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Full Paper

Activation of chloride channels and promotion of bowel movements by heat-killed *Bifidobacterium longum* CLA8013

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Constipation is strongly associated with the deterioration of quality of life (QOL), and patients with constipation desire clear spontaneous defecation without the feeling of incomplete evacuation, rather than improved defecation frequency. The use of common osmotic or stimulant laxatives has not been shown to lead to a satisfactory improvement of bowel movements. In addition, softening of stools by increasing their water content has been reported to increase the frequency of spontaneous defecation and improve hard stools, straining during defecation, and abdominal symptoms, such as abdominal bloating, thereby leading to improvement of QOL deterioration caused by constipation. Thus, the present study screened bacterial strains *in vitro* using intestinal epithelial T84 cells, aiming to identify one that activates chloride channels involved in water secretion into the intestinal tract. As a result, the conditioned medium of *Bifidobacterium longum* CLA8013 was found to induce ion transport. Also, this effect was suppressed by cystic fibrosis transmembrane conductance regulator (CFTR) (inh)-172, a CFTR chloride channel inhibitor. Furthermore, both live and heat-killed CLA8013 similarly induced ion transport, suggesting that bacterial cell components are responsible for the effect. In addition, the administration of heat-killed CLA8013 to loperamide-induced constipation rats resulted in an increase in fecal water content and promoted defecation. These results suggest that the active components in CLA8013 act on CFTR chloride channels in the intestinal tract, promote water secretion into the intestinal tract, and soften stools, thereby promoting bowel movements.

Key words: constipation, chloride channels, *Bifidobacterium longum* CLA8013, CFTR (inh)-172

INTRODUCTION

Stool is a parameter of health, and constipation is strongly associated with the deterioration of quality of life (QOL). In recent years, there has been an increase in the number of patients with constipation due to abnormal function of the large intestine caused by decreased food intake, lack of exercise, stress, or aging. Constipation symptoms are often managed by the use of foods and over-the-counter (OTC) medications, as well as improvement of lifestyle habits. Moreover, osmotic and stimulant laxatives are mainly used to treat constipation, but they have the problems of adverse effects, habituation, and tolerance. In addition, patients with constipation tend to place more importance on clear spontaneous defecation without the feeling of incomplete evacuation (complete spontaneous bowel movement; CSBM) rather than on improved defecation frequency [1].

For anti-constipation drugs, novel ingredients that promote bowel movements through the softening of stools by increasing their water content are being developed. One such ingredient is lubiprostone, which activates chloride channel 2 (CLC-2) in the small intestine epithelium to move chloride ions into the intestinal tract, promotes water secretion, and enhances fecal transport capacity in the intestinal tract, thereby promoting bowel movements [2]. Thus, the administration of lubiprostone was shown to increase the frequency of spontaneous defecation and exhibit effectiveness against abdominal symptoms, such as hard stools, straining during defecation, and abdominal bloating [3], and it is expected to lead to the improvement of QOL deterioration caused by constipation. However, lubiprostone is a prescription drug and cannot be easily obtained and taken like food or OTC medications, and its use is contraindicated for pregnant women, as it was found to transfer to the fetus in animal studies.

Found in ethical drugs, OTC medications, and foods, probiotics are highly safe, have no adverse effects, and are widely used among all generations. Bifidobacteria and lactic acid bacteria are common probiotics that have been shown to exhibit various physiological activities, including constipation improvement [4], in addition to diarrhea improvement [5], infection prevention [6], immunostimulation [7], and anti-allergic effects [8]. With regard to their mechanisms of action, many authors have reported that

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bifidobacteria and lactic acid bacteria improve the microbiota [9]. Also, they have been shown to act on ion transporters in the intestinal tract, and it has been reported that they may be effective against diarrhea and constipation due to facilitated water movement in association with ion movement [10]. In fact, it has been reported that bifidobacteria and lactic acid bacteria act on ion transport in intestinal epithelial cells and that they suppress enhanced intestinal transport in mice [11]. However, there have been no reports to date that bifidobacteria and lactic acid bacteria promote the secretion of chloride ions, enhance intestinal transport, or improve constipation.

In addition to probiotic-containing foods, there are many foods containing paraprobiotics, which are represented by heat-killed probiotic bacteria, and paraprobiotics are also used as functionality-related ingredients in foods with functional claims. Probiotics also have problems, such as restrictions on storage temperature and dosage form to maintain viable bacterial counts, contamination of production lines, and changes in flavor. In contrast, paraprobiotics do not have these problems, enabling the expansion of their commercialization range.

With this as the background, the present study aimed to develop a probiotic- or paraprobiotic-containing product that has a higher degree of safety and no adverse effects and improves not only defecation frequency but also QOL deterioration in constipation patients. Thus, we conducted an *in vitro* test to search for a bacterial strain that acts on ion transporters in the intestinal tract, promotes water secretion into the intestinal tract, and softens stools, thereby improving defecation frequency and abdominal symptoms, leading to the improvement of QOL deterioration caused by constipation. Furthermore, we investigated the *in vivo* effects of the identified bacterial strain in the loperamide-induced constipation rat model.

MATERIALS AND METHODS

Bacterial strains used for screening and preparation of conditioned medium

This study screened 47 strains of *Bifidobacterium* and 24 strains of *Lactobacillus* (Fig. 1). Each cryopreserved bacterial strain was anaerobically cultured in 10 mL of GAM medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 1% glucose and 0.1% polysorbate 80 at 37°C for 18–30 hr. After culture, the bacteria were centrifuged (3,000 × g, 10 min, R.T.), and the supernatant was discarded. After being washed with isotonic phosphate buffer solution (PBS), the bacteria were resuspended in 10 mL of DMEM/F-12 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and allowed to stand at 37°C for 24 hr under anaerobic conditions. After centrifugation, the supernatant was filtered (0.22 μm), and the filtrate was used as a conditioned medium (CM) in the short-circuit current test.

Preparation of cultured and heat-killed Bifidobacterium longum *CLA8013*

Cryopreserved CLA8013 strain was anaerobically cultured in 100 mL of GAM medium (Nissui Pharmaceutical Co. Ltd.) containing 1% glucose and 0.1% polysorbate 80 at 37°C for 30 hr. After culture, the bacteria were centrifuged (3,000 × g, 10 min, R.T.), and the supernatant was discarded. After being washed with PBS, the bacteria were resuspended in 10 mL of DMEM/F-12, which was then used as cultured CLA8013 in the short-circuit

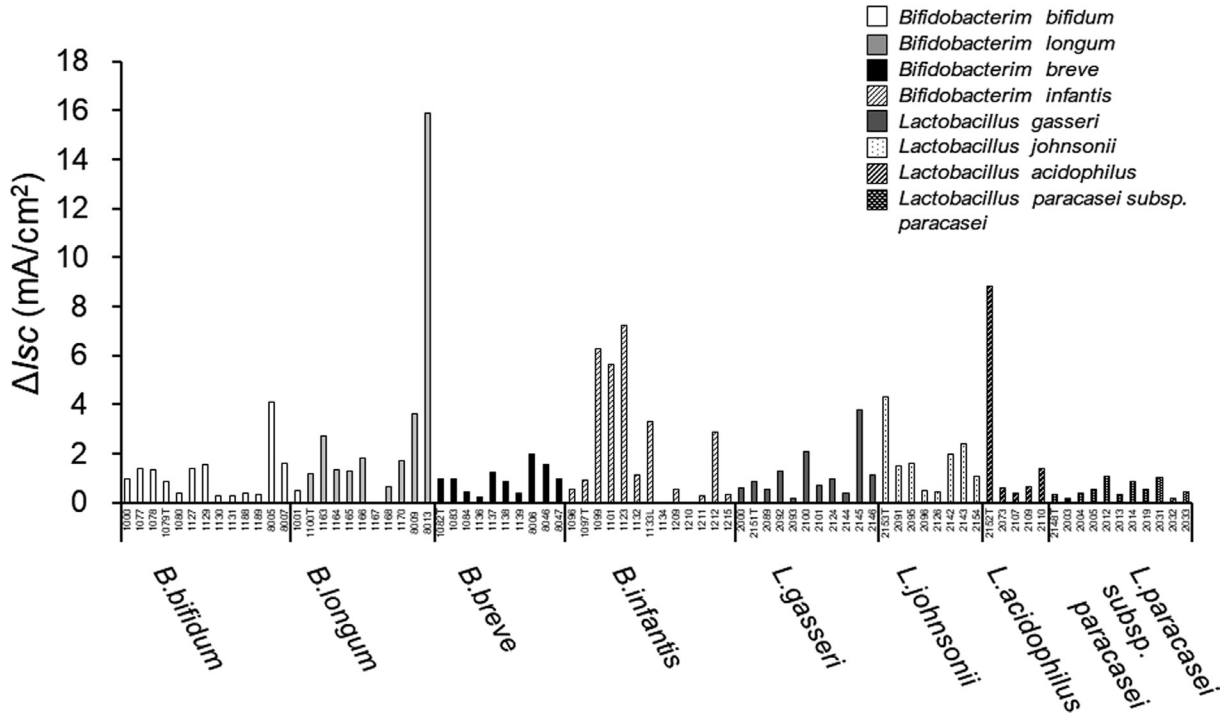

Fig. 1. Screening of bacterial strains based on short-circuit current changes (*ΔIsc)* in T84 cells using an Ussing chamber system. A Snapwell filter on which T84 cells had formed a monolayer was set vertically in the Ussing chamber. The two compartments of the chamber separated by the T84 cell monolayer were filled with 5 mL of 37°C Krebs–Ringer solution and allowed to stand for at least 20 min. The largest changes in short-circuit current (*ΔIsc*) were recorded.

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current test.

Cultured CLA8013 was heat treated at 100°C for 30 min, lyophilized, and then used as heat-killed CLA8013. For the short-circuit current test, 0.4 g of heat-killed CLA8013 was suspended in 5 mL of PBS. For the loperamide-induced constipation model study, 60, 120, and 240 mg of heat-killed CLA8013 was suspended in 10 mL of PBS (6, 12, and 24 mg/mL).

Separation of sonicated heat-killed CLA8013 using a dialysis membrane

Ten grams of heat-killed CLA8013 suspended in 150 mL of water was sonicated and centrifuged (10,000 × g, 10 min, R.T.), after which the centrifugation supernatant was repeatedly freeze-dried. One gram of the obtained lyophilized matter was dissolved in 200 mL of water and sealed in a 100-500D dialysis membrane. The dialysis membrane was placed in water (5 L) and stirred for 24 hr. After 24 hr, the water was exchanged, and the membrane was stirred for 48 hr. The 48 hr of stirring was carried out twice. The exchanged water (15 L) was concentrated using a rotary evaporator (N-3100, EYELA) at 40°C, 100 rpm, and 20–50 hPa, resulting in 225.6 mg of lyophilized matter. The solution in the dialysis membrane was dried, resulting in 687.1 mg of lyophilized matter. Finally, 41.22 mg of in-membrane lyophilized matter and 13.536 mg of out-of-membrane lyophilized matter were each suspended in 1 mL of water and used in the short-circuit current test.

Cell culture

A human colonic epithelial cell line, T84, was seeded at 4×10^4 cells/cm^2 in DMEM/F-12 containing 10% FBS and penicillin/streptomycin and passaged every 5–7 days. For the short-circuit current test, the cells were seeded at 5×10^5 cells/well on a Snapwell filter (Costar, 1.13 cm^2, Corning). The cells were cultured for 5–7 days with the medium exchanged every 3 days, and the cultured cells were then used in the short-circuit current test.

Short-circuit current test

In the short-circuit current test, measurements were conducted using an Ussing chamber system (VCC MC6, Physiologic Instruments, San Diego, CA, USA). A Snapwell filter on which T84 cells had formed a monolayer was set vertically in the Ussing chamber. The two compartments of the chamber separated by the T84 cell monolayer were filled with 5 mL of 37°C Krebs–Ringer solution (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose) and allowed to stand for at least 20 min. The test started after the short-circuit current (*Isc*) across the T84 cell monolayer had stabilized. A total of 100 µL of CLA8013 CM, 100 µL of a bacterial sample (cultured or heat-killed CLA8013), or 300 µL of a sample separated by dialysis membrane (in-membrane or out-of-membrane fraction) was added to the mucosal side of the T84 cells. Subsequent changes in *Isc* (*ΔIsc*) were monitored, and changes in transcellular ion secretion were compared. *ΔIsc* was defined as the difference between the value of *Isc* with the largest change after sample addition and the value of *Isc* at the time of sample addition. In addition, 10 mM of the chloride channel inhibitor CFTR (inh)-172 (C2992, Sigma; dissolved in DMSO) was added to the mucosal side of the T84 cells at 5 µL/5 mL Krebs–Ringer solution (final concentration: 10 µM) [12], and after 2 min, 100 µL of CLA8013 CM was added to the mucosal side of the T84 cells.

Animals

Six-week-old SD male rats (Japan SLC, Inc., Shizuoka, Japan) were used in the loperamide-induced constipation model study. The rats were subjected to the study after a one-week acclimation period. They were individually housed in five consecutive cages under a room temperature of 22 ± 3°C, a humidity of 55 ± 5%, and 12-hr lighting (7:00–19:00) in an SPF environment. The CE-2 diet (CLEA Japan, Inc., Tokyo, Japan) was given *ad libitum* as the standard diet, and tap water was freely available from a water supply bottle. The study was conducted according to the Guide for the Care and Use of Laboratory Animals after being reviewed and approved by the Animal Care and Use Committee of Biofermin Pharmaceutical Co., Ltd. (approval number: 134-010, 134-011).

Bowel movement-promoting effect of CLA8013 on loperamide-induced constipation rats (dose response)

SD rats were divided into the following five groups: 3, 6, and 12 mg/rat heat-killed CLA8013 administration groups, a normal group, and a control group. The 3, 6, and 12 mg/rat heat-killed CLA8013 administration groups were administered a single dose of heat-killed bacterial suspension at different concentrations (6, 12, or 24 mg/mL) by gavage at 0.5 mL/rat. The normal and control groups were administered the same volume of PBS. Then, the control group and heat-killed CLA8013 administration groups were forcibly administered 5 mg/5 mL/kg of saline-dissolved loperamide hydrochloride subcutaneously, while the normal group was forcibly administered the same volume of PBS subcutaneously. As a fecal marker, carmine red dissolved in 0.5% carboxymethylcellulose (60 mg/mL/rat) was administered by gavage. Subsequently, the excretion of feces was examined every 30 min, and the time elapsed until the excretion of feces stained red with carmine red (total intestinal transport time) was measured.

Measurement of fecal counts and fecal water content in loperamide-induced constipation rats

The 12 mg/rat heat-killed CLA8013 administration group was administered heat-killed bacterial suspension (24 mg/mL) by gavage at a dose of 0.5 mL/rat once a day for 7 days. The normal and control groups were administered the same volume of PBS by gavage once a day for 7 days. After the 7-day gavage administration, loperamide hydrochloride solution was administered subcutaneously twice a day for 3 consecutive days. CLA8013 was continuously administered by gavage for three days from the start of administration of the loperamide hydrochloride solution to the end of the test (days 0–3). Fecal counts and fecal water content were measured once a day from the start of the administration of loperamide hydrochloride solution to the end of the test (days 0–3). For fecal water contents, fresh feces were collected by gently compressing the abdomens, immediately weighed, and dried at 90°C for 24 hr. Fecal water content was determined as the difference in fecal weight before and after drying.

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Statistical analysis

Experimental results are expressed as the mean ± standard error. Homogeneity of variance was assessed using Bartlett's test, and significance of differences was tested using the Steel test for unequal variances and Dunnett's test for equal variances.

RESULTS

Bacterial strain screening by the short-circuit current test

In the screening of the CMs of the 47 strains of *Bifidobacterium* and 24 strains of *Lactobacillus* by the short-circuit current test, the CM of CLA8013 showed the highest *ΔIsc* (15.9 mA/cm^2). The next highest value was that of *Lactobacillus acidophilus* 21533^T, followed by *Bifidobacterium infantis* 1123. The average short-circuit current value of all the confirmed bacteria was 1.555 mA/cm^2, but the result for *B. longum* CLA8013 was substantially higher (Fig. 1).

Effect of chloride channel inhibitor on short-circuit current changes induced by CLA8013

The *ΔIsc* induced by the addition of CLA8013 CM was 26.61 mA/cm^2, while it was 12.13 mA/cm^2 when cells were pretreated with the chloride channel inhibitor CFTR (inh)-172. Additionally, the value for the treatment with CFTR (inh)-172 when the CLA8013 CM was set as 100% was 41.2% ± 2.64% (n=4), indicating significant inhibition (58.8%) by the CFTR (inh)-172 treatment (Fig. 2).

Comparison of short-circuit current changes induced by the CLA8013 CM and bacterial suspensions (cultured and heat-killed CLA8013)

We compared the reactivity in short-circuit current upon addition of the CLA8013 CM and bacterial suspensions (non-treated (8.8 × 10^6 CFU/mL) and heat-killed CLA8013). The reaction peaked approximately 4 min after the addition of the CLA8013 CM and approximately 7 min after the addition of heat-killed CLA8013. However, the reaction occurred slowly after the addition of cultured CLA8013, and it peaked approximately 27 min after addition (Fig. 3).

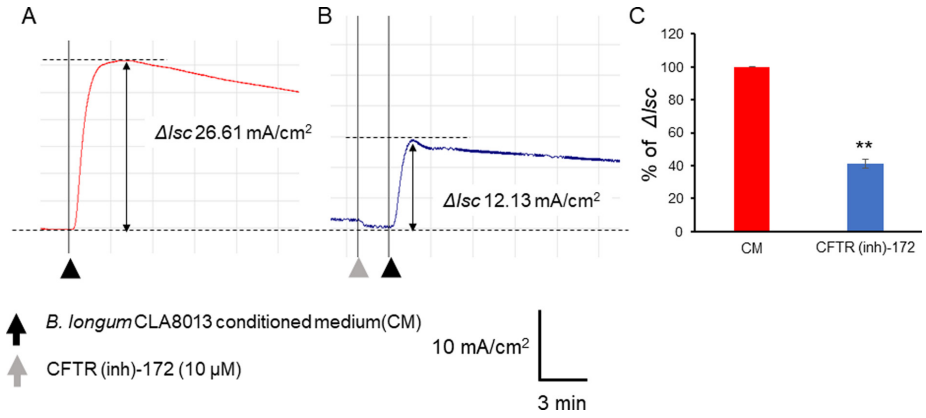

Fig. 2. Effect of the CFTR chloride channel inhibitor CFTR (inh)-172 on *ΔIsc* in T84 cells induced by the CLA8013 CM in an Ussing chamber system. A: Representative chart for CLA8013 CM only, B: Representative chart for 2-min pretreatment of CLA8013 CM with CFTR (inh)-172, C: percent of *ΔIsc*. Values are means ± standard error. **p<0.01 (n=4).

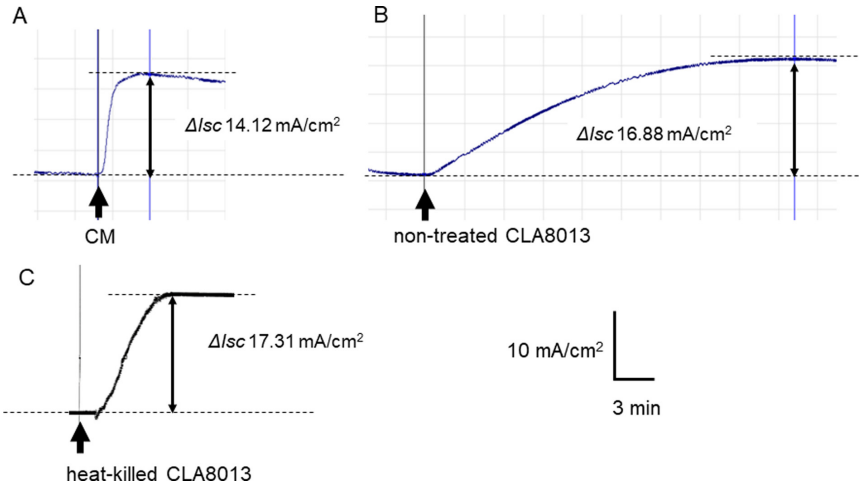

Fig. 3. *ΔIsc* in T84 cells induced by CM or non-treated/heat-killed CLA8013 in an Ussing chamber system. A: CM, B: non-treated CLA8013, C: heat-killed CLA8013.

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Bowel movement-promoting effect of heat-killed CLA8013 on loperamide-induced constipation rats

Compared with the normal group (8.30 ± 0.23, n=10), the control group showed a significant prolongation of total intestinal transport time (13.35 ± 0.33, n=10). The administration of 3, 6, and 12 mg/rat/day of heat-killed CLA8013 shortened the total intestinal transport time in a dose-dependent manner, and a significant reduction in total intestinal transport time (11.00 ± 0.33, n=10) was observed at 12 mg/rat/day (Fig. 4).

Effect of heat-killed CLA8013 on fecal counts and fecal water content in loperamide-induced constipation rats

Compared with the normal group (days 1, 2, and 3, 64 ± 3.38, 66 ± 2.00, and 65 ± 3.14, respectively; n=8), the control group had significantly decreased fecal counts at days 1, 2, and 3 (25 ± 2.81, 30 ± 3.26, 39 ± 2.88, respectively; n=8). The administration of 12 mg of heat-killed CLA8013 for 7 days before loperamide administration resulted in a significant increase in fecal counts on days 1, 2, and 3 (40 ± 1.20, 42 ± 2.32, and 53 ± 2.63, respectively; n=8) after the administration of loperamide hydrochloride solution. Also, the control group (days 1, 2, and 3, 54.13 ± 0.80, 54.75 ± 0.97, and 54.37 ± 1.03, respectively) showed a significant decrease in fecal water content (normal group: days 1, 2, and 3, 65.27 ± 1.53, 61.52 ± 0.78, and 63.41 ± 1.23, respectively), and the administration of 12 mg/rat/day of heat-killed CLA8013 for 7 days before loperamide administration resulted in a significant increase in fecal water content on days 1 (58.52 ± 0.62) and 3 (57.29 ± 0.84) after the administration of loperamide hydrochloride solution (Fig. 5).

Short-circuit current changes upon the addition of sonicated heat-killed CLA8013 separated by a dialysis membrane

Sonicated heat-killed CLA8013 was separated into in-membrane and out-of-membrane fractions by a dialysis membrane, and the activities of the fractions were examined by the short-circuit current test. The values for *ΔIsc* induced by the in-membrane and out-of-membrane fractions were 21.43 and 1.1 mA/cm^2, respectively.

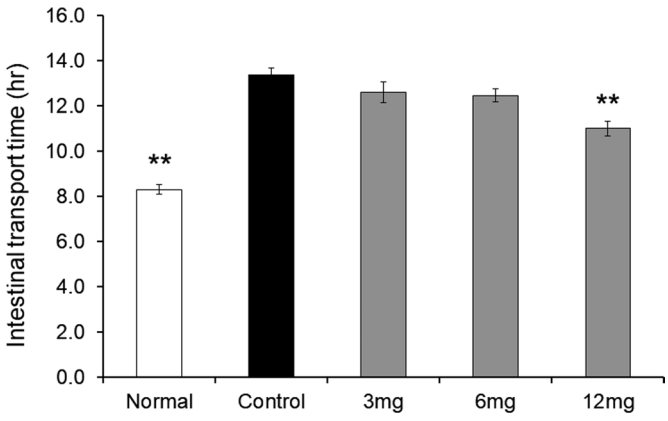

Fig. 4. Effect of heat-killed CLA8013 on intestinal transport time in loperamide-induced constipation rats. Intestinal transport time was evaluated as the time during which stool stained with the carmine dye was excreted. Values are means ± standard error of 10 animals. **p<0.01 vs. control.

DISCUSSION

The present study identified a novel bacterial strain, CLA8013, which strongly activates chloride channels in the intestinal tract. Furthermore, this bacterial strain was shown to increase fecal water content and promote bowel movements in loperamide-induced constipation rats.

First, we screened bacterial strains *in vitro* based on the measurement of short-circuit current changes in intestinal epithelial cells, aiming to identify one with high ion-transport capacity. Among the strains screened, the CM of CLA8013 induced the largest increase in *Isc* (Fig. 1), suggesting that CLA8013 acts on ion transporters in the intestinal epithelium and has a particularly high ion-moving capacity. In the screening tests, other strains were shown to have short-circuit changes, but not to the extent of CLA8013. Thus, one or both of the following may apply to CLA8013: (1) other strains have similar active compounds, but CLA8013 can secrete a high amount; or (2) other strains have active compounds, but CLA8013 has a high content.

In the intestinal tract, there exist transporters with various roles, such as Na^+/H^+ exchange transporters and epithelial sodium channels (ENaC), which regulate pH and osmotic

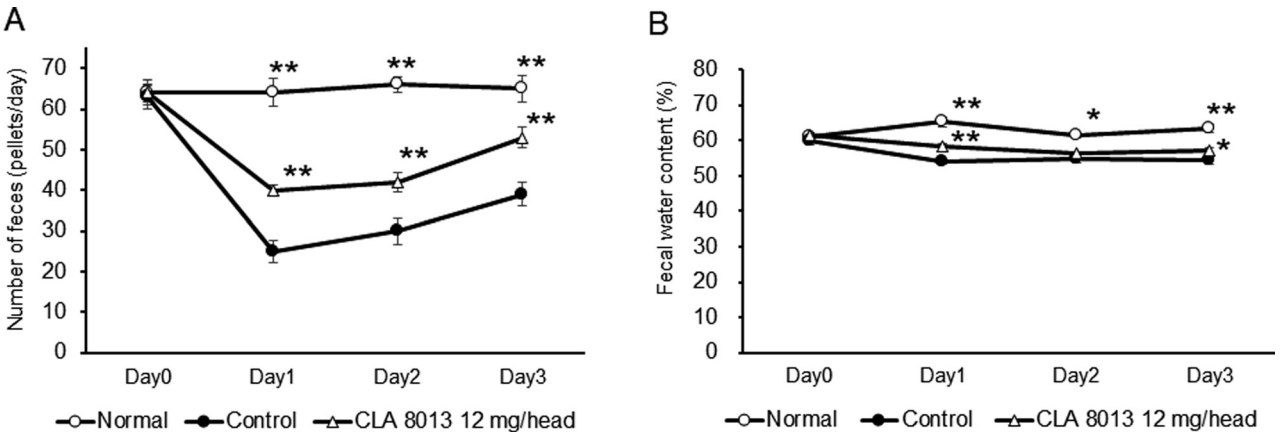

Fig. 5. Effect of heat-killed CLA8013 on fecal counts and fecal water content in loperamide-induced constipation rats. (A) Numbers of feces and (B) fecal water contents were measured every experimental day. Values are means ± standard error of 8 animals. *p<0.05 vs. control, **p<0.01 vs. control.

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pressure. Activation of chloride channels is involved in water secretion into the intestinal tract [13], and it is thought to lead to the improvement of constipation. The types of chloride channels present in intestinal epithelial cells include CFTR and CLC-2. Thus, to examine whether the CLA8013 CM was involved in water secretion into the intestinal tract, we pretreated cells with CFTR (inh)-172, a CFTR chloride channel inhibitor, and this resulted in the suppression of the short-circuit current changes induced by the CLA8013 CM by 58.8% (Fig. 2). This indicates that the CLA8013 CM activates CFTR chloride channels and promotes the movement of chloride ions toward the intestinal lumen. However, the short-circuit current changes induced by the CLA8013 CM were not completely suppressed by CFTR (inh)-172, suggesting that the CLA8013 CM also acts on other ion transporters. Since probiotics have been reported to act on ion transporters, including Na-K-2Cl co-transporters and down-regulated in adenoma (DRA) [10], the CLA8013 CM may have acted on these transporters, inducing changes in short-circuit current.

There have been no reports to date that bifidobacteria and lactic acid bacteria act on chloride channels in the intestinal epithelium or promote the secretion of chloride ions, and the newly identified CLA8013 is the first probiotic strain found to have such capacity.

The CLA8013 CM examined in this study likely contains components that activate CFTR chloride channels, and these components could be bacterial metabolites or cell components. In recent years, the effectiveness of bacterial metabolites [14] and heat-killed bacteria [15] that act directly without the intervention of the microbiota has been demonstrated, and many foods containing them are commercially available. In the present study, the CLA8013 CM, live CLA8013, and heat-killed CLA8013 all induced short-circuit current changes in T84 cells. Comparing the reactivity, live CLA8013 induced a slower reaction over time than the CM or heat-killed CLA8013 (Fig. 3). This suggests that the active components in live CLA8013 were gradually exposed or released to the Krebs–Ringer solution in the Ussing chamber system and acted on T84 cells. On the other hand, the reaction in T84 cells was induced immediately after the addition of heat-killed CLA8013, as with the CM, suggesting that the active components are found even in heat-killed bacteria and are in a state of being readily exposed or released. Therefore, a chloride channel activator was thought to be secreted from CLA8013.

While all of the CMs and the live/heat-killed CLA8013 induced short-circuit current changes in T84 cells, heat-killed CLA8013 is considered a material that can be easily commercialized, as it is highly versatile and handled with ease. Therefore, we examined the bowel movement-promoting effect of heat-killed CLA8013 in an animal model. The loperamide-induced constipation model is often used to examine the effectiveness of a test substance against slow transit constipation. The bowel movement-promoting effect of bifidobacteria administration has been demonstrated using the loperamide-induced constipation model, and there have been reports on the improvement of dysbiosis and the involvement of organic acids and neurotransmitters [16]. However, no studies have examined the bowel movement-promoting effect of water secretion associated with the activation of chloride channels. In the present study, the administration of heat-killed CLA8013 to loperamide-induced constipation rats resulted in a reduction in the delayed intestinal transport time as well as an increase in fecal counts and fecal water content (Figs. 4 and 5).

The results of our *in vitro* and *in vivo* examinations suggest that the active components in CLA8013 act on CFTR chloride channels in the intestinal tract, promote water secretion into the intestinal tract, and soften stools, thereby promoting bowel movements.

We are currently working to identify the active components in CLA8013. In this study, when heat-killed CLA8013 was separated by molecular weight using a dialysis membrane, the fraction inside the dialysis membrane induced short-circuit current changes in T84 cells, indicating that the active components have a molecular weight of greater than 100-500D. Many substances are involved in ion transport in the intestinal epithelium, including forskolin, *Escherichia coli* toxins, intestinal peptides, and carbachol. With reference to information on the structures and properties of these substances, the identification of active components in CLA8013 through repeated fractionation and analysis is expected to lead to the development of novel ingredients in the future.

Additionally, in a clinical study of heat-killed CLA8013, the administration of 25 billion cells of CLA8013 (heat killed) to constipation-prone healthy individuals significantly increased defecation frequency and stool volume and significantly improved stool consistency, straining during defecation, and pain during defecation, which affect QOL [17].

Despite being a probiotic, the risk of bacterial translocation in compromised hosts cannot be eliminated if the probiotic is live bacteria. Meanwhile, if there is no viable bacterial activity as in the case of CLA8013, then it is thought that there is at least no risk from viable bacterial activity. Furthermore, Takami *et al.* have clinically proven the safety of CLA8013 [18]. Therefore, heat-killed *B. longum* CLA8013 can be used with a high degree of safety and is valuable as a new food or pharmaceutical material that improves not only bowel movements but also QOL deterioration caused by constipation.

CONFLICT OF INTEREST

None.

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