h. Approximately 18 mL 0.05 M phosphate buffer (pH 6.8) and 2 mL 1 N sodium bicarbonate were added, and the flask was shaken for an additional 3 h. Finally, 6 mL of phosphate buffer (pH 7.4) and 2 mL 1 N sodium bicarbonate were added, and shaking was continued for 2 h. Samples were collected every 30 min and amylase activities were measured by the DNS method.

Bacterial adhesion capacity to HT-29 cells

Adhesion assays were performed as described previously (Sanae et al. 2013), with some modifications. The human colon adenocarcinoma cell line HT-29 (Korean Cell Line Bank, Seoul, Korea) was used to assay the adhesion capacity of the *B. subtilis* CBD2 strain. HT-29 cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (WELGENE Inc., Daegu, Korea) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco, USA) at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were seeded at a concentration of 1×10^5 cells/well in 24-well cell culture plates. Monolayers were formed after 24 h incubation. The HT-29 monolayer was washed twice with phosphate-buffered saline (PBS, pH 7.4) before use. One milliliter of each suspended sample (grain fermented by *B. subtilis* CBD2) was added to wells containing HT-29 monolayers in 1 mL of growth medium. The inoculated cell culture plates were incubated under 5 % (v/v) CO_2 at 37 °C for 4 h. The monolayers were washed 3 times with PBS (pH 7.4) to remove bacteria that did not attach to the HT-29 cells. Subsequently, the HT-29 cells were lysed using 0.05 % (v/v) Triton X-100, which did not lyse the *B. subtilis* CBD2 strain, and the number of viable adherent bacteria was determined by serial dilutions on nutrient agar plates. This assay was performed with triplicate wells. The adhesion percentage was calculated according to the following equation: Adhesion (%) = $(N_1/N_0) \times 100$ %, where N_1 is the number of adherent bacterial cells, and N_0 is the number of added bacterial cells.

Care and use of animals

Four-week-old male BALB/c mice were purchased from Central Lab Animal, Inc. (Seoul, Korea). All animals were acclimated to the experimental animal facility for 1 week. Mice were housed under a controlled temperature (22 ± 2 °C) and 50 ± 15 % humidity on a 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 (Approval Number BHCC-IACUC-2014-15).

In vivo experimental design and treatment

At 5 weeks of age, mice were randomly divided into four groups ($n = 8$). The normal (NO) group and the constipated (CO) control group received a powdered normal commercial diet (Purina rodent feed, Cargill Agri Purina Inc., Seongnam, Korea). Experimental group 1 (EX-1) and experimental group 2 (EX-2) mice were fed a powdered diet containing a 3:1 mixture of normal commercial diet and non-fermented grain (NonF; EX-1) or *B. subtilis* CBD2 strain-fermented SD-P2A2 (EX-2, 4.53 ± 0.06 log CFU/(g day), respectively, for 7 days.

One day after the last feed with the experimental diets (day 8), constipation was induced in the CO, EX-1, and

EX-2 groups by subcutaneous injection of loperamide hydrochloride (4 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) in 0.5 % Tween-20 (Sigma-Aldrich) twice a day for 5 days. The non-constipated NO group was subcutaneously injected with 0.5 % Tween-20 alone (Shimotoyodome et al. 2000). The induction of constipation was evaluated by measuring fecal parameters (frequency, wet/dry weight, and water content).

Measurement of body weights and food intake

Alterations in mouse body weights and food intake were determined at day 1 (before the experiment), day 8 (after the last feed with the experimental diets), and day 13 (after the last injection of loperamide hydrochloride), using a CP423S balance (Sartorius AG, Germany). All measurements were performed in triplicate to ensure accuracy.

Fecal parameters and GIT ratios

Fecal output was assayed by counting the number of fecal pellets produced in 1 h on days 1, 8, and 13 and by assessing wet and dry fecal weights, as described previously (Devries et al. 2010). Dry weights were measured after the pellets had been heated at 70 °C for 24 h in a laboratory dry-oven. The percentage of water content was calculated as follows: (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 contained within. Other mice ($n = 3$ per each group) were used to measure GIT ratios.

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

Harmful enzyme activities

Ceca from sacrificed mice were removed and suspended 1:10 in cold 0.1 M phosphate buffer (pH 7.0). Non-bacterial debris was removed by centrifugation at 6000 rpm for 10 min, and the cecal supernatants were assayed for β-glucosidase, tryptophanase, and nitroreductase activity. Harmful enzyme activities were expressed as the percentage of the absorbance compared to that observed with the NO group.

β-glucosidase activity was measured in 2 mL reaction mixtures containing 800 μL of 2 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) and 200 μL of cecal supernatant. Reactions were incubated for 30 min at 37 °C and stopped by the addition of 1 mL 0.5 N NaOH. Subsequently, reaction mixtures were centrifuged at 3000 rpm for 10 min, and β-glucosidase activities were determined by measuring the absorbance at 405 nm (Lee et al. 2011).

Tryptophanase activities were assayed in 2.5 mL reaction mixtures containing 200 μL of complete reagent solution (2.75 mg pyridoxal phosphate, 19.6 mg disodium EDTA dehydrate, 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 cecal supernatant. Reactions were incubated for 1 h at 37 °C and stopped by the addition of 2 mL of color reagent (14.7 g p-dimethylaminobenzaldehyde dissolved in 52 mL H_2SO_4 and 948 mL 95 % ethanol). Reaction mixtures were centrifuged at 3000 rpm for 10 min, and tryptophanase activities were determined by measuring the absorbance at 550 nm (An et al. 2010).

Nitroreductase activities were assayed in 750 μL reaction mixtures containing 400 μL of pre-heated (37 °C) substrate solution (0.025 g of m-nitrobenzoic acid in 100 mL of 0.1 M potassium phosphate buffer, pH 7.0) and 100 μL of the cecal supernatant. Reactions were incubated for 1 h at 37 °C and then stopped by the addition of 250 μL of 10 % trichloroacetic acid. To measure color formation, the reaction mixtures were centrifuged at 5000 rpm for 5 min, and 200 μL of each supernatant was transferred to a new tube. Next, 200 μL of 0.1 % sodium nitrite was added to new tubes containing reaction mixture supernatants and incubated for 3 min at room temperature. Subsequently, 200 μL of 0.5 % ammonium sulfamate was added to the mixtures and incubated for 2 min at room temperature. Finally, 100 μL of 0.1 % N-1-napthylethylenediamine dihydrochloride was added to the mixtures and nitroreductase activities were determined by measuring the absorbance at 550 nm (Gudiel-Urbano and Goni 2002).

Quantitative real-time polymerase chain reactions

RNA was extracted from the small intestines of five mice per group using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA, USA), and RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). A total of 5 μg of each RNA was converted to cDNA using the Sprint RT Complete-Oligo(dT)18 Kit (Takara Bio Inc., Shiga, Japan), according to the manufacture's guidelines. The cDNAs were quantified using the iQ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA, USA). For each transcript level investigated, a mixture of the following reaction

Table 1 Comparison of viable bacteria cells, amylase activities, yields, and particle sizes in fermented grain after either freeze-drying or spray-drying

Group[1)]	Viable bacteria cell (log CFU/g)	Amylase activity (units/g)	Yield (%)	Particle size (μm)
FD	7.01 ± 0.09[a, 2)]	506.02 ± 19.88[bc]	98.53 ± 0.45[a]	357.87 ± 10.83[a]
SD-P2	4.66 ± 0.05[b]	995.71 ± 23.86[a]	89.97 ± 3.15[b]	36.94 ± 0.64[b]
SD-P2A0.5	4.36 ± 0.11[d]	697.96 ± 24.58[b]	89.69 ± 2.45[b]	45.36 ± 0.29[c]
SD-P2A1	4.73 ± 009[b]	416.93 ± 12.82[c]	88.65 ± 1.94[b]	46.66 ± 1.19[cd]
SD-P2A2	4.53 ± 0.06[c]	365.62 ± 11.07[c]	88.11 ± 2.11[b]	47.93 ± 0.64[d]

[1)] *FD* freeze-dried powder, *SD-P2* spray-dried powder with 2 % pectin added, *SD-P2A0.5* spray-dried powder 2 % pectin and 0.5 % alginic acid added, *SD-P2A1* spray-dried powder 2 % pectin and 1 % alginic acid added, *SD-P2A2* spray-dried powder with 2 % pectin and 2 % alginic acid added

[2)] In each line, different letters mean statistical significant differences ($p < 0.05$) between samples

components was prepared containing a forward primer and a reverse primer (final concentration for each primer: 10 pM) and iQ SYBR Green Supermix. Briefly, 20 μL of master mix was added to each well of an iCycler 96-well PCR plate, and 5 μL cDNA (125 ng) was added as the PCR template. The plate was closed, centrifuged, and placed into an iQ™5 Real-Time PCR Machine (Bio-Rad). Fold-changes were calculated using the delta–delta Ct method (Schmittgen and Livak 2008). Primers with the following sequences were used: muscarinic acetylcholine receptor M2 (mAChR M2; forward: 5′-GCT GCG TGG GTT CTT TCC T-3′; reverse: 5′-CCC CTA CGA TGA ACT GCC AG-3′), muscarinic acetylcholine receptor M3 (mAChR M3; forward: 5′-CCA TCT GGC AAG TGG TCT TC-3′; reverse: 5′-TGC CAC AAT GAC AAG GAT GTT G-3′), and GAPDH (forward: 5′-GGA GGA ACC TGC CAA GTA TG-3′; reverse: 5′-TGG GAG TTG CTG TTG AAG TC-3′).

Statistical analysis

Data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to evaluate mean differences among the experimental groups. Duncan's multiple-range test was used to adjust for multiple-comparisons, and null hypotheses were rejected at the 0.05 level. All data were analyzed using SPSS/Windows software (Version 12.0, SPSS Inc., Chicago, IL, USA).

Results and discussion

Characterization of fermented grain: yield, particle size, adhesion, and dissolution parameters

The fermentation characteristics of FG were investigated. After 72 h of fermentation, the viable count of *B. subtilis* CBD2 in a fermented sample was 6.32 CFU/mL. During the fermentation process, the pH decreased significantly. Amylase activity produced by *B. subtilis* CBD2 in FG supernatant was 194.00 ± 12.89 units/mL (data not shown). Thus, we confirmed changes on the stability of bacteria and amylase activities by microencapsulation of FG. The viable bacterial cell counts in FD powder and SD powders containing different agents (SD-P2, SD-P2A0.5, SD-P2A1, and SD-P2A2) are shown in Table 1. Compared to the findings for FD, the viable bacterial cell counts in the SD powders were lower. An increase of the alginic acid concentrations in the SD powder significantly inhibited amylase activity. Significantly higher amylase activities were evident in SD-P2 (995.71 units/g).

Our findings demonstrated that microencapsulation of the probiotic and the amylase enzyme using different microencapsulation agents by the SD technique is possible. The use of alginic acid is limited due to its low stability in the presence of chelating agents and in acidic conditions below pH 2.0 (María et al. 2010). Adding pectins to alginic acid-based encapsulant formulations may confer multiple benefits, including the enhanced protection of core substances and increased dietary fiber consumption and product nutritional value (Fernandez 2001).

The yield of spray-dried powders was all lower than that of freeze-dried powder (Table 1). The yield of spray-dried powders varied from 88.11 to 89.97 %; this value is known to be significantly influenced by the inlet and outlet drying air temperatures, the droplet formation mechanism, and the composition and concentration of feed solution (Anandharamakrishnan et al. 2007). The FD powder and SD powders exhibited a wide range of particle sizes, which varied from 36.94 to 357.87 μm. The microcapsule particle size is a key factor because small-sized particles are preferred in food formulations to ensure homogeneity and quality (Burgain et al. 2011). Compared to FD powder, SD powders exhibited a smaller mean diameter with a narrow range of particle size distribution. The size of the microcapsules is an

important parameter that affects the sensory properties of foods. In general, spray-drying produces microcapsule sizes ranging between 5 and 80 µm. The microcapsule size also affects the adhesion properties of probiotics, and the optimal size is 50 µm in diameter (Nazzaro et al. 2012).

The adhesion values of SD powders to epithelial HT-29 cells were 3.17–6.06 % of the initial cell counts, whereas FD showed lower adhesive ability (0.07 %), as shown in Fig. 1A. The addition of alginic acid resulted in increased adhesion capacity of the *B. subtilis* CBD2 strain. Thus, high adhesion was observed for SD-P2A2. These results demonstrated the possibility of producing a dry probiotic bacterial preparation using SD. The adhesion rate of SD-P2A2 was higher than that of *B. subtilis* H7 (0.35 %) (Rowan et al. 2001).

The dissolution profiles observed with the FD powder and the SD powders are shown in Fig. 1B. No release of amylase was detected at pH 1.2 over a 2 h incubation period with any of the microencapsulation-coated samples. At a pH of 6.8, a rapid release of amylase activity was observed, with little difference observed as a function of the forming agent or the drying method used. Amylase activity values observed for the dissolution of FD, SD-P2, SD-P2A0.5, SD-P2A1, and SD-P2A2 were 152.57 ± 14.86, 194.50 ± 17.35, 248.63 ± 20.88, 330.88 ± 16.74, and 367.09 ± 8.35 units/g, respectively.

In recent years, microencapsulation has also been found to be useful for the stabilization of probiotic cells in functional food applications. Microencapsulation can enhance the viability of probiotic cells during processing, storage, and subsequent consumption (Krasaekoops et al. 2003). The above results showed that microencapsulation using alginic acid and pectin materials is a feasible approach for microencapsulating probiotics. The functional properties of probiotic microorganisms, such as gastrointestinal tolerance and the adhesion to intestinal epithelium, are critical factors in probiotic efficacy (Ranadheera et al. 2014). Probiotics are defined as microbial cell preparations or components of microbes that have a beneficial effect on the health and well-being of a host. Probiotic bacteria such as *Lactobacillus* produce organic acids such as lactic acid and acetic acid, which can lower the pH of the colon, enhancing peristalsis and reducing colonic transit times (Jayasimhan et al. 2013). *Bacillus* spores are commonly used as probiotics for human and animal consumption due to their immunostimulatory properties on the gastrointestinal immune system (Salinas et al. 2005). SD-microencapsulated samples exhibited a significantly higher capability of adhesion to HT-29 cells than did the FD sample. Considering these results, SD-P2A2 was selected as a diet supplement for in vivo experiments.

Changes of body weight and food intake in constipated mice

We examined body weights and food-intake levels in mice fed experimental diets prior to loperamide treatment. As shown in Table 2, food intake did not differ significantly among the experimental groups on day 1 (before the experiment), day 8 (after the last day of feeding on the experimental diets), and day 13 (after the last injection of loperamide). However, body weight was significantly increased by 4.1 ± 3.9 % in the CO group compared to the NO group at day 13 ($p < 0.05$).

Body weight and food intake are important factors for the evaluation of constipation. However, constipated animal models developed by loperamide treatment have shown conflicting effects on body weights and food-intake levels. For example, the results from a previous study demonstrated that loperamide treatment reduced body weight and food intake (Chumakova et al. 2011), whereas another study did not report changes of these factors following loperamide treatment (Lee et al. 2012). In our study, although the degree of food intake did not differ among the experimental groups, the gain in body weight was significantly increased in the CO group compared to the untreated NO group after loperamide treatment. This may be due to the accumulation of fecal pellets in their intestines, thus accounting for the extra weight. A similar observation was reported by Wintola et al. (2010).

Changes of fecal frequency, wet weight, water content, and fecal pellet numbers in constipated mice

To determine the constipation-preventative effects of experimental diets, the frequency of defecation, fecal weight, and fecal water content were analyzed in mice pretreated with experimental diets for 7 days prior to the induction of constipation with loperamide. The injection of loperamide (4 mg/kg) significantly reduced the excretion frequency, fecal weight, and fecal water content to 37.4 ± 18.2, 38.7 ± 13.3, and 7.2 ± 15.9 % of those observed in the NO group, respectively ($p < 0.05$, $n = 8$; Fig. 2A–C). The ingestion of an experimental diet containing SD-P2A2 prevented loperamide-induced constipation, with mice in the EX-2 group showing significantly increased fecal frequency, wet weight, and water content (44.6 ± 27.1, 48.3 ± 20.5, and 15.9 ± 14.0 %, respectively) compared to CO group ($p < 0.05$, $n = 8$). In contrast, the EX-1 group did not demonstrate significant differences in fecal parameters, compared with the CO group ($n = 8$ for each group). On the last day of the experiment, five mice in each group were sacrificed, and the distal colon was analyzed for the presence of fecal

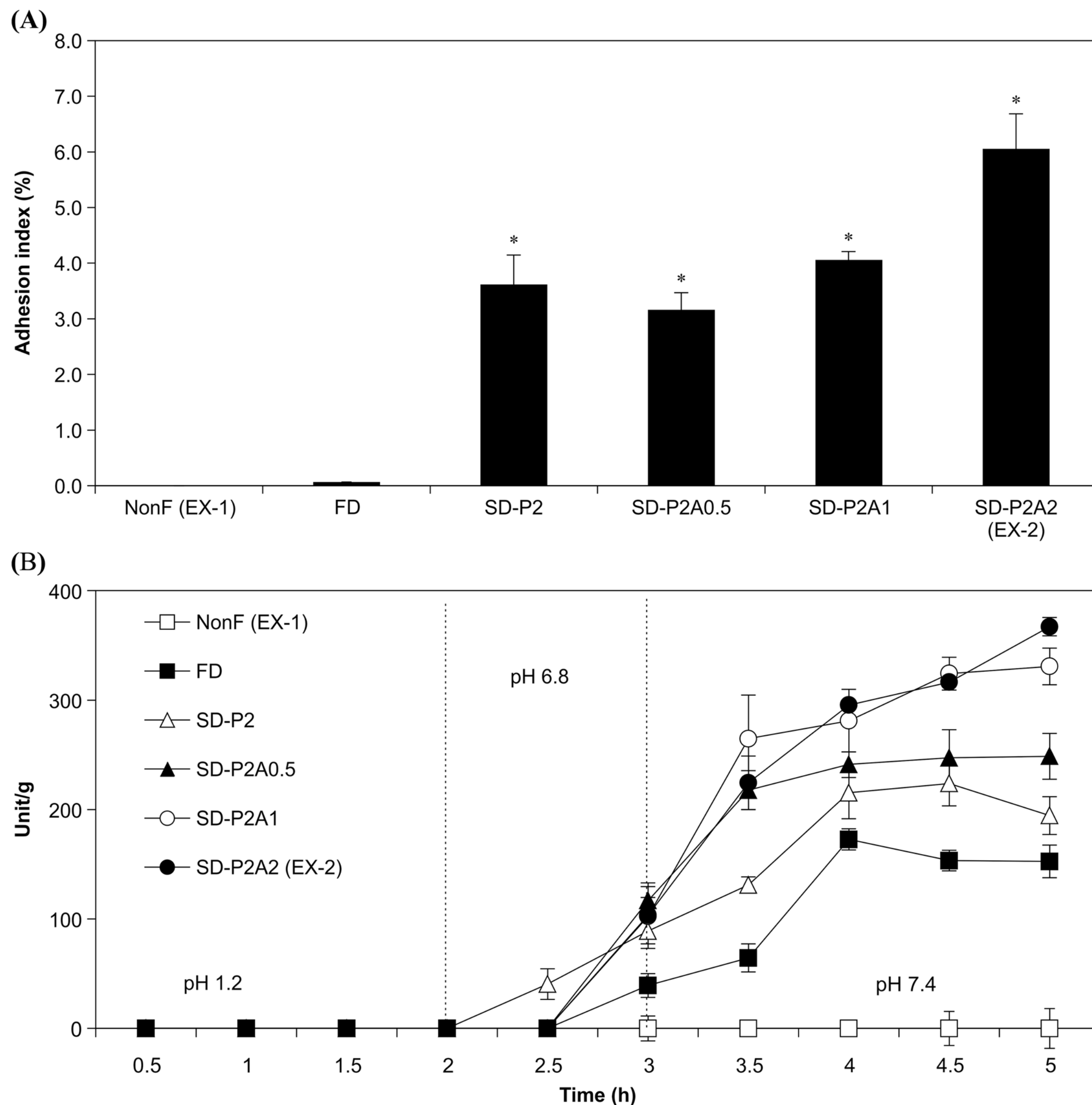

Fig. 1 Properties of in vitro-fermented grain. (**A**) Adhesion of *B. subtilis* CBD2 in fermented grain to intestinal epithelial HT-29 cells. (**B**) Dissolution behavior at pH 1.2, pH 6.8, and pH 7.4. *NonF* non-fermented grain, *FD* freeze-dried powder, *SD-P2* spray-dried powder with 2 % pectin added, *SD-P2A0.5* spray-dried powder with 2 % pectin and 0.5 % alginic acid added, *SD-P2A1* spray-dried powder with 2 % pectin and 1 % alginic acid added, *SD-P2A2* spray-dried powder with 2 % pectin and 2 % alginic acid added. The data are expressed as the mean ± SD. *$p < 0.05$, significant difference from the freeze-dried control group, as determined by Duncan's multiple-range test

pellets. The mice of the EX-2 group had fewer fecal pellets in the distal colon: 33.3 ± 21.1 %, compared to CO group ($p < 0.05$; Fig. 2D). However, no significant difference was found in the EX-1 group.

Loperamide is an opioid-receptor agonist that inhibits the activity of μ-opioid receptors in the intestinal myenteric plexus which in turn inhibits the contractility of circular and longitudinal smooth muscles in the intestinal wall (di Bosco et al. 2008; Tan-No et al. 2003). Loperamide also induces constipation by disturbing intestinal motility and increasing the contact time, inhibiting intestinal fluid secretion, and stimulating salt and water absorption (Read 1983; Schiller et al. 1984). Fecal-related markers such as the number, weight, and water content of fecal pellets were significantly decreased in loperamide-induced constipation animal models (Kim et al. 2013). An alteration of fecal parameters is regarded as one of the important markers of constipation. The significant changes of fecal parameters in mice fed an experimental diet containing SD-P2A2 prior to loperamide administration

Table 2 Effects of experimental diets containing microencapsulated mixtures of *B. subtilis* CBD2-fermented grain on body weights and food intake in constipated mice

Group[1)]	Body weight (g)			Food intake (g/day)		
	Day 1	Day 8	Day 13	Day 1	Day 8	Day 13
NO	20.3 ± 0.8[a, 2)]	23.1 ± 0.9[a]	24.8 ± 0.9[a]	4.8 ± 0.1[a]	5.1 ± 0.1[a]	5.4 ± 0.1[a]
CO	19.6 ± 0.8[a]	23.4 ± 1.3[a]	25.9 ± 1.0[b]	4.7 ± 0.1[a]	5.1 ± 0.1[a]	5.3 ± 0.1[a]
EX-1	20.0 ± 1.0[a]	23.5 ± 1.0[a]	25.6 ± 1.0[ab]	4.8 ± 0.1[a]	5.1 ± 0.1[a]	5.4 ± 0.1[a]
EX-2	20.0 ± 1.1[a]	23.8 ± 0.8[a]	25.6 ± 0.6[ab]	4.8 ± 0.1[a]	5.1 ± 0.1[a]	5.3 ± 0.1[a]

[1)] *NO* normal group, *CO* constipated control group, *EX-1* experimental group fed a powdered experimental diet containing a 3:1 mixture of commercial diet and non-fermented grain, *EX-2* experimental group fed a powdered experimental diet containing a 3:1 mixture of commercial diet, and *B. subtilis* CBD2 strain-fermented grain spray-dried with 2 % pectin and 2 % alginic acid. The data are expressed as the mean ± SD ($n = 8$ for all groups)

[2)] Superscripted lowercase letters indicate significant differences ($p < 0.05$, one-way ANOVA followed by Duncan's multiple-range test) observed among the indicated groups in the column

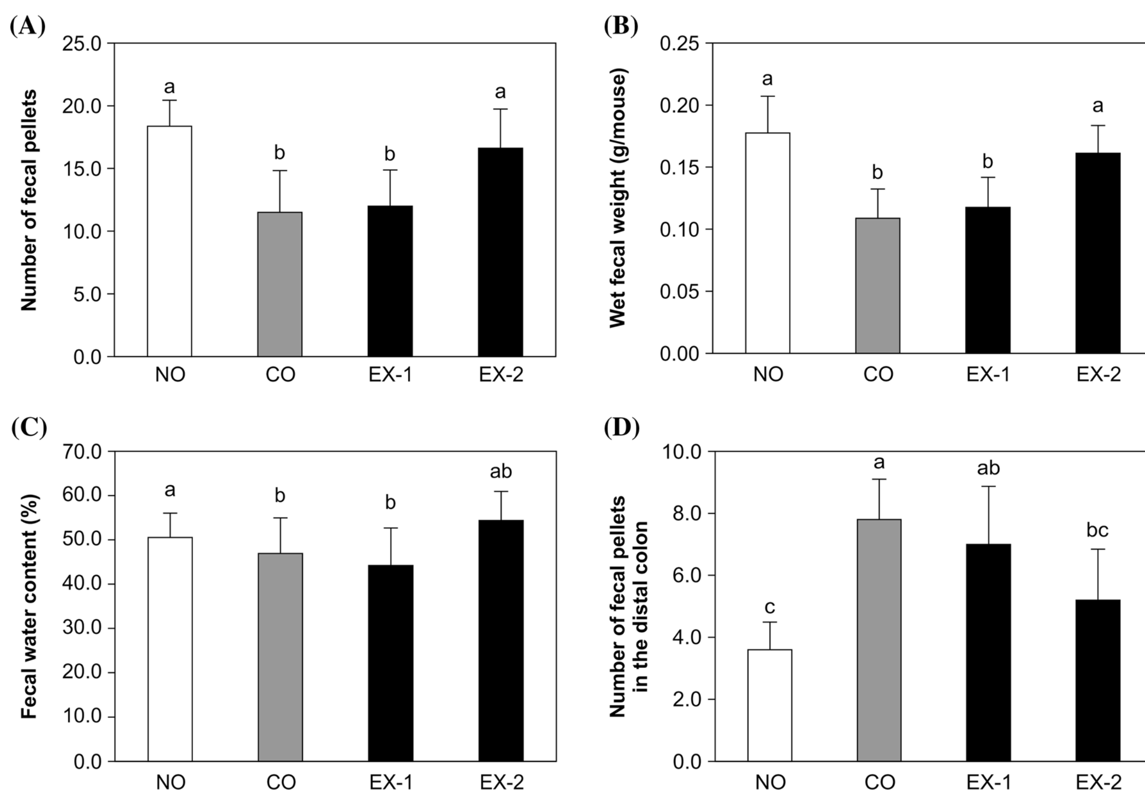

Fig. 2 Effect of experimental diet on feces-related parameters in constipated mice. Excretion frequencies (**A**), wet fecal weights (**B**), and fecal water content (**C**) were assayed after collecting fecal pellets produced in 1 h on day 13 (1 day after the last injection of loperamide; $n = 8$ per group). At the end of the analysis, the mice were sacrificed ($n = 5$ per group), and fecal pellets were counted in the distal colon (**D**). The data are expressed as the mean ± SD. *Superscripted lowercase letters* indicate significant differences at $p < 0.05$

showed that SD-P2A2 can ameliorate constipation. An alteration of fecal parameters is regarded as one of the important markers of constipation.

Changes of the GIT ratio in constipated mice

To examine the effects of experimental diets on gastrointestinal function, we used the barium sulfate method (Fig. 3). The GIT ratio was assessed as the length from the pylorus to the most distal point of barium sulfate migration divided by the length from the pylorus to terminal ileum. Loperamide injection reduced the GIT ratio, resulting in GIT ratio in the CO group was 40.4 ± 7.3 % lower than that of the NO group. The ingestion of the experimental diet containing SD-P2A2 significantly accelerated GIT, resulting in GIT ratio in the EX-2 group that was

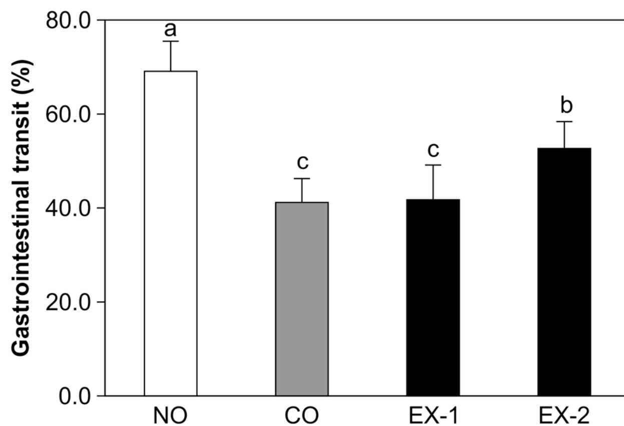

Fig. 3 Effect of experimental diet on gastrointestinal transit (GIT) in constipated mice. The data are expressed as the mean ± SD ($n = 3$). *Superscripted lowercase letters* indicate significant differences ($p < 0.05$). GIT ratios (%) = (A/B) × 100 %, where A is the length from the pylorus to the most distal point of barium sulfate migration, and B is the length from the pylorus to terminal ileum (B)

27.9 ± 13.8 % higher than that of the CO group ($p < 0.05$). However, ingestion of the experimental diet containing non-fermented grain (NonF; EX-1 group) failed to induce a significant acceleration in GIT.

Transit through the gastrointestinal tract reflects the overall gastrointestinal motility, and measurement of the GIT ratio is very useful for diagnostic screening of constipation (Wintola et al. 2010), with a decrease in the GIT ratio indicating constipation (Choi et al. 2014). In contrast, increases in the GIT ratio following feeding on the experimental diet (EX-2 group) provided clear evidence of beneficial laxative effects. Loperamide treatment reduced the GIT ratio, and this constipation symptom was ameliorated by pre-feeding mice with an experimental diet containing SD-P2A2. This result demonstrated that SD-P2A2 exerts a beneficial effect in mice in the context of loperamide-induced constipation.

Changes of harmful intestinal enzyme activation in constipated mice

Various anaerobic intestinal microflora, which are dominated by obligate anaerobes, can modify a wide range of environmental chemicals, either directly or indirectly through the enterohepatic circulation (Kinouchi et al. 1987). The harmful enzyme activities of intestinal microflora can promote the enterohepatic circulation of toxic and carcinogenic substances (Corinne et al. 1998). In particular, β-glucosidase, tryptophanase, and nitroreductase have been associated with carcinogenesis in intestinal tract (Chung et al. 1975; Chadwick et al. 1992; Shen et al. 2009).

Harmful enzyme activities in the cecum of loperamide hydrochloride-induced constipated mice are shown in Fig. 4. The activities of harmful enzymes such as β-

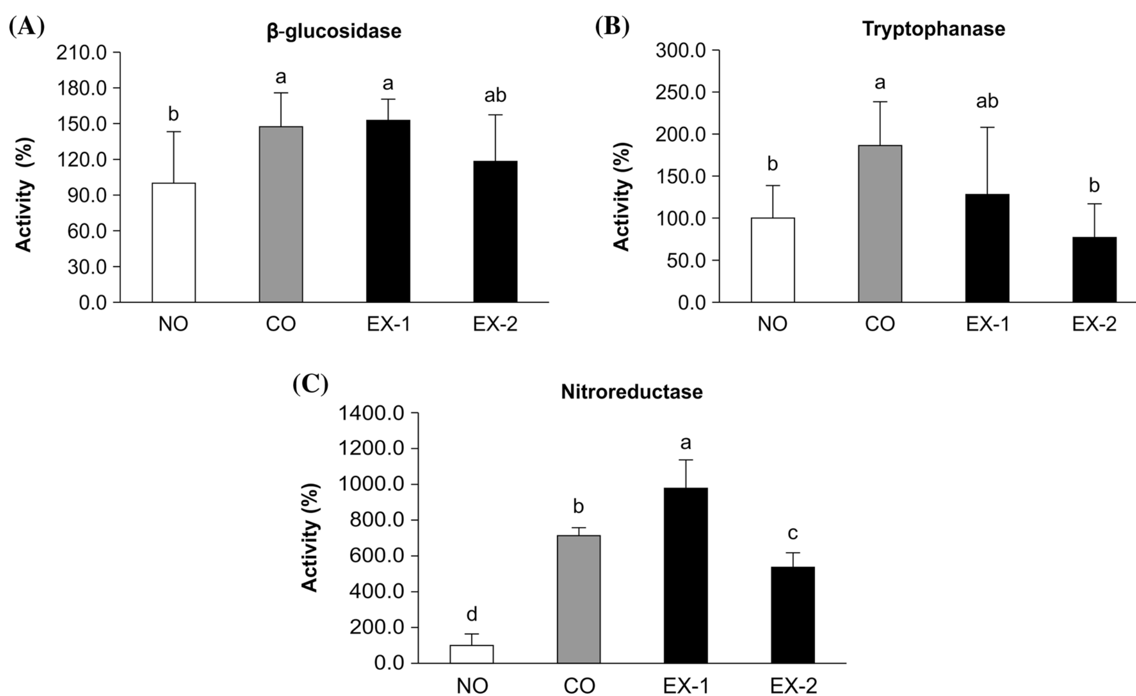

Fig. 4 Effect of experimental diets containing β-glucosidase (**A**), tryptophanase (**B**), or nitroreductase (**C**) on the activities of harmful enzymes in the ceca of constipated mice. The data are expressed as the mean ± SD. *Superscripted lowercase letters* indicate significant differences ($p < 0.05$)

glucosidase, tryptophanase, and nitroreductase were significantly elevated in the CO group following loperamide treatment, compared to that observed in the NO group ($p < 0.05$). β-glucosidase, tryptophanase, and nitroreductase activities in the EX-2 group were decreased by 19.7, 58.6, and 24.6 %, respectively, compared to those observed in the CO group. However, ingestion of the experimental diet containing NonF (EX-1 group) failed to significantly inhibit the upregulation of harmful enzyme activities.

Previous studies have demonstrated that a decrease in the activity of harmful enzymes improves the gastrointestinal environment and relieves constipation (Sengottuvelan and Nalini 2006; Lee et al. 2009; An et al. 2010). In poultry studies, controlled trials have shown that oral administration of *B. subtilis* reduces infection by *Salmonella enterica* serotype *Enteritidis*, *Clostridium perfringens*, and *Escherichia coli 078:K80* (La Ragione et al. 2001; La Ragione and Woodward 2003). In addition, the addition of *B. subtilis* in feeds improved the performance of animals, enhanced their intestinal probiotic microorganism populations, and suppressed intestinal *E. coli* numbers, without affecting the colonization of pathogenic cecal *Salmonella* and *Campylobacter* in animals (Jin et al. 1996).

The results of the present study show that the experimental diet containing SD-P2A2 inhibited the activity of β-glucosidase, tryptophanase, and nitroreductase, identified as harmful enzymes of the intestinal microflora. These results confirmed that SD-P2A2 has beneficial probiotic effects in mice with loperamide-induced constipation.

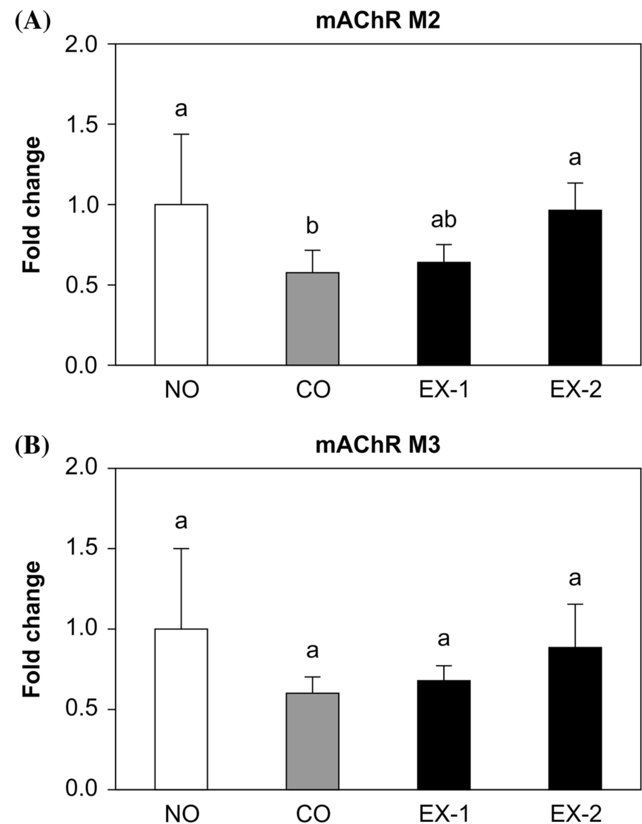

Fig. 5 Effect of experimental diet on expression of intestinal muscle contraction-related genes in constipated mice. The data are expressed as the mean ± SD. *Superscripted lowercase letters* indicate significant differences ($p < 0.05$)

Variation of the expression levels of mAChRs in constipated mice

Acetylcholine, the most common neurotransmitter, is released from primary excitatory motor neurons and mediates smooth muscle contraction (Goyal and Hirano 1996; Furness 2000). Cholinergic signaling is regulated by the mAChRs expressed on the surfaces of neuron and other cells, including heart and smooth muscle cells (Eglen 2001; Ishii and Kurachi 2006). mAChRs are classified into five subtypes (M1–M5) according to their tissue-distribution and signal-transduction mechanisms (Caulfield and Birdsall 1998). In particular, mAChR M2 and M3 play a key role in the contraction of gastrointestinal smooth muscle (Ehlert et al. 1999). Therefore, we selected mAChR M2 and M3 as target genes to investigate the mechanism behind the probiotic effects of experimental diets.

Our results indicated that mAChR M2 mRNA expression levels were significantly decreased in loperamide-treated mice (42.4 ± 14.0 % of that observed in NO group mice; $p < 0.05$), whereas ingestion of experimental diet containing SD-P2A2 significantly inhibited the decrease of mAChR M2 expression (67.6 ± 29.3 % that observed in the NO group; $p < 0.05$; Fig. 5A). Similarly, mAChR M3 mRNA expression was decreased in loperamide-treated mice, whereas ingestion of experimental diet containing SD-P2A2 inhibited mAChR M3 downregulation. However, no significant differences were found between these results (Fig. 5B). The ingestion of experimental diet containing NonF (EX-1 group) failed to significantly inhibit the decrease in mAChR M2 and mAChR M3 mRNA expression levels. The mAChR M2 mRNA expression level was significantly decreased by loperamide treatment, whereas ingestion of experimental diet containing SD-P2A2 significantly inhibited the decrease of mAChR M2. These results suggested that the probiotic effects induced by SD-P2A2 are corrected by mAChR M2 downregulation in the small intestine of mice with loperamide-induced constipation. In this study, microencapsulation was performed with *B. subtilis* CBD2-fermented grain (rice and amaranth) and forming agents containing pectin and alginic acid. SD samples microencapsulated with SD-P2A2 exhibited a significantly higher rate of adhesion to HT-29 cells than did FD and other SD samples. Furthermore, our in vivo data

indicated that the experimental diet containing SD-P2A2 positively affected intestinal function by suppressing the activity of harmful enzymes and by positively regulating the expression of genes related to intestinal muscle contraction, resulting in the amelioration of constipation. The probiotic effects of the experimental diet containing SD-P2A2 were compared with those observed in mice fed a control diet of non-fermented grain. Constipation symptoms were significantly ameliorated in mice fed the experimental diet with SD-P2A2. In our study, EX-2 mice were fed a powdered diet containing a 3:1 mixture of normal commercial diet and *B. subtilis* CBD2 strain-fermented SD-P2A2 (4.53 ± 0.06 log CFU/[g day]), respectively, for 7 days. Commonly, the food intake of laboratory mice is approximately 15 g per 100 g of body weight per day. Therefore, we suggest that the optimum dosage of SD-P2A2 to prevent constipation in mice is approximately 5 g per 100 g of body weight per day. The results of this study suggest that *B. subtilis* CBD2-fermented grain microencapsulated with SD-P2A2 can be used as a functional food for improving gastrointestinal health and shows potential as a preventive strategy for constipation.

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