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Spore-forming *Bacillus coagulans* SNZ 1969 improved intestinal motility and constipation perception mediated by microbial alterations in healthy adults with mild intermittent constipation: A randomized controlled trial

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ABSTRACT

The spore-forming *Bacillus coagulans* has attracted attention for their therapeutic action in the colon. However, the mechanism of this action remains unclear. In this study, healthy subjects with mild intermittent constipation were supplemented with *B. coagulans* SNZ 1969 (BC) or the placebo for 8 weeks ($n = 80$). Then, we assessed colonic transit time (CTT), weekly complete spontaneous bowel movement (CSBM) scores, bowel discomfort symptom (BDS) scores, and 16S rRNA fecal microbiome profiles. The association between the critically altered gut microbiome and clinical outcomes was analyzed using redundancy analysis (RDA) and validated by receiver operating characteristic (ROC) curves. BC supplementation significantly improved CTT ($p = 0.031$), CSBM at weeks 2 ($p = 0.045$) and 9 ($p = 0.038$), and BDS at weeks 3 ($p = 0.019$) and 6 ($p = 0.029$) compared with the placebo, while altering the community composition of the gut microbiota. We also confirmed that BC was effectively delivered to the gut. Finally, the multivariate redundancy analysis concluded that BC-induced enrichment of Lactobacillales and diminishment of Synergistales were related to CTT improvements. This study provides important new data on how spore-forming *B. coagulans* SNZ 1969 contributes to improving gut motility and presents evidence supporting the use of *B. coagulans* SNZ 1969 in adults with mild intermittent constipation and habitual low intake of fruit and vegetables.

1. Introduction

Constipation is a common chronic problem worldwide. It is not a disease but a general term characterized by unsatisfactory defecation due to infrequent stools, difficult stool passage, or both (Gallegos-Orozco et al., 2012). However, chronic constipation has a sizeable negative impact on the quality of life due to abdominal pain, bloating, headache, nausea, and poor appetite. It may also contribute to the development of pelvic organ prolapse and psychological distress if not adequately managed (Suares & Ford, 2011). Moreover, it renders

Abbreviations: AUC, area under the curve; BC, *Bacillus coagulans* SNZ 1969; BDS, bowel discomfort symptom; CSBM, complete spontaneous bowel movement; CTT, colonic transit time; ITT, intention-to-treat; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; RDA, redundancy analysis; RFS, recommended food score; ROC, receiver operating characteristic.

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difficulties in treating chronic constipation because of its wide variety of primary and secondary etiologies. Primary causes are defined as intrinsic defects in colonic function or malfunction of the defecation process, whereas secondary causes are associated with organic disease, systemic disease, or medications (Andrews & Storr, 2011). The well-known chemical therapeutics mainly target the primary causes of constipation: psyllium as a bulking agent, docusate sodium as a stool softener, senna as a stimulant laxative, and lactulose as an osmotic laxative (Alexandre et al., 2016). Nevertheless, overall satisfaction rates were somewhat disappointing due to the lack of effectiveness or severe side effects from these agents (Johanson & Kralstein, 2007). Therefore, further research is required to address unmet needs concerning the practical and safe management of chronic constipation.

Probiotics demonstrate immense promise as a treatment option for chronic constipation (Ojetti et al., 2014; Zhao & Yu, 2016). In particular, spore-forming organisms have received notable attention as gut probiotics because of their capacity to withstand the acidic condition of the stomach and bile acids (Bora et al., 2009; Hong et al., 2005). Potential mechanisms of gut probiotics on constipation may include alterations in intestinal microbiota, improvements in colonic transit time (CTT), or pH reduction due to enhanced short-chain fatty acids production (Chmielewska & Szajewska, 2010). Clinical studies involving the administration of *Bacillus coagulans* SANK 70258 isolated from green malt (Ara et al., 2002), *B. coagulans* lilac-01 isolated from the petals of *Syringa vulgaris* (lilac) (Minamida et al., 2015), and *B. coagulans* Unique IS2 obtained by the fermentation process (Madempudi et al., 2020) have shown improved CTT. In a more recent study, Maity et al. (Maity et al., 2020) reported a positive impact of *B. coagulans* LBSC on human gut microbiome modulation. However, the link between gut microbiota alteration and intestinal motility remains to be elucidated.

This study hypothesized that daily supplementation of *B. coagulans* SNZ 1969 (BC) enhances gut motility and suppresses constipation symptoms by modulating gut microbiota. To test this hypothesis, we investigated whether BC supplementation could induce alterations in gut microbiota, modifying CTT and bowel function perception of healthy volunteers with mild intermittent constipation in a randomized controlled trial. Furthermore, we examined possible links of significantly altered microbiota with the host clinical parameters using redundancy analysis (RDA). RDA is a multivariate statistical analysis that investigates how a set of response variables is related to a set of explanatory variables (Everitt, 2021). Our result provides novel insight into how gut microbiota modulation plays a critical role in the probiotic-dependent amelioration of intestinal motility.

2. Material and methods

2.1. Test materials

Both BC and the placebo materials in capsules were provided by CTCBIO, Inc. (Seoul, Korea). Treatment capsules were a mix of BC and maltodextrin, supplying a dose of 1.0×10^9 CFU. Placebo capsules contained maltodextrin only.

2.2. Study design

This study was an 8-week, randomized, double-blind, placebo-controlled, parallel-group trial with two arms. A sample size of at least 30 subjects per group was determined to detect a difference in the stool frequency or the *Bifidobacterium* composition with 80% statistical power at $p < 0.05$ (Ara et al., 2002; Jayasimhan et al., 2013). This sample size was increased to 40 subjects per group to account for a 25% dropout rate. Subjects living in Seoul, Korea, were recruited between March 2017 and March 2018 through a research company and posted advertisements. This study was conducted at the Seoul Metropolitan Government-Seoul National University Boramae Medical Centre (Seoul, Korea) and approved by the Institutional Review Board of Boramae Medical Center, Seoul National University (IRB No. 20161020/16-2016-136/111). It was also registered on the WHO International Clinical Trials Registry Platform (KCT0002226, http://apps.who.int/, date of registration: 14/12/2017).

2.3. Inclusion/Exclusion criteria

Inclusion criteria for this study were age over 20 years and meeting the modified Rome III functional constipation criteria (Drossman, 2006). Exclusion criteria included (1) the use of probiotics, prebiotics, or antibiotics within 1 week of enrollment, (2) regular use of a high-fiber diet, as measured by the recommended food score (RFS), (3) regular use of medications affecting bowel habits, such as irritable bowel syndrome, functional bloating, and functional diarrhea, (4) suffering from diseases that could cause secondary constipation, such as hypothyroidism, diabetes, Alzheimer's, Parkinson's disease, and dementia, (5) medical history of cardiovascular, liver, or renal diseases; alcoholics, (6) hypersensitivity to probiotics or the ingredients used in this study, (7) pregnancy or breastfeeding, and (8) participation in another clinical trial in the 1 month before enrollment. All participants provided written informed consent.

2.4. Procedures

An independent researcher randomly assigned eligible subjects to the test or placebo group by using computer-generated randomization. Group allocations were blinded for both investigators and participants. Participants were asked to consume either one placebo or BC capsule with water each day for 8 weeks. The follow-up lasted 2 weeks after the final supplementation. Excessive fruit (>2 times/day) and vegetable (>6 times/day) consumption was prohibited because of the high-fiber content. Data on vital signs, anthropometric measurements, drinking/smoking, 3-day dietary records, sleep quality, depression level, and physical activity were obtained at baseline and the end of the study using a mobile phone app.

2.5. CTT measurement

The CTT was measured at weeks 0 and 8 using radio-opaque markers (Bouchoucha et al., 1998). Briefly, subjects ingested one capsule containing 20 circular radio-opaque markers (Kolomark^{TM}, M.I. Tech, Pyeongtaek, Korea) on three consecutive days shortly after breakfast. On both the 4th and 7th day, markers were localized and counted in each segment of the large bowel (the right colon, the left colon, and the rectosigmoid colon) by abdominal radiography in the supine position (Tomita et al., 2011). One marker corresponds to 1.2 h (20 markers for 24 h) of transit time. The total and segmental CTT were derived by multiplying these numbers by 1.2. Then, responders were defined as individuals who had total CTT reduced by more than 25% compared with the individual baseline values after treatment for 8 weeks (Magro et al., 2014; Polymeros et al., 2014).

2.6. Perception of bowel function

Subjects were required to complete their daily bowel movements in a paper diary during the 8-week treatment and 2-week follow-up. The complete spontaneous bowel movement (CSBM) is a summary value for spontaneous bowel movements associated with a sensation of complete evacuation (Bharucha et al., 2008). We determined the changes in the CSBM scores per week by comparing it with a pre-treatment baseline. Weekly bowel discomfort symptom (BDS) scores were also determined by totaling the rates (0 = no and 1 = yes) of four items in the bowel movement diary, including stool form, straining, bothersome constipation, and the use of rescue operations (Hong et al., 2009; Kim et al., 2010).

2.7. Metagenomic analysis of gut microbiome

Stool samples were collected at weeks 0 and 8 using a stool collection kit (SPL Life Sciences, Gyeonggi-do, Korea). We determined the fecal moisture content by measuring fecal weight decrease after drying by the vacuum drying oven (Daihan WOV-30, Gangwon-do, Korea). The fecal pH was measured using pH meter (Inolab pH 720, Weilheim, Germany). Metagenomic analysis was performed at MD Healthcare (Seoul, Korea). Briefly, each fecal suspension in phosphate-buffered saline was filtered through a cell strainer. Genomic DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) and quantified using QIAxpert (QIAGEN). Sequence analysis of the V3–V4 region of the 16S ribosomal RNA (rRNA) gene was performed using the MiSeq System Guide (Illumina, San Diego, CA, USA). Illumina adapter sequences in paired-end reads were trimmed by Cutadapt version 1.1.6 (https://cutadapt.readthedocs.io) (Martin, 2011). Overlapping paired-end Illumina fastq files were merged using CASPER and then filtered by Phred quality score ($Q \geq 20$) and read length. Any reads < 350 bp or > 550 bp were rejected. Chimeric sequences introduced by PCR or amplification were identified and removed using VSEARCH with the SILVA Gold database (Rognes et al., 2016). The remaining sequences were binned into operational taxonomic units (OTUs) with 97% homology using VSEARCH with the *de novo* clustering algorithm, allowing taxonomical assignment with UCLUST (parallel_assign_taxonomy_uclust.py script on QIIME 2) under default parameters (Caporaso et al., 2010). OTUs containing one sequence in only one sample were excluded from further analysis. The relative abundance of each OTU was determined as a proportion of the sum of sequences for each sample following the generation of taxonomic relative abundance profiles at the phylum, class, order, family, and genus levels.

2.8. Statistical analysis of clinical and pyrosequencing data

All statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) to compare clinical variables. The MicrobiomeAnalyst web platform (Dhariwal et al., 2017) and the Galaxy website (Segata et al., 2011) were used for comprehensive statistical and visual analysis of microbiome data. R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.r-project.org/index.html) was used to integrate the clinical and microbiome data. Data analyses were conducted using the intention-to-treat (ITT) population. Differences in baseline characteristics collected at week 0 were assessed using Student's *t*-tests or Chi-squared tests. Efficacy comparisons of CTT, CSBM, and BDS score between the placebo and BC groups were analyzed using a linear mixed-effects model (LMM). Fixed effects (treatment, week, and interaction of treatment and week), random effect (participant), and random error (within-participant) were considered after adjusting for dietary fat intake at week 8 and weekly stool weight. Data are presented as least-square means ± standard errors. Finally, a Chi-square test was used to compare the percentage of responders with improved CTT in the two groups. A value of $p < 0.05$ was considered statistically significant.

Alpha-diversity was measured by the Chao1 index (richness) and Shannon diversity (evenness) and statistically evaluated by the Student's *t*-tests. An overview of gut microbial dynamics was provided by principal coordinate analysis (PCoA), followed by statistical testing using permutational multivariate analysis of variance (PERMANOVA) based on the Bray–Curtis distance. Linear discriminant analysis (LDA) coupled with effect-size measurement (LEfSe) was performed on the Galaxy website (http://huttenhower.sph.harvard.edu/galaxy/) with the Kruskal–Wallis test. The $p < 0.05$ and logarithmic LDA score > 1.0 were used to detect features significantly different in abundance between the placebo and BC groups (Segata et al., 2011). Finally, to explore the critical gut microbiome associated with the clinical outcome responses, we performed multivariate redundancy analysis (RDA) via the 'rda' function of the 'vegan' package in R on *z*-score standardized data (Prehn-Kristensen et al., 2018). Receiver operating characteristic (ROC) curves were used to validate the accuracy of the relationship between the gut microbiome and clinical outcomes using the 'roc' function of the 'pROC' package in R (Robin et al., 2011). The following binary cut-off values were used: 70 h for total CTT (Husni-Hag-Ali et al., 2003), 4 times for CSBM (FDA, 2012), and 0 for BDS score (no = 0, yes = 1).

3. Results and discussion

3.1. Baseline characteristics

Fig. 1 shows the Consolidated Standards of Reporting Trials (CONSORT) flow diagram for this study. A total of 98 subjects were recruited for the trial. Of the 80 subjects included in each group, 73 completed the study. In the placebo group, six subjects were lost due to withdrawal of consent ($n = 3$), participation in other clinical studies ($n = 2$), and recurrence of functional dyspepsia symptoms ($n = 1$). In the test group, one subject with high blood pressure was eliminated. Post-intervention follow-up data were available only for 49 participants (24 in the placebo group and 25 in the BC group) because the post-intervention follow-up visit was optional.

The baseline demographic and biochemical characteristics for the ITT population are presented in Table 1. Study groups were well matched, with no significant differences between the two groups. Subjects included in this study were characterized as adults aged 20–65 years with an average body mass index of 22.7 kg/m^2, stool frequency of 3.4/week, Bristol Stool Form Scale (BSFS) of 3.3, Constipation Assessment Scale (CAS) of 7, and RFS of 18.5. The BSFS refers to an ordinal scale of stool types, which ranges from 1 (the hardest) to 7 (the softest) (Blake et al., 2016). The CAS is a tool for measuring both the presence and severity of constipation, with ranges from 0 (no constipation) to 16 (severe constipation) (Coffin & Causse, 2011). The RFS evaluates the overall diet quality of the Korean diet and ranges from 0 to 47 points (Kim et al., 2011). Therefore, subjects included in this study represent typical adults with a tendency toward constipation with a low intake of fruit and vegetables.

The compliance was excellent in both arms (99.2% in the placebo group *vs.* 99.5% in the BC group). There were no cases of violating exclusion restriction, including the use of probiotics, high fiber diet, or medications affecting bowel habits. The LMM analysis revealed no significant differences between groups for nutrient intakes at all visits, except for dietary fat intake (**Supporting information Table S1**). Thus, we included dietary fat intake as a covariate in the efficacy evaluation.

3.2. BC supplementation increased colonic motility and bowel function perception

We first assessed changes in the total and segmental transit times using radiopaque markers and X-rays, a well-known secure and objective technique for investigating the intervention effects on colonic motility. This method requires specialized facilities and several days to perform. However, it is straightforward to distinguish subgroups with different bowel movement levels (Kim & Rhee, 2012). As shown in Fig. 2**A**, the total CTT decreased by 13.6 h in the BC-treated group during the experimental period. However, it increased by 6.1 h in the placebo-treated group. As a result, the treatment × week interactions were statistically significant ($p = 0.031$), suggesting a considerable improvement of colonic motility by 8-week BC supplementation. Moreover, when we define responders as individuals who had total CTT reduced by more than 25% as compared to the individual baseline values after 8-week treatment, the percentage of responders was also significantly higher in the BC-treated group than in the placebo-treated group (58% *vs.* 32%, $p = 0.046$) (Fig. 2**B**).

In one study comparing total and segmental CTT, transit time in the right segment was significantly higher in functional constipation subjects than in healthy subjects (Bhate et al., 2015). In another study

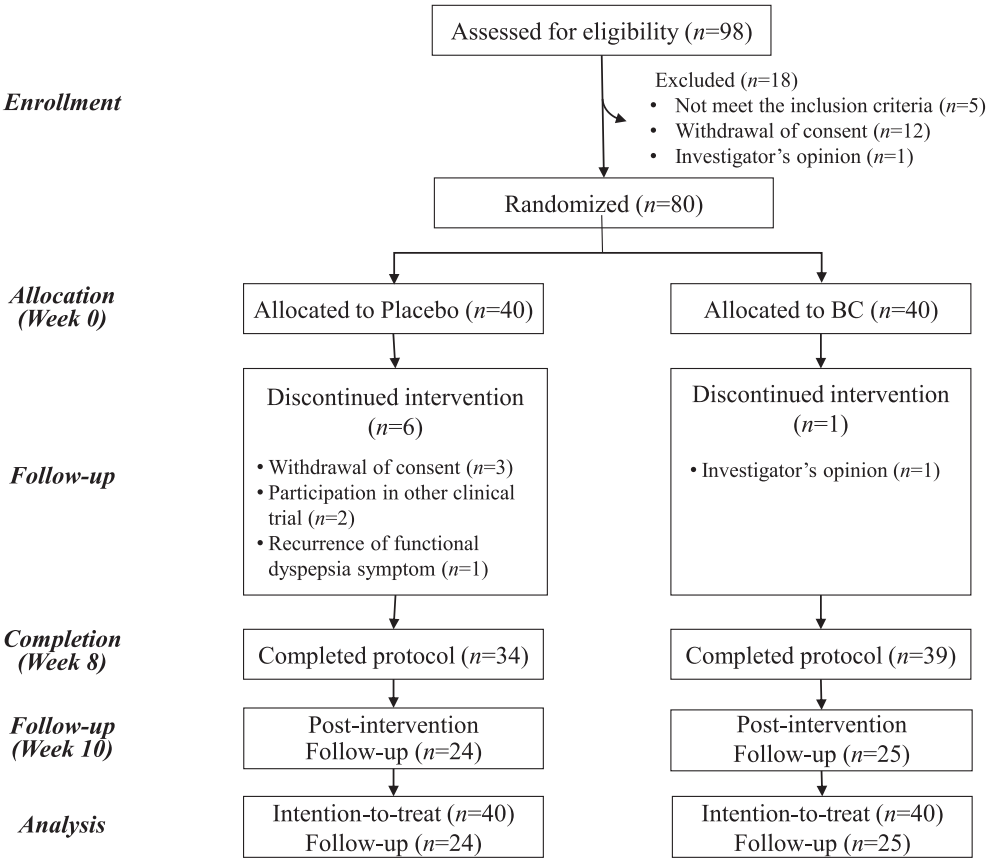

Fig. 1. CONSORT flow diagram of the study from enrollment to data analysis. The primary reasons for exclusion are also described. The intention-to-treat population comprised all participants randomized at the allocation visit and who had consumed at least one dose of the study product. CONSORT, Consolidated Standards of Reporting Trials; BC, *Bacillus coagulans* SNZ 1969.

Table 1
Baseline demographic and biochemical characteristics of the intent-to-treat participants.

Variable	Placebo (n = 40)	BC (n = 40)	p-value
Age (years)	45.3 ± 1.8	44.4 ± 2.2	0.756
Gender (male/female)	5/35	5/35	1.000
Menstruation (Y/N)	23/12	21/14	0.621
Alcohol consumption (Y/N)	17/23	12/28	0.245
Cigarette smoking (Y/N)	0/40	1/39	1.000
Body weight (kg)	60.9 ± 1.7	57.7 ± 1.2	0.131
BMI (kg/m^2)	23.2 ± 0.5	22.2 ± 0.4	0.112
Waist circumference (cm)	74.3 ± 1.5	74.4 ± 1.2	0.953
Blood pressure (mmHg)			
BP	113.8 ± 1.6	115.8 ± 1.7	0.386
DBP	73.2 ± 1.1	75.3 ± 1.2	0.205
RFS	17.6 ± 1.1	19.4 ± 1.1	0.239
Physical activity (MET-min/week)	1753.1 ± 318.3	2130.7 ± 346.1	0.424
Sleep quality (PSQI)	5.7 ± 0.5	6.1 ± 0.5	0.579
Depression level (BDI)	9.9 ± 1.1	8.1 ± 1	0.219
Frequency of defecation (times/week)	3.4 ± 0.3	3.4 ± 0.2	0.995
Average BSFS (1–7)	3.4 ± 0.1	3.3 ± 0.1	0.797
CAS (0–16)	7.1 ± 0.5	6.8 ± 0.4	0.658

All data are expressed as the mean ± standard error. Differences between the groups were compared using Student's t-tests for continuous variables and Chi-square tests for categorical variables. BC, *Bacillus coagulans* SNZ 1969; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RFS, recommended food score; PSQI, Pittsburgh Sleep Quality Index; BDI, Beck Depression Inventory; BSFS, Bristol Stool Form Scale; CAS, Constipation Assessment Scale.

investigating the effects of a food supplement on CTT in healthy subjects, transit time in the right segment was significantly different between the treatment and control groups (Alexandre et al., 2016). Similar to these previous studies, we also demonstrated that the most significant improvement in the BC-treated group occurred in the right segment of the colon ($p = 0.003$). The greatest advantage of the CTT method is that it can provide insights into the mechanism of action (Alexandre et al., 2016). The right ascending colon has a role in absorbing the remaining water and other nutrients from the indigestible materials, solidifying it to form stool (Azzouz & Sharma, 2019). Thus, we assumed that BC might potentially enhance colonic motility by altering water reabsorption and bacterial fermentation in the colon (Dimidi et al., 2017). However, contrary to our initial hypothesis, changes in fecal moisture content (0.34 ± 2.2% *vs.* −0.51 ± 2.11%) and pH (-0.002 ± 0.125 *vs.* −0.153 ± 0.123) were not significantly different in stools of placebo and BS groups. It might be because interindividual variations observed in fecal moisture and pH mask the subtle changes induced by BC supplementation. Besides, the fecal collection method, which is desirable for gut microbiome analysis, may not be appropriate for determining fecal moisture and pH value. Finally, the lack of a significant change in the fecal pH might be attributed to the fact that short-chain fatty acid produced in the colon does not remain in feces to lower fecal pH, although it can lower intestinal pH (Asano et al., 2004).

In addition to CTT analysis, we examined daily variations in bowel movement frequency and bowel symptoms from diary recordings during the 8-week intervention and 2-week post-intervention periods. Constipation is generally defined as fewer than three bowel movements per week (Jamshed et al., 2011). However, for accurate assessment of intervention efficacy in constipation, the U.S. Food and Drug Administration (FDA) issued a guidance document concerning the CSBM,

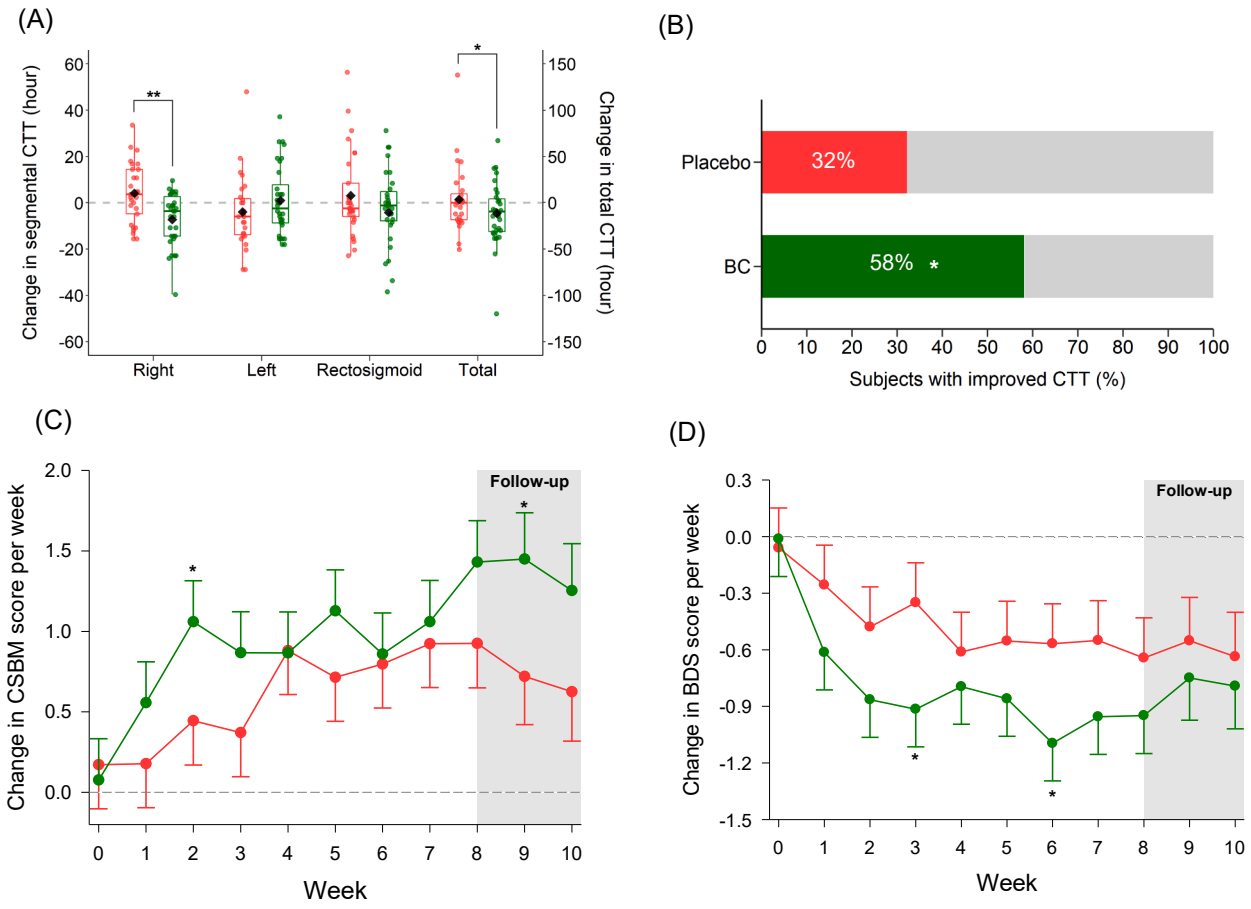

Fig. 2. Changes in colonic motility and bowel function perception in the placebo (red) and BC (green) groups. (**A**) Segmental and total CTT. Dots represent individual subjects. Black diamonds indicate the least-square mean. (**B**) Percentage of responders to total CTT. (**C**) Changes in weekly CSBM. (**D**) Changes in weekly BDS score. For (A), (C), and (D), data are expressed as the least-square mean ± standard error, and statistical significances were determined by the linear mixed-effects model. For (B), the *p*-value for responders of CTT was obtained from the Chi-square test. $*p < 0.05$, $**p < 0.01$. BC, *Bacillus coagulans* SNZ 1969; CTT, colonic transit time; CSBM, complete spontaneous bowel movement; BDS, bowel discomfort symptom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

defined as a bowel movement with a complete evacuation sensation without laxative (FDA, 2012). As a result, the CSBM has been widely used in investigating intervention effects on constipation (DeMicco et al., 2017; Mori et al., 2019). In the current study, both groups showed improvements in the weekly mean CSBM score than the pre-treatment condition, demonstrating the placebo effect (Fig. 2**C**). We note that strong placebo effects are often observed in clinical trials of gut health (Udani & Bloom, 2013). However, in the present study, the CSBM scores increased more than double in the BC group over the study period, showing significant differences compared with the placebo treatment at weeks 2 ($p = 0.045$) and 9 ($p = 0.038$). In many drug studies of patients with constipation, the abdominal pain or discomfort intensity was determined using a Likert scale (Johnston et al., 2010) or visual analog scale (Ondo et al., 2012) in parallel with the CSBM results. However, considering that participants in our study were not patients but healthy subjects with mild intermittent constipation, we considered that the use of multiple markers might be comparatively more comprehensive and reliable. Therefore, we used the BDS score, a total score of symptom-based Rome III criteria recorded by the binary method (yes or no). As a result, we found that the BDS scores reduced significantly in subjects with BC supplementation than those with placebo treatment at weeks 3 ($p = 0.019$) and 6 ($p = 0.029$, Fig. 2**D**).

3.3. BC supplementation induced alterations in the gut microbiome

The studies on the gastrointestinal microbiota in constipation consistently demonstrated a decrease in bifidobacteria and lactobacilli levels compared with controls (Zhu et al., 2014). We thus postulated that BC supplementation might stabilize the dysbiotic condition in constipation subjects by beneficial modulation of the gut microbiota. The V3 − V4 region of the 16S rRNA gene was sequenced in the fecal samples to identify alterations in the gut microbiota due to BC supplementation. The Chao1 and Shannon indices (α-diversity) and PCoA (β-diversity) indicated no significant difference in the gut microbiota diversity between the two groups (**Supporting information Figure S1**). These results are consistent with findings across many studies. In a systematic review of seven randomized controlled trials of the alterations in fecal microbiota composition by probiotic supplementation, Kristensen et al. (Kristensen et al., 2016) noted that the absence of effects on α- and β-diversities of the bacterial community was a common finding.

However, LEfSe analysis identified 26 bacterial taxa that were differentially abundant between the placebo and BC groups (Fig. 3**A**). The BC supplementation enriched 14 bacterial taxa (two orders, three families, and nine genera) and diminished 12 bacterial taxa (two orders, four families, and six genera) compared with the placebo treatment. The dominant bacteria at the order level were Bacillales ($p = 0.007$) and Lactobacillales ($p = 0.014$), and those at the corresponding genus level were *Bacillus* ($p = 0.021$), *Gemella* ($p = 0.018$), and *Streptococcus* ($p = 0.015$). In consistent with our findings, a previous clinical trial of constipation patients showed enrichment of Bacillales (order), Bacillaceae (family), and *Bacillus* (genus) after 1-month treatment with synbiotics (Huang et al., 2018). We also found that order Synergistales ($p = 0.006$)

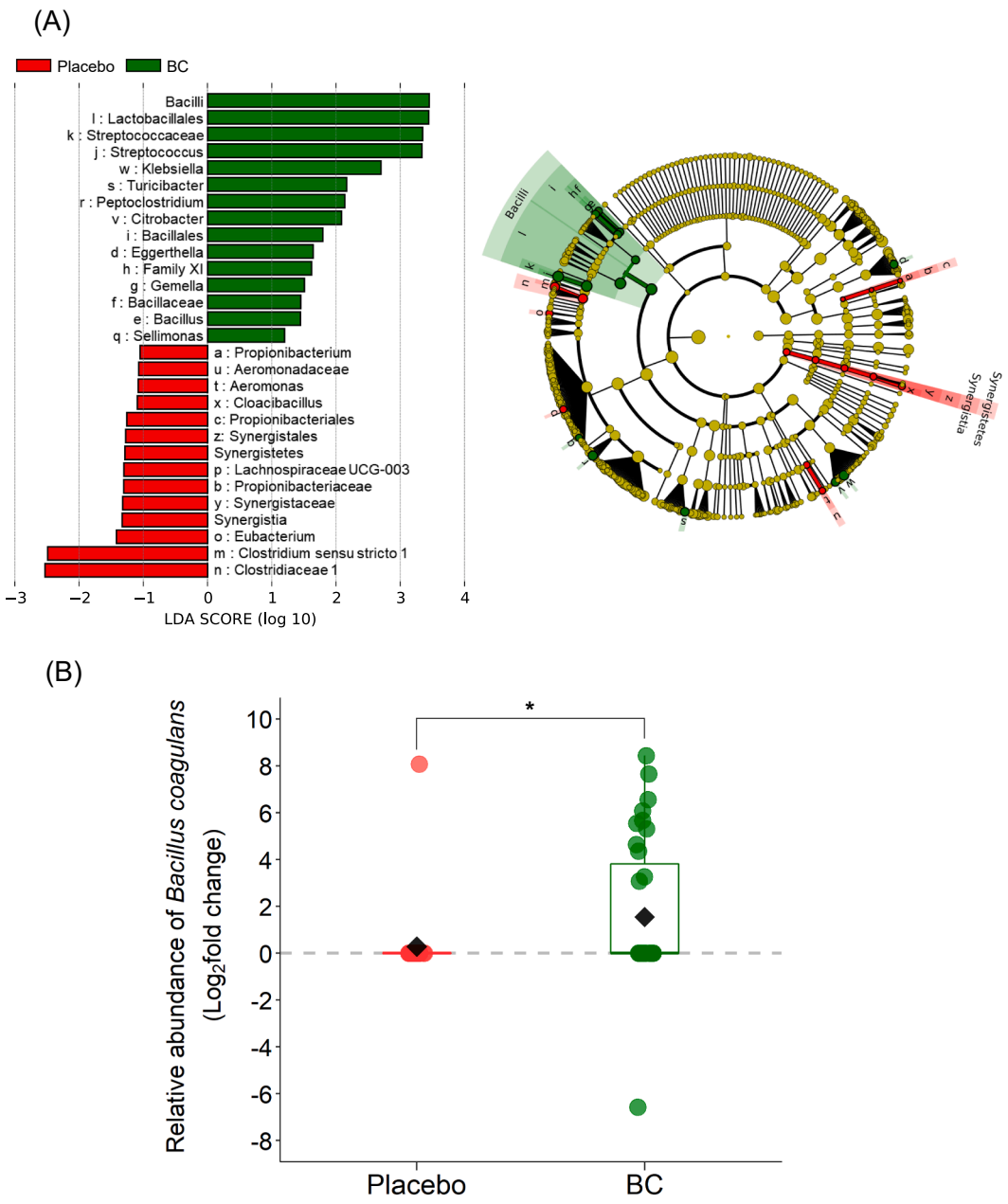

Fig. 3. Changes in fecal microbiomes by BC supplementation. (**A**) Taxonomic cladogram and histogram of the LDA scores coupled with effect-size measurements observed for individual taxa that passed the LEfSe significance threshold. Taxa with enriched levels in BC samples are shown in green, and those with enriched levels in the placebo samples are shown in red. (**B**) Log_2-fold change in the relative abundance of *Bacillus coagulans* from baseline. Dots represent individual subjects. Black diamonds indicate mean. The Wilcoxon rank-sum test was used to compare the difference between the two groups. $^*p < 0.05$. BC, *Bacillus coagulans* SNZ 1969; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and its decedent genus *Cloacibacillus* ($p = 0.047$) were the dominant bacteria differentially diminished in the BC group. Similarly, a previous study of patients with irritable bowel syndrome found higher relative abundances of Synergistetes (phylum), Synergistia (class), Synergistaceae (family), and *Cloacibacillus* (genus) compared with the healthy subjects (Ng et al., 2013).

As the bacterial V3 − V4 region of the 16S rRNA gene has a limited resolution to identify bacterial species, we made a concerted effort to confirm the presence of *B. coagulans* species in feces. As expected, the relative abundance of BC in the treatment group (1.54 ± 3.07) was significantly increased compared with the placebo group (0.28 ± 1.50), as expressed by the log_2-fold changes ($p = 0.018$) (Fig. 3**B**). Taken together, we could conclude that spore-forming bacterium BC effectively influenced to stabilize the dysbiotic condition in constipation by enriching Bacillales and Lactobacillales and diminishing Synergistales when delivered via the oral − intestinal route.

3.4. Relationship between dominant microbes and key clinical outcome variables

The role of gastrointestinal microbiota in modulating gut motility has been highlighted using germ-free mice (Abrams & Bishop, 1967). This study used multivariate RDA to search the relevance between the differential microbial species and intestinal motility/perception. The RDA graph showing these correlations is presented in Fig. 4**A**. The first and second RDA axes explained 74.8% and 19.6% of the variance,

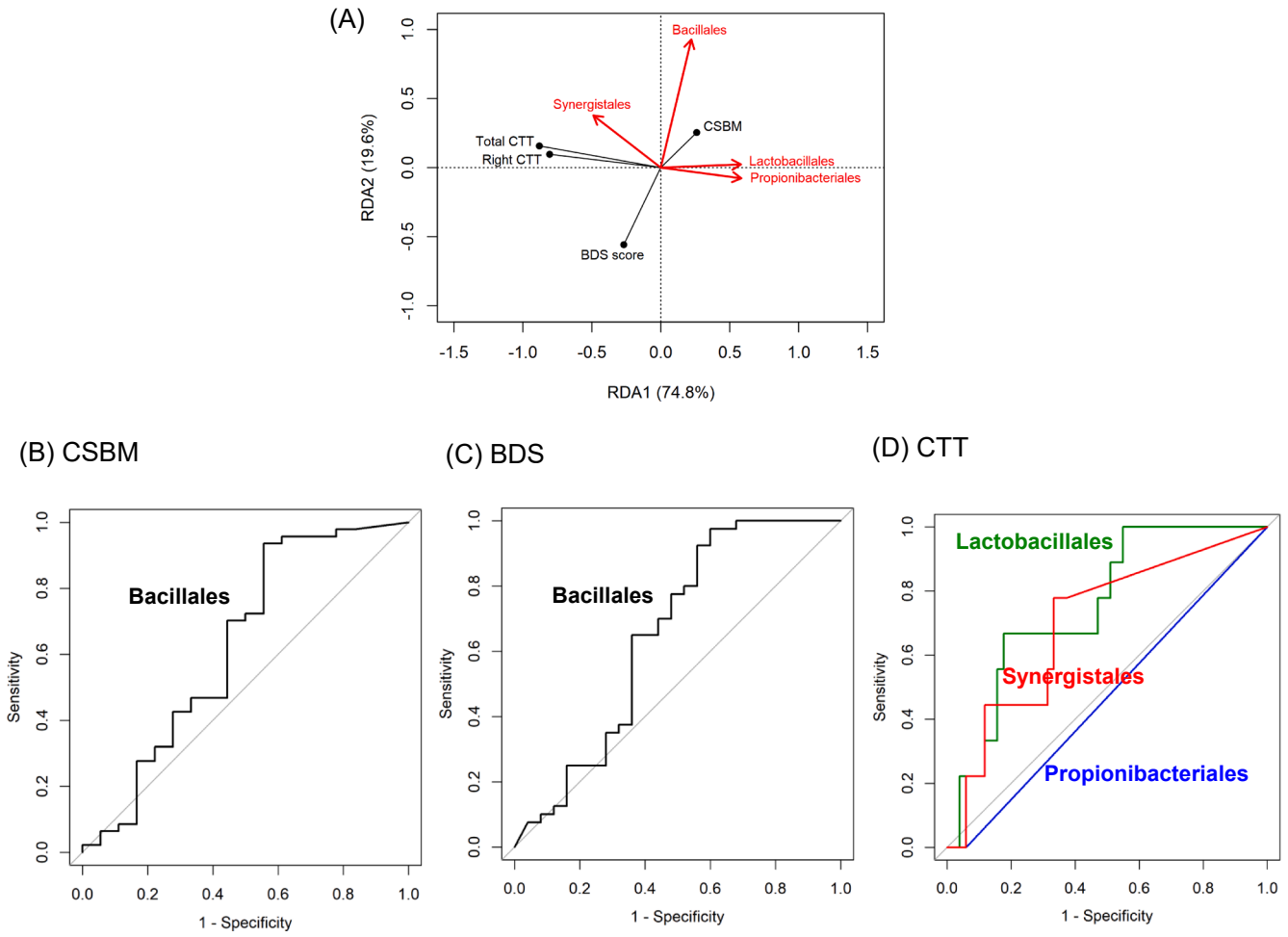

Fig. 4. Association and its validation between differential microbiota and the clinical outcome changed by BC supplementation. (**A**) RDA plot illustrating the relationships between the abundant microbiota at the order level and clinical outcome. Red arrows indicate abundant taxa. Black dots and lines indicate clinical outcomes. ROC curves for (**B**) CSBM predicted by order Bacillales. (**C**) BDS score CSBM predicted by order Bacillales. (**D**) CTT predicted by order Lactobacillales (green), order Synergistales (red), and order Propionibacteriales (blue). RDA, Redundancy analysis; ROC, receiver operating characteristic; BDS, bowel discomfort symptom; CSBM, complete spontaneous bowel movement; CTT, colonic transit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. The angle between the variables in the RDA plot represents their correlation. The smaller angle indicates the closer correlation; an acute angle denotes a positive correlation; an obtuse angle signifies a negative correlation; and a right angle indicates no correlation (Legendre & Legendre, 2012). Based on the RDA graph, we could conclude that members of the order Bacillales are dominant microbes closely related to the bowel function perception, showing a positive correlation with the CSBM and a negative correlation with the BDS score. The members of the orders Lactobacillales and Propionibacteriales are the dominant microbes that exhibited negative correlations with CTT. Conversely, the order Synergistales was positively correlated with CTT.

Although RDA gives an interesting approximation of correlation between explanatory and response variables, some approximation can be inaccurate. Hence, we performed ROC analysis to validate the above differential microbiota's association with the clinical outcomes. Bacillales was related to CSBM and BDS, with an area under the curve (AUC) of 0.63 (sensitivity 93.6% and specificity 44.4%, Fig. 4**B**), and 0.65 (sensitivity 97.5% and specificity 40.0%, Fig. 4**C**), respectively. The AUCs for Lactobacillales, Synergistales, and Propionibacteriales with total CTT were 0.75 (sensitivity 66.7% and specificity 82.4%), 0.70 (sensitivity 77.8% and specificity 66.7%), and 0.47 (sensitivity 0.0% and specificity 0.0%), respectively (Fig. 4**D**). The area below the ROC curve (AUC) results are considered failed if AUC values are between 0.5 and 0.6 and fair if AUC values are between 0.6 and 0.8 (Mandrekar, 2010). Therefore, our results confirmed an acceptable association between Lactobacillales/Synergistales and CTT, concluding that BC-induced CTT improvement might be mediated by the enrichment of Lactobacillales and diminishment of Synergistales.

It is worth noting that this study has some limitations. Although this study was carefully designed as a randomized, double-blind trial with a 2-week run-in period, we still observed a pronounced placebo response. Patel et al. (Patel et al., 2005) suggested that more stringent entry criteria and an increased number of office visits are required to decrease the placebo response. Next, BC-induced gut microbiota alteration may affect host metabolism, and circulating metabolites can exert intermediary effects on host biology (Wu et al., 2020). Therefore, a combination of metabolome and microbiome may be a promising strategy for a better understanding of the probiotics intervention at the molecular level (Lv et al., 2020). However, in this study, we did not include metabolomics analysis for detecting serum metabolites. Future studies are needed to provide detailed information on the interactions between host metabolism and gut microbiota.

4. Conclusion

The discovery of the relationships between the vital microbiota

composition and clinical outcomes provided an understanding of the underlying mechanisms. The results obtained in this study indicated that *B. coagulans* SNZ 1969, a spore-forming probiotic strain, reaches the colon intact and ameliorates intestinal motility and gut microbiota composition compared with the placebo group. The following multivariate redundancy analysis and receiver operating characteristic validation concluded that *B. coagulans* SNZ 1969 effectively improved intestinal motility by enhancing Lactobacillales and diminishing Synergistales. This study is the first to report the interplay between the gut microbiome and bowel movement/perception when *B. coagulans* SNZ 1969 were delivered via the oral-intestinal route in adults with both mild intermittent constipation and a habitual low intake of fruit and vegetables.

CRediT authorship contribution statement

Seunghee Kang: Formal analysis, Writing - original draft, Visualization. **Min Young Park:** Formal analysis, Writing - original draft, Visualization. **Isabel Brooks:** Writing - original draft. **Jaekyung Lee:** Conceptualization, Methodology, Investigation. **Su Hwan Kim:** Conceptualization, Methodology, Investigation. **Ji Yeon Kim:** Conceptualization, Methodology. **Bumjo Oh:** Conceptualization, Methodology, Investigation. **Ji Won Kim:** Conceptualization, Methodology, Project administration. **Oran Kwon:** Conceptualization, Methodology, Writing - original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110428.

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