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Impact of the probiotic *Bacillus coagulans* on loperamide-induced delayed bowel movement in Sprague–Dawley rats†

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This study investigated the effects of *Bacillus coagulans* on alleviating loperamide-induced constipation. To evaluate the efficacy of *B. coagulans* in Sprague–Dawley (SD) rats, fecal parameters, the intestinal transit rate, and changes in intestinal mucosal cells were measured through histological analysis. Additionally, serotonin levels, water absorption, tight junction-related gene expression, and the cecal short-chain fatty acid (SCFA) content were analyzed. The administration of *B. coagulans* significantly altered the fecal weight and moisture content and improved gastrointestinal transit in rats with loperamide-induced constipation. Furthermore, *B. coagulans* supplementation restored the thickness of both muscular and mucosal layers that had been reduced by loperamide and significantly increased the area of intestinal cells, including Cajal and crypt cells. *B. coagulans* administration upregulated the expression levels of tryptophan hydroxylase and aquaporin genes, which were downregulated by loperamide. As the dose of *B. coagulans* increased, there was a corresponding upregulation in the expression of tight junction-related genes, including occludin (*OCLN*), zonula occludens 1 (*ZO-1*), and claudin 1 (*CLDN1*). Additionally, the levels of c-kit, AQP 3, and OCLN proteins, which were elevated by loperamide treatment, were reduced with higher concentrations of *B. coagulans*. Loperamide decreased the acetic acid content; however, high doses of *B. coagulans* increased it, leading to a significant increase in the total cecal SCFA content. Thus, *B. coagulans* shows potential as a probiotic for improving constipation.

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1. Introduction

Constipation is a prevalent gastrointestinal condition characterized by infrequent or difficult bowel movements, often accompanied by symptoms such as bloating, abdominal pain, and a sensation of incomplete evacuation. It can arise from a variety of factors including a low fiber diet and inadequate fluid intake, both of which affect stool consistency. Other contributing factors include physical inactivity, certain medications, and various health conditions. The management of chronic constipation typically requires dietary adjustments, regular exercise, and the use of fiber supplements, osmotic agents, stool softeners, and over-the-counter laxatives.[1,2] Notably, prescription medications can cause various side effects including diarrhea, bloating, gas, nausea, stomach pain, and dependence. Therefore, to improve the quality of life, the effective management of constipation requires sustained lifestyle changes.[3]

Prebiotics, such as lactulose, inulin, and various oligosaccharides, alleviate constipation by promoting the growth and activity of beneficial gut bacteria.[4] Probiotics, particularly those from the *Lactobacillus* or *Bifidobacterium* genera, are known to increase the frequency of evacuation and stool quality by improving the gastrointestinal transit time.[5] Prebiotics, probiotics, and synbiotics alleviate constipation symptoms by increasing the population and diversity of beneficial gut bacteria, thereby promoting colonic muscle contraction and regulating intestinal motility through the production of short-chain fatty acids (SCFAs).[6] SCFAs trigger the release of peptide YY, glucagon-like peptide-1, tryptophan 5-hydroxylase, and mucin from enteroendocrine and epithelial cells, thereby affecting intestinal motility and strengthening the epithelial barrier.[7–9]

Numerous studies have investigated the environmental tolerance and probiotic properties of spore-producing *Bacillus*

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coagulans strains. *B. coagulans* produce enzymes that enhance intestinal excretion and digestion, suppress pathogenic bacterial growth, and interact with the host immune system by modulating metabolites and immune cell function.[10,11] Clinical trials have demonstrated that supplementation with *B. coagulans* improves patients' quality of life by alleviating abdominal pain, reducing bloating, and modifying stool frequency.[12,13] Additionally, *B. coagulans* has demonstrated potential in managing comorbid major depressive disorder exacerbated by persistent irritable bowel syndrome (IBS) symptoms, as supported by previous clinical study findings.[14]

Our study aimed to evaluate the effectiveness of *B. coagulans* in alleviating constipation and to elucidate its specific mechanisms of action using an animal model. To assess the effect of administering *B. coagulans* on alleviating constipation, the intestinal transit rate, fecal parameters, and intestinal peristalsis-related factors were analyzed in loperamide constipation-induced Sprague–Dawley (SD) rats.

The research investigates *Bacillus coagulans*, a probiotic known for its ability to improve gut health, focusing on its specific effects in a loperamide-induced constipation model. This highlights its potential as a novel treatment for constipation, distinct from traditional laxatives or other interventions. Constipation is a widespread issue, particularly in chronic cases. The study adds value by exploring a non-pharmacological intervention that could be safer and more sustainable than current treatments, such as laxatives, which often come with side effects or dependency issues.

This research introduces *B. coagulans* as a promising therapeutic for constipation, enhancing the literature with mechanistic details and suggesting future avenues for human trials.

2. Materials and methods

2.1. Materials

Loperamide was supplied by Sigma-Aldrich (St. Louis, MO, USA). Lactulose, an osmotic laxative, was sourced from JW Pharmaceutical Co., Ltd (Seoul, Korea). *B. coagulans GBI-30, 6086* (*BC30*) was procured from Neo Cremar Co., Ltd (Seoul, Korea).

The manufacturing process for *Bacillus coagulans GBI-30, 6086* includes fermentation, followed by a recovery phase, and then either spray-drying or freeze-drying. The recovery phase, which involves centrifugation, is designed to collect and concentrate *Bacillus coagulans* after the fermentation process.

2.2. Animals and experimental design

Six-week-old male SD rats (160–180 g) were supplied by Orient Bio (Seongnam, Republic of Korea). Following a 1-week adaptation period in a controlled environment (23 ± 1 °C, 55 ± 2% relative humidity, and 12-hour light/dark cycle), constipation was induced by administering 5 mg kg^{-1} of loperamide orally twice a day in all groups except for the normal control (NC) group. The rats were divided into five groups (eight rats per group): NC, control (CON, loperamide only, 5 mg kg^{-1}), positive control (PC, lactulose 2010 mg kg^{-1} with loperamide), low-dose *B. coagulans* (BCL, 1×10^7 colony-forming units [CFU] with loperamide), and high-dose *B. coagulans* (BCH, 2×10^9 CFU with loperamide). All treatments were administered daily at 10 AM for 4 weeks. Sample sizes were determined based on a power analysis to detect statistically significant differences, using 8 animals per group across 5 groups (n = 40 in total). The intestines were harvested immediately after euthanasia.

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2023-0028). All surgeries were performed under anesthesia, and every effort was made to minimize the suffering of the animals used in this study.

2.3. Analysis of fecal parameters and intestinal transit rate

The fecal parameters and intestinal transit rates were evaluated using the method previously described by Inatomi *et al.*[15] Fecal samples were collected weekly at 11 AM, starting two weeks after the administration of loperamide, lactulose, and/or *B. coagulans* as required. The samples were dried at 75 °C for 18 hours, and their water content was determined by measuring the weight difference.

Before the end of the experiment, the rats were fasted for 12 hours and orally administered a 5% charcoal suspension. Subsequently, the rats were sacrificed within 30 min, and the total intestinal length, including the distance traversed by charcoal, was measured to determine the intestinal transit rate.

2.4. Histological analysis

Hematoxylin–eosin (H&E) staining and immunohistochemistry (IHC) were performed as described in a study by Jang and Kim *et al.*[16] The H&E staining was conducted on sections of the colon from the cecum to the rectum, while the IHC staining was performed using the colon od SD rat. The stained intestinal mucosal cells and interstitial cells of Cajal (ICC) were examined under a light microscope (ZEISS Axiovert S100, Jena, Germany). Crypt and intestinal mucosa cells were examined using an optical microscope with Leica Application Suite software (Leica Microsystem, Heerbrugg, Switzerland).

2.5. Quantitative real-time reverse-transcription polymerase chain reaction

The total ribonucleic acid (RNA) was extracted from rat intestines using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Genomic deoxyribonucleic acid (DNA) was removed from the RNA samples by treatment them with Q1 RNase-free DNase (Promega, Madison, WI, USA). Subsequently, complementary DNA (cDNA) was synthesized using SuperScript® III (Invitrogen) reverse transcriptase and the 1μg of the resulting cDNA was analyzed through quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) using a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). The expression levels of each target gene were nor-

malized to the NC group using a previously described ΔΔCt method.[17]

2.6. Western blot analysis

Target protein levels were analyzed using the western blotting method described by Jang and Kim *et al.*[16] Intestinal tissue (100 mg) was mixed with lysis buffer to create a homogenate, which was then centrifuged to separate the supernatant (10 000*g*, 10 min, 4 °C). Protein concentrations in the supernatant were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The primary antibody (β-actin, C-kit, AQP3, and OCLN, 1 : 1000) and the secondary antibodies (anti-rabbit IgG, 1 : 1000) were obtained from Cell Signaling Technology (Beverly, MA, USA). Protein bands were detected using a FluorChem M Fluorescent Western Imaging System (ProteinSimple, Santa Clara, California, USA).

2.7. Short-chain fatty acid analysis

SCFAs in the cecum were analyzed following the method described by Kim *et al.*,[18] selected for its proven accuracy and efficacy in the quantification of SCFAs in biological samples. Cecal content (200 mg) was extracted using 2 mL of methanol and was subsequently filtered through a 0.45 μm Millipore filter (Millipore, USA). The resulting extracts were analyzed through gas chromatography (GC, Agilent Technologies, CA, USA) equipped with an HP-FFAP (nitroterephthalic acid modified polyethylene glycol high polarity designed) column (50 m × 0.32 mm × 0.50 μm) and a flame ionization detector. The sample injection volume was set at 1 μL, with inlet and detector temperatures maintained at 200 °C and 240 °C, respectively.

2.8. Statistical analysis

The experimental results are expressed as means ± standard errors of the mean (SEM). Statistical analyses were performed using the SPSS software version 24.0 (SPSS Inc., USA). Comparisons among experimental groups were performed using Tukey's multiple-range test after an analysis of variance with the significance level set at a p value of <0.05.

3. Results

3.1. Changes in fecal parameters caused by *B. coagulans* administration in loperamide-treated SD rats

Analysis of the changes in body weight, dietary intake, organ weight, and serum biochemical parameters owing to *B. coagulans* administration revealed no significant differences between the NC and CON groups (Fig. S1–S3†). However, the number, weight, and water content of fecal pellets were significantly reduced in the CON group compared with those in the NC group (Fig. 1A, B and C; $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively). Loperamide caused a notable reduction in the gastrointestinal transit ratio compared with the NC group (Fig. 1D, $p < 0.01$). The combined treatment of lactulose (PC) and a high dose of *B. coagulans* (BCH, 2.0×10^9 CFU per day) significantly increased the number of fecal pellets compared with the CON group (Fig. 1A, $p < 0.01$, and $p < 0.05$, respectively). Both the weight and water content of the fecal pellets were significantly higher in the BCL and BCH groups compared with the CON group (Fig. 1B and C, $p < 0.001$, respectively). Mice in the PC group did not experience significant improvements in the gastrointestinal transit ratio compared with those in the CON group, while mice in the BCL and BCH groups demonstrated significant enhancements (Fig. 1D, $p < 0.001$).

3.2. Changes in intestinal architecture caused by *B. coagulans* administration in loperamide-treated SD rats

The thickness of the intestinal muscular and mucosal layers significantly decreased in the CON group compared to the NC group (Fig. 2A–D; $p < 0.001$, $p < 0.05$). In contrast, the PC group, treated with lactulose, showed a marked increase in both layers relative to the CON group ($p < 0.001$, $p < 0.05$). Low and high doses of B. coagulans (BCL, BCH) significantly increased the muscular layer thickness ($p < 0.01$, $p < 0.001$, respectively), while the mucosal layer thickness increased significantly in the BCH group ($p < 0.05$). Loperamide-induced constipation also reduced crypt cell and ICC area, but both were restored in the PC and BCH groups ($p < 0.05$, $p < 0.001$) area (Fig. 3D, $p < 0.05$ and $p < 0.001$, respectively).

3.3. Changes in the expression of serotonin and mucin-related genes caused by *B. coagulans* administration in loperamide-treated SD rats

Loperamide treatment (CON) significantly reduced serotonin transporter (SERT) expression, crucial for serotonin reuptake, while neither lactulose nor *B. coagulans* affected its levels (Fig. 4A). Additionally, loperamide suppressed tryptophan hydroxylase (TPH) expression, vital for neurotransmitter synthesis. In contrast, lactulose and *B. coagulans* increased TPH1 and TPH2 expression, with TPH1 showing a dose-dependent increase (Fig. 4B and C).

Loperamide also reduced mucin (MUC) 2 and MUC4 expression, essential for the intestinal mucus barrier ($p < 0.001$, Fig. 4D and E). Lactulose treatment significantly increased MUC4 expression ($p < 0.05$, $p < 0.001$), and *B. coagulans* elevated both MUC2 and MUC4 levels (Fig. 4E). Notably, MUC2 expression was higher in *B. coagulans*-treated mice, while MUC4 expression was more significantly elevated by lactulose. Overall, *B. coagulans* reversed loperamide-induced decreases in TPH and MUC expression.

3.4. Changes in the expression of intestinal mucosal barrier- and tight junction-related genes in loperamide-treated SD rats caused by *B. coagulans* administration

Absorption and reduced intestinal secretion, resulting in dry stools. Loperamide (CON) upregulated these genes, while lactulose (PC) and *B. coagulans* (BCL, BCH) significantly downregulated their expression (Fig. 5A–C).

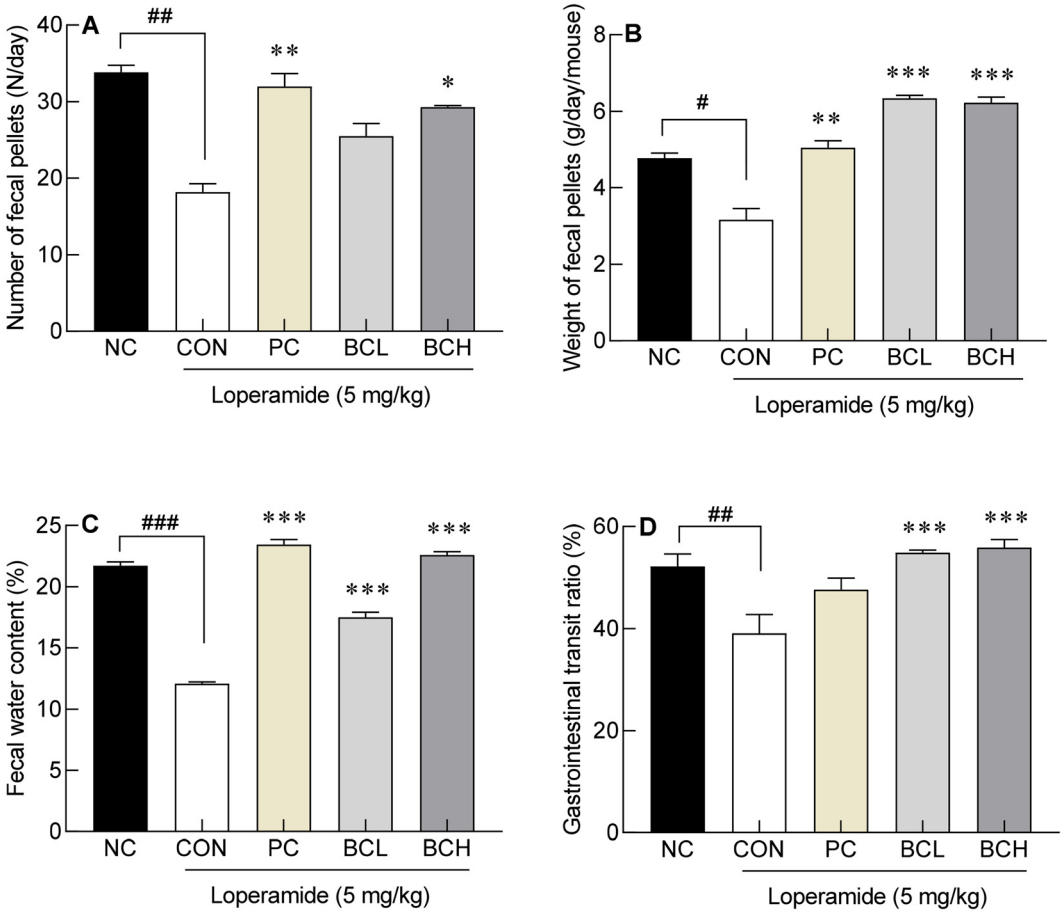

Fig. 1 Effect of *Bacillus coagulans* on the animal fecal and gastrointestinal transit ratio. (A) Number of fecal pellets, (B) weight of fecal pellets, (C) fecal water contents, and (D) gastrointestinal transit ratio in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* NC, and $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose treated, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0×10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0×10^9 CFU per day).

Loperamide also decreased the expression of OCLN, CLDN1, and ZO-1, key genes involved in maintaining the intestinal barrier ($p < 0.001$, $p < 0.01$, $p < 0.001$, respectively). *B. coagulans* restored OCLN and ZO-1 levels in a dose-dependent manner (Fig. 5D and F). While CLDN1 remained unchanged with lactulose (PC) and low-dose *B. coagulans* (BCL), high-dose *B. coagulans* (BCH) significantly increased its expression ($p < 0.001$, Fig. 5E). Overall, *B. coagulans* restored tight junction integrity and reversed the effects of loperamide on the intestinal mucosal barrier.

3.5. Changes caused by *B. coagulans* administration in protein levels of loperamide-treated SD rats

Loperamide treatment showed a non-significant trend towards decreasing c-Kit protein levels in the SCF/c-Kit pathway. However, c-Kit levels significantly increased with lactulose (PC) and high-dose *B. coagulans* (BCH) compared to the CON group (Fig. 6A). Loperamide also significantly increased AQP3 protein levels ($p < 0.01$, Fig. 6B), consistent with gene expression results. In contrast, both lactulose and *B. coagulans* significantly reduced AQP3 levels ($p < 0.05$ and $p < 0.001$, respectively), with *B. coagulans* showing a dose-dependent effect. For OCLN protein, the reduction caused by loperamide was significantly reversed by lactulose ($p < 0.001$), while *B. coagulans* increased OCLN protein, though the change was not statistically significant (Fig. 6C).

3.6. Changes in SCFA content caused by *B. coagulans* administration in loperamide-treated SD rats

Loperamide-induced constipation significantly reduced total SCFA content, which is crucial for maintaining intestinal barrier integrity and motility (Fig. 7, $p < 0.01$). Specifically, acetic acid was significantly decreased by loperamide ($p < 0.01$), while propionic and butyric acid levels were unaffected. Treatment with lactulose (PC) and high-dose *B. coagulans* (BCH) significantly restored acetic acid levels ($p < 0.05$ and $p < 0.01$, respectively), leading to a significant increase in total SCFA content, reversing the effects of loperamide (Fig. 7).

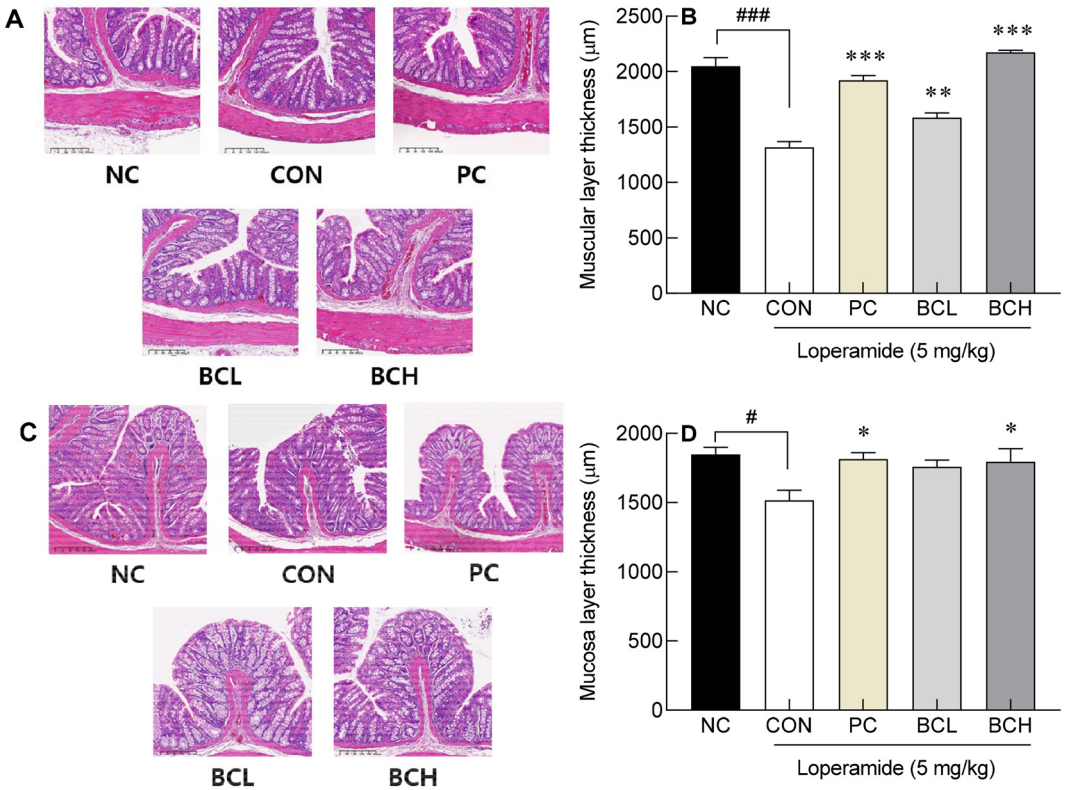

Fig. 2 Effect of *Bacillus coagulans* on intestinal muscular (A and B) and mucosal (C and D) layer thickness in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs.* NC and $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^{7} CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^{9} CFU per day).

4. Discussion

Constipation, affecting 5%–20% of the general population, is one of the most prevalent gastrointestinal issues. However, its symptoms vary widely among patients and often present as a cluster of related issues. This makes it challenging to objectively define it.[1] Factors such as lifestyle, medications, pelvic floor muscle weakness, and changes in the colon or rectal tissues contribute to constipation. Dietary control and appropriate physical activity are essential preventive measures. Recent studies have suggested that the probiotic *B. coagulans* can alleviate the symptoms of constipation and IBS. However, limited research exists on the molecular and intestinal morphological effects of *B. coagulans* in relieving constipation.

In the present study, we assessed the effects of different doses of *B. coagulans* on fecal parameters and gastrointestinal transit in rats with loperamide-induced constipation (Fig. 1). Constipation can arise from various causes, with long-term issues often linked to imbalances in intestinal microbiota. Previous studies have reported that probiotics like *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Clostridium tabificum* can enhance gastrointestinal transit in germ-free mice.[19] The results of several clinical trials further support the efficacy of probiotics in improving bowel movements and alleviating chronic constipation with benefits often persisting even after treatment discontinuation.[20,21] Therefore, in-depth research on the clinical effects of probiotics is essential.

Using H&E and IHC staining, we assessed the impact of *B. coagulans* on the intestinal architecture in rats with the loperamide-induced constipation (Fig. 2 and 3). Constipation and IBS often disrupt the gastrointestinal barrier owing to changes in the epithelium and mucosal layers, which express mucin glycoproteins. Loperamide-induced constipation typically results in reduced mucus production by crypt epithelial cells and a thinner mucosal surface layer.[22] Probiotics like *Lactobacillus* and *Bifidobacterium* have enhanced mucus production and thickness in drug-induced constipation models.[23] Additionally, these probiotics increased the mucus layer's thickness and prevented defects in models of dextran sulfate sodium (DSS)-induced colitis and obesity.[24] Furthermore, while constipation reduces crypt cells and ICCs, thereby slowing intestinal fluid passage and peristalsis,[25,26] Wnt/β-catenin signaling promotes stem cell renewal in the crypts, and probiotics boost crypt height, intestinal stem cells, Paneth cells, and goblet cells in the small intestine.[27,28]

Serotonin (5-hydroxytryptamine or 5-HT) plays a crucial role in regulating intestinal peristalsis and influences muscle contraction and relaxation. It is involved in gastrointestinal moti-

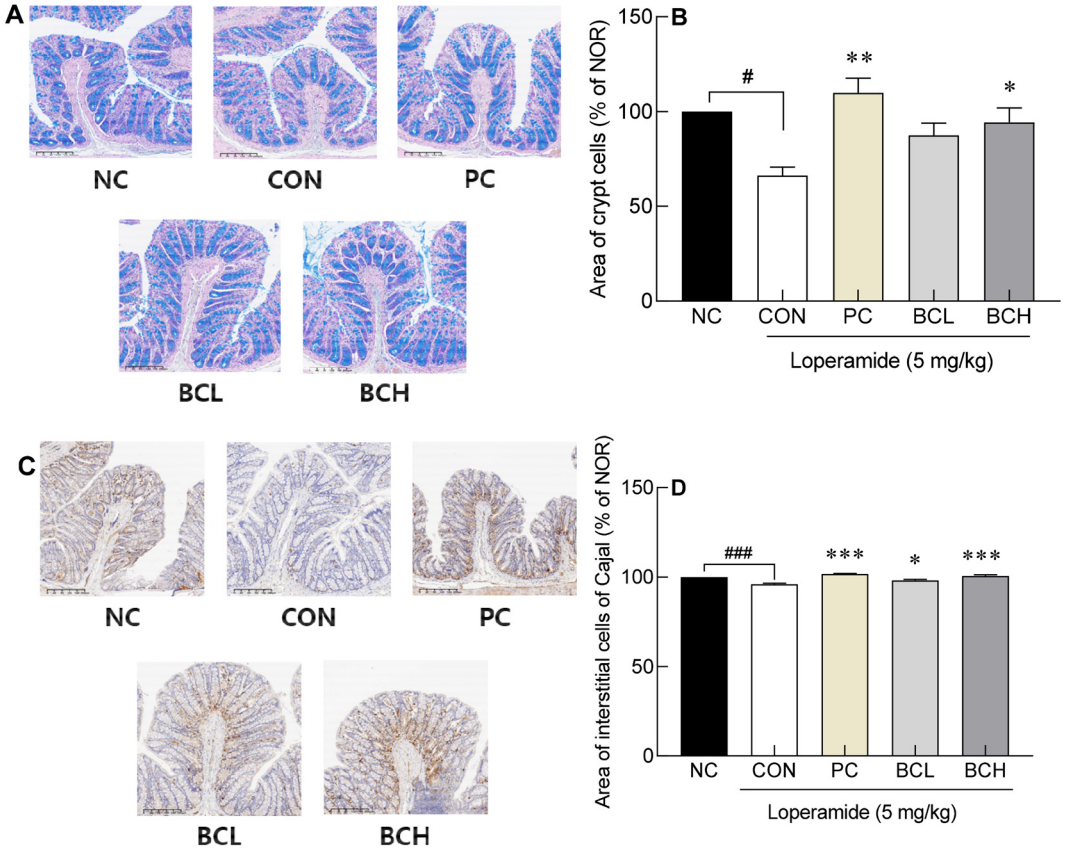

Fig. 3 Effect of *Bacillus coagulans* on the area of crypt cells (A and B) and intestinal cells of Cajal (C and D) in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs.* NC and $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^9 CFU per day).

lity, secretion, and inflammation in disorders such as IBS and constipation, making it a target for treatment.[29,30] Alterations in *SERT* transcript expression have been linked to different types of IBS, and increased *TPH1* expression in enterochromaffin cells influences intestinal motility.[31,32] The intake of *B. coagulans* elevates the expression levels of *SERT*, *TPH1*, and *TPH2*, which were reduced by loperamide treatment (Fig. 4).

In loperamide-treated rats, constipation is associated with a decrease in luminal mucin, which protects the colonic mucosa. However, we demonstrated that the administration of *B. coagulans* significantly elevated the expression of *MUC2* and *MUC4* (Fig. 4). Moreover, prebiotics such as sulfated polysaccharides and the probiotic formula VSL#3 enhanced the epithelial mucin production, *MUC2* expression, and mucus layer thickness, thereby improving fecal excretion through enhanced intestinal motility.[33,34] Additionally, single bacterial strains such as *L. plantarum* 299v, *Escherichia coli* Nissle 1917, and *L. casei* GG increase mucin gene expression in HT-29 and Caco-2 cell lines, with *L. plantarum* 299v exerting protective effects by modulating the caspase-dependent apoptotic pathway.[35–38]

B. coagulans administration influenced the expression of intestinal mucosal barrier genes (*AQP3*, *AQP8*, and *PKA*) in the qRT-PCR analysis (Fig. 5). AQPs facilitate water transport and help maintain the intracellular water balance. The abnormal expression of AQPs is linked to constipation, as it can result in excessive water absorption and reduced intestinal fluid secretion.[39,40] Loperamide exacerbates constipation by activating the vasoactive intestinal peptide (VIP)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/aquaporin 3 (AQP3) signaling pathway.[41] Additionally, the role of PKA in phosphorylating neuronal nitric oxide synthase and promoting ileal relaxation is associated with slow-transit constipation.[42] Prebiotics and probiotics, such as *Lactococcus lactis* subsp. lactis HFY14 and high specific volume polysaccharides, alleviate constipation symptoms by modulating this signaling pathway.[43,44]

The expression levels of *OCLN*, *CLDN1*, and *ZO-1*, which are essential components of tight junctions, were significantly elevated in the *B. coagulans*-treated group compared with those in the loperamide-induced constipation model (Fig. 5). These tight junction proteins, located beneath the mucus layer that

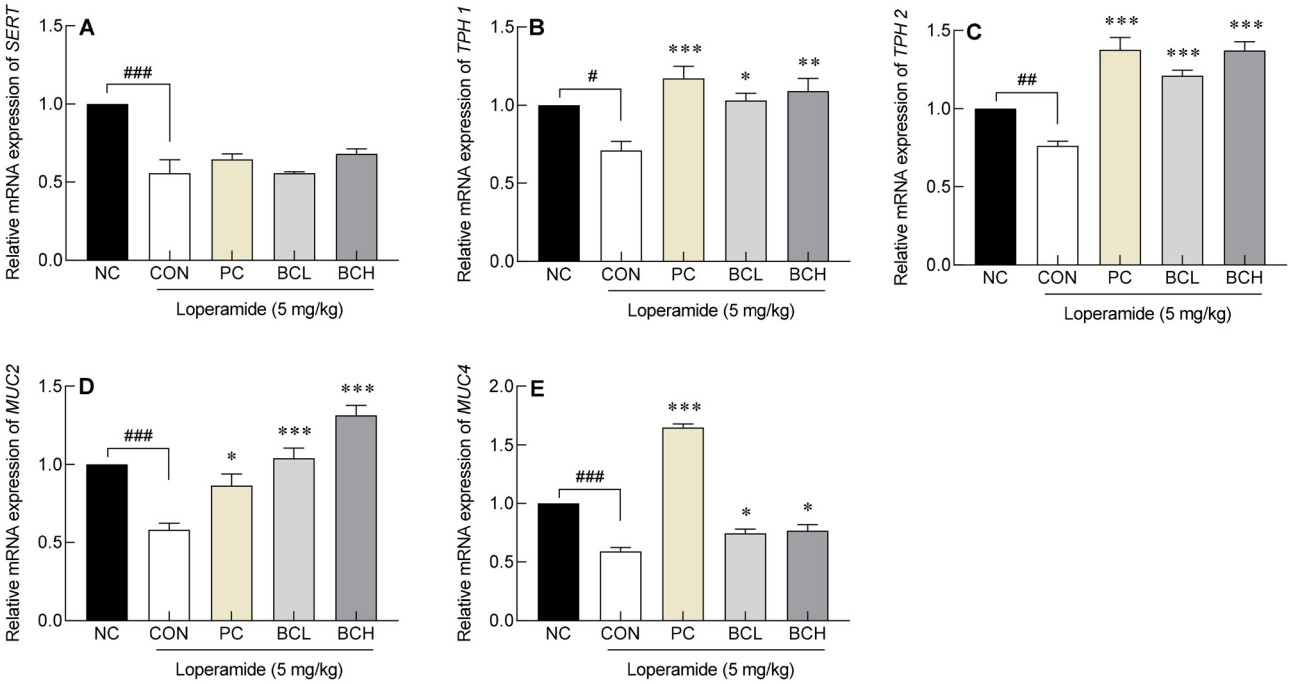

Fig. 4 Effects of *Bacillus coagulans* on the mRNA expression of (A) *SERT*, (B) *TPH 1*, (C) *TPH 2*, (D) *MUC2*, and (E) *MUC4* in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 *vs.* NC and p < 0.05, p < 0.01, p < 0.001 *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^9 CFU per day). SERT: serotonin transporter, TPH1: tryptophan hydroxylase.

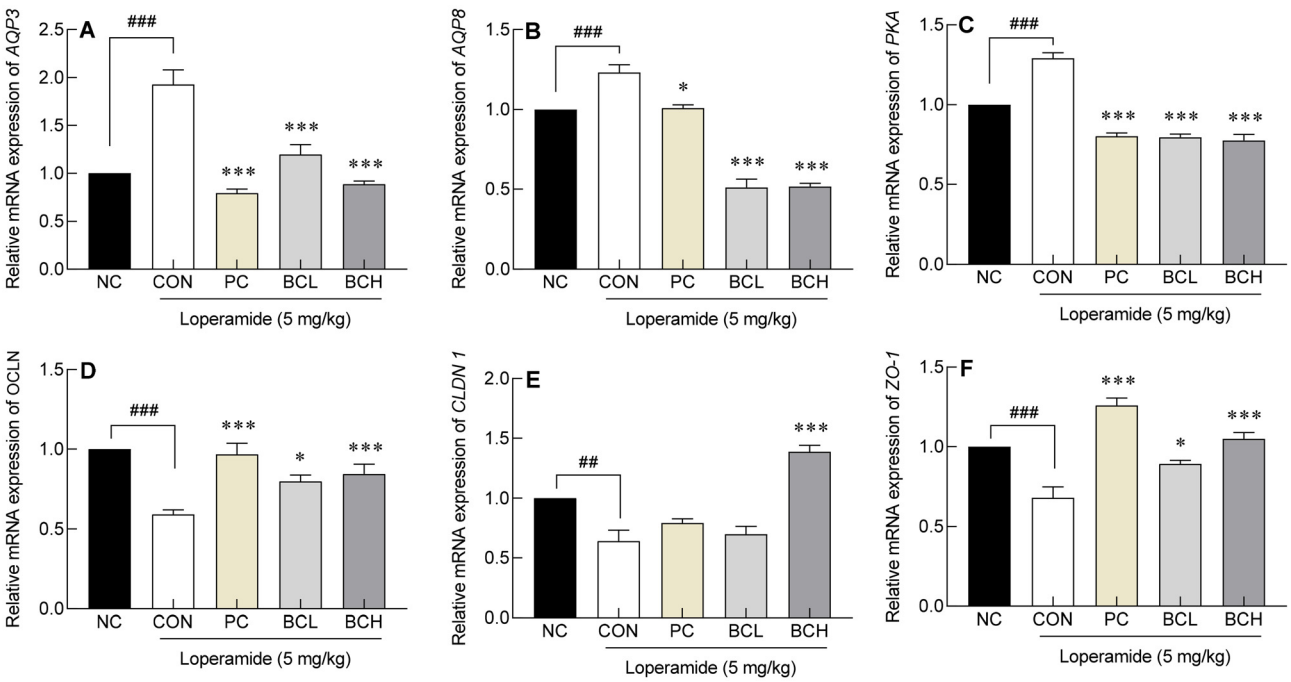

Fig. 5 Effects of *Bacillus coagulans* on the mRNA expression of (A) *AQP3*, (B) *AQP8*, (C) *PKA*, (D) *OCLN*, (E) *CLDN1*, and (F) *ZO-1* in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#\#\#}p$ < 0.001 *vs.* NC and p < 0.05, p < 0.001 *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^9 CFU per day). MUC: mucin.

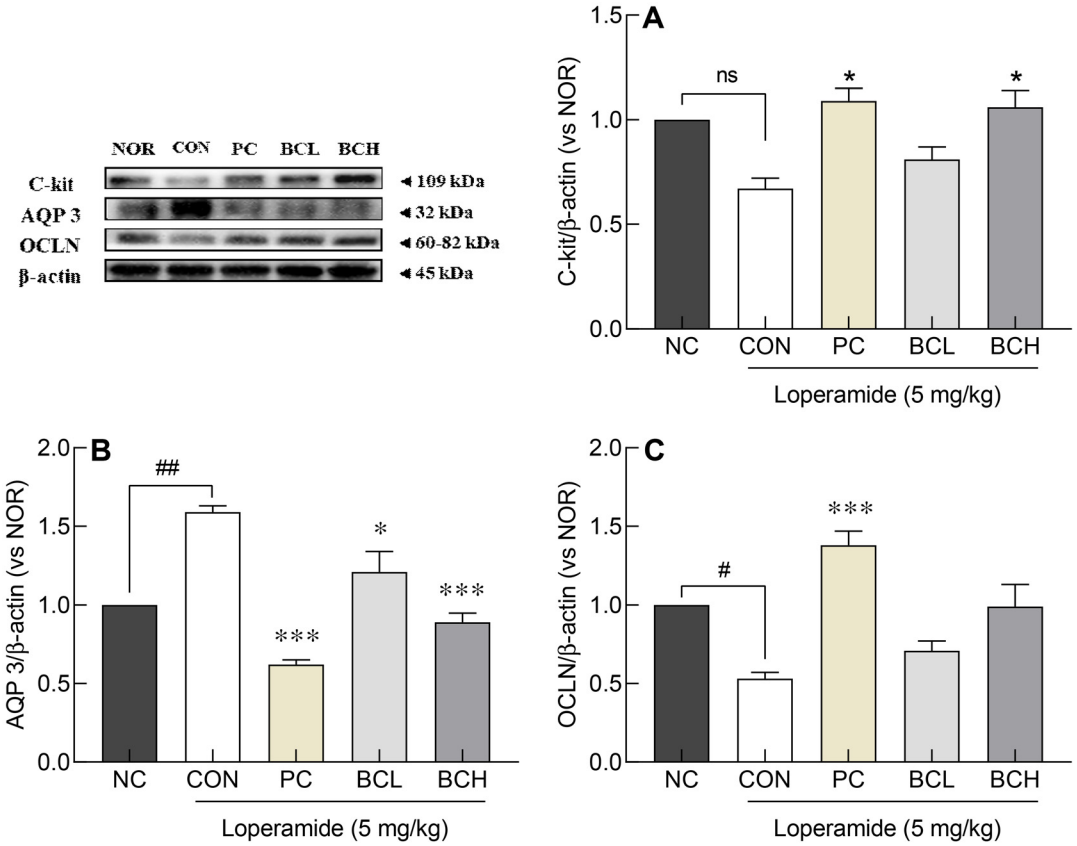

Fig. 6 Effect of *Bacillus coagulans* on the protein levels of (A) C-kit, (B) AQP 3, and (C) OCLN in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (n = 6). The following symbols indicated significance: $^{\#\#}p < 0.01$ *vs.* NC and $p < 0.05$, $p < 0.001$ *vs.* CON according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^9 CFU per day). C-kit: receptor tyrosine kinase protein, AQP 3: aquaporin 3, OCLN: occludin.

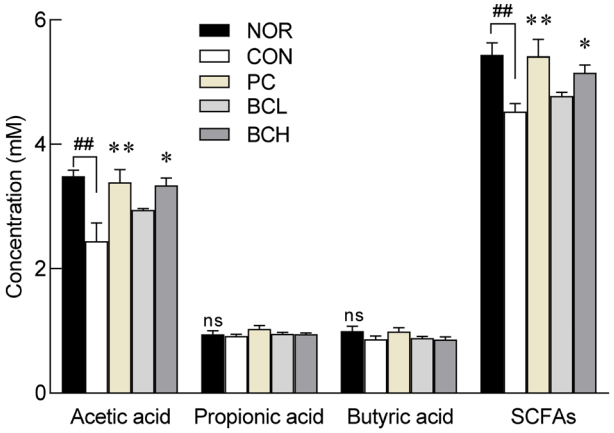

Fig. 7 Effects of *Bacillus coagulans* on SCFAs in SD rats with loperamide-induced constipation. Data are expressed as the means ± standard error of the mean (S.E.M) (n = 6). The following symbols indicated significance: $^{\#\#}p < 0.01 < 0.001$ *vs.* NC and $p < 0.05$, $p < 0.01$ *vs.* CON group according to Tukey's test. NC: normal group; CON: loperamide treated-control group (5 mg kg^{-1}); PC: positive control group (lactulose, 2010 mg kg^{-1}); BCL: low-dose *Bacillus coagulans*-treated group (1.0 × 10^7 CFU per day); BCH: high-dose *Bacillus coagulans*-treated group (2.0 × 10^9 CFU per day). SCFAs: short chain fatty acids; ns: not significant.

protects the intestines, form complexes with cytoplasmic adaptors, such as ZO-1 to maintain the intestinal epithelial barrier.[45] In piglets, a synbiotic mix of lactulose and *B. coagulans* improved the LPS-induced intestinal damage by regulating tight junction protein expression in the jejunum and ileum.[46] Similarly, *B. coagulans* SCC-19 mitigates cadmium-induced damage in common carp by increasing the mRNA levels of tight junction proteins and preserving intestinal barrier function.[47]

Our results revealed that *B. coagulans* administration increased the protein levels of C-kit, AQP3, and OCLN, as well as the SCFA content, which were collectively decreased with loperamide treatment (Fig. 6 and 7). c-kit, a receptor involved in gastrointestinal motility, alleviates constipation when its expression is enhanced with *B. coagulans.*[4] The SCF/C-kit signaling pathway influences gastrointestinal muscle activity by regulating the ICC, which are crucial for motility and constipation relief.[26] Probiotics alleviate constipation by modifying the gut microbiota, improving transit time, and increasing the SCFA content, which support colonic epithelial cell proliferation and mucosal growth.[48] For instance, *Bifidobacterium* enhances acetate levels, correlating with reduced consti-

pation,[49] while *B. coagulans* X26 boosts SCFA content and improves the intestinal structure in laying hens.[50]

Here, we demonstrated the effectiveness of *B. coagulans* in alleviating constipation. Moving forward, we will further investigate the gut microbiome to analyze the role of *B. coagulans* on the abundance of beneficial gut microbiota and evaluate the various changes it introduces in the gut environment.

Meanwhile, there are several studies that have explored *Bacillus coagulans* as a potential treatment for constipation in humans. For example, one clinical study investigated the effects of *B. coagulans* Unique IS2 in adults with functional constipation. The results showed that participants who received the *B. coagulans* supplement had significant improvements in bowel movements compared to the placebo group, suggesting its potential as a therapeutic agent for managing constipation.[51] Another randomized controlled trial demonstrated that *B. coagulans* SNZ 1969 improved intestinal motility and reduced constipation symptoms by modulating the gut microbiota. These findings point to the probiotic's ability to influence gut health and alleviate constipation symptoms, though further research, particularly involving larger human trials, is needed to establish its long-term efficacy and safety across different populations.

5. Conclusion

B. coagulans alleviates loperamide-induced constipation symptoms. These effects occur through changes in intestinal environment-related factors. In summary, this study demonstrates the efficacy of *B. coagulans* as a probiotic for managing constipation. *B. coagulans* influences the secretion of neurotransmitters and glycoproteins, as well as the expression levels of genes and proteins that are crucial for maintaining a balance in the intestinal environment. Additionally, it alleviates constipation symptoms by altering the content of SCFAs in response to the changes in the gut microbiota.

Author contributions

Joo Hyun Jang: conceptualization, formal analysis, data curation, writing – original draft, funding acquisition. Yeok Boo Chang: methodology, formal analysis, investigation, writing – original draft. Sang Min Kim: methodology, formal analysis, writing – review & editing. Kisoo Han: methodology, formal analysis. Sim Wan Scu: methodology, formal analysis. Ki-Bae Hong: conceptualization, methodology. Hyung Joo Shu: conceptualization, methodology. Sung Heel Han: conceptualization, methodology, writing – review & editing, supervsion, funding acquisition.

Data availability

The data are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare no conflict of interest.

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