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Full Paper

# Silk-derived sericin/fibroin mixture drink fermented with plant-derived *Lactococcus lactis* BM32-1 improves constipation and related microbiota: a randomized, double-blind, and placebo-controlled clinical trial

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**We previously showed through clinical trials that one plant-derived lactic acid bacteria (LAB) can improve constipation. We preliminarily found that the plant-derived LAB *Lactococcus lactis* BM32-1 can grow in a mixture of sericin and fibroin, which are extracted from silk and have been reported to help promote health. Thus, in the present study, we evaluated the favorable effect of a sericin/fibroin mixture (S/F-M), which was extracted from silk prepared from cocoons reared in an aseptic rearing system using an artificial diet, fermented with the BM32-1 strain through a clinical trial. The trial was conducted at Hiroshima University from June to October 2022 as a double-blind, placebo-controlled, randomized parallel-group comparative study with 50 eligible subjects (aged 23–71) who had an average defecation frequency of less than 5 times per week. The subjects were instructed to drink 100 mL of fermented S/F-M or placebo every day. After the 12 weeks of the clinical trial period, the average defecation frequency increased significantly—1.4 times higher than that at baseline in the test group—as compared with the placebo group. Furthermore, the fecal microbiota was also compared before and after treatment, revealing that intake of the fermented S/F-M significantly multiplied the relative abundance of the genera *Enterococcus* and *Clostridium*, which have been reported to contribute to the amelioration of constipation by improving the gut microbiota and producing butyric acid, respectively. In conclusion, the S/F-M fermented using the BM32-1 strain improves defecation frequency through alteration of the gut microbiota.**

**Key words: constipation, sericin, fibroin, lactic acid bacteria, clinical trial**

## INTRODUCTION

Sericulture, the rearing of silkworms (*Bombyx mori*) to produce silk, is an important industry in many countries that can be traced back at least thousands of years [1, 2]. In modern days, in addition to manufacturing silk clothing, genetic engineering of the silkworm has led to great achievements in the development of pharmaceutical and medical applications, such as the production of recombinant human proteins, enzyme drugs, and vaccines [3–6] Reportedly, silkworms have also been farmed and used as edible insects around the world [7]. As compared with the breeding of domestic animals, insect farming has advantages for environmental sustainability, such as saving water resources and greenhouse gas emissions [8]. Furthermore, due to the growing demand for meat caused by the increase in global population and rapid climate change, attention has been paid to edible insects as alternative protein and fat sources [9–11].

Raw silk is a protein biopolymer that mainly consists of two types of silk protein, fibroin and sericin, as the fiber and glue, respectively [12]. While silk fibroin has been used in textiles and surgical sutures as a natural fibrous protein, silk sericin has been largely removed from the fibroin to improve the quality of the fibers and discarded in wastewater during the scouring process [13–15]. In recent years, however, the useful characteristics of sericin have attracted attention for cosmetic and biotechnological applications [15–18]. Sericin also has been reported to be resistant to proteases; therefore, it is considered to be a resistant protein, which is defined as the remnants of proteins no longer

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digestible in the gut [19, 20]. Resistant protein exerts health-promoting properties similar to those of dietary fibers. In fact, an improvement in constipation and modulation of the intestinal immune and barrier functions have been reported with sericin intake [20, 21]. In addition, pharmacological effects of fibroin have also been reported in wound healing, skin regeneration, and inflammatory colitis [22, 23]. Thus, we have aimed to develop a new application for these interesting silk proteins for preparing health-promoting foods in combination with probiotic lactic acid bacteria (LAB), which have also been reported to benefit gastrointestinal conditions, including improving constipation and the gut microbiota [24, 25].

The generic name LAB is given to generally non-pathogenic and Gram-positive bacteria that produce large amounts of lactic acid during homo- or heterofermentative metabolism and are recognized as probiotics beneficial for human health [24–27]. We have isolated more than 1,200 LAB strains from plants, because plant-derived strains have the advantage of being resistant to digestive fluid in the gastrointestinal tract as compared with dairy strains [28]. Therefore, screening and characterizing the various plant-derived LAB strains will contribute significantly to the discovery of excellent probiotic activities. Indeed, our previous clinical studies have revealed that some strains are useful for preventive medicine [29–33]. In one of our studies, one of the isolates, the banana leaf-derived LAB strain named SN13T, was found to improve constipation and liver function [32]. We have also shown that intake of the SN13T strain alters the composition of the gut microbiota while improving liver function in subjects with mild liver dysfunction [33]. Interestingly, we found in a preliminary that a plant-derived LAB, *Lactococcus lactis* BM32-1, can grow in a protein mixture of sericin and fibroin extracted from silk, named sericin/fibroin mixture (S/F-M; unpublished data). In the present study, therefore, we conducted a clinical study involving subjects with an average defecation frequency of less than 5 times per week with the aim of showing whether S/F-M fermented using the BM32-1 strain can improve constipation and the gut microbiota.

## MATERIALS AND METHODS

### *Bacterial strain and culture conditions*

The BM32-1 strain was isolated in our laboratory from a flower of *Euonymus sieboldianus* var. *sieboldianus*. Briefly, pieces of the flower (about 1 $cm^2$) were suspended in a de Man, Rogosa, and Sharpe (MRS) medium (Merck KGaA, Darmstadt, Germany) and cultured at 28°C. After 2–3 days of cultivation, an aliquot of the culture broth was spread on a fresh MRS agar plate to generate single colonies. After colony purification, the productivity of organic acid and catalase-negative properties in each colony were checked, and then the LAB candidates were identified by 16S rRNA-encoding gene sequencing.

### *Chromosomal DNA preparation and LAB strain identification*

Chromosomal DNA from LAB was extracted as in our previous study, with slight modifications [34]. Briefly, the LAB cells were collected by centrifugation from the culture broth and washed twice with a glucose-ethylenediaminetetraacetic acid (EDTA) buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) containing 100 µg/mL ribonuclease A (Nacalai Tesque, Kyoto, Japan). The cells were resuspended in the same buffer supplemented with 4.5 mg/mL lysozyme (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 0.45 mg/mL Achromopeptidase (FUJIFILM Wako Pure Chemical Corporation) and then incubated at 28°C for 1 hr. The cells were lysed by adding a 0.1 volume of 10% (w/v) sodium dodecyl sulfate (SDS), and a 0.25 volume of 5 M sodium perchlorate was added to the lysate. The denatured protein was removed by the chloroform/isoamyl alcohol (24/1) extraction method, and the chromosomal DNA was obtained by polyethylene glycol-induced precipitation.

To identify the BM32-1 strain, its entire 16S rRNA-encoding gene sequence was determined as described previously [35–37] with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The obtained nucleotide sequence was analyzed using the ATGC and GENETYX software (GENETYX Corporation, Tokyo, Japan) and compared with that of typical LAB registered in the non-redundant database provided by the National Center for Biotechnology Information (NCBI) using the BLAST algorithm-based homology search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [38].

### *Acute toxicity and mutagenicity tests*

An acute oral toxicity test was performed by Japan Food Research Laboratories (Tokyo, Japan) according to the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (Guideline 420, 2001). Ten Slc:Wistar/ST male rats (5 weeks old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were divided into two groups of five rats each and housed in polycarbonate cages. One group was assigned to be a reference group and the other an LAB-fed group. All rats were maintained with free access to drinking water and a Labo MR stock diet (Nosan Co., Yokohama, Japan) under conditions of 23 ± 3°C and a 12 hr light–dark cycle. The experimental protocol was approved by the ethics committee established in the company.

After a 1 week acclimation period, a 50 mL/kg dose of the BM32-1 cell suspension prepared in 15% (w/v) skim milk ($2 \times 10^{10}$ cells/mL) was administered to the LAB-fed group of rats using a sterile stomach tube. Instead of the cell suspension, 15% (w/v) skim milk without cells was administered in the reference group. During the experiment, the activity, behavior, and health status of the rats were recorded every day, and their body weights were measured on days 1, 7, and 14. After the experimental period, the rats were euthanized, and some extracted organs were analyzed histologically. A Student's or Welch's t-test was used to analyze the body weight difference among rats using IBM SPSS Statistics 22 (IBM Japan, Tokyo, Japan). The same research was carried out using female rats.

The mutagenicity test (*umu* test) [39] of the BM32-1 culture broth was performed using an Umulac AT-F kit (Protein Purify Co., Ltd., Gunma, Japan) according to the manufacturer's instructions in the presence and absence of the S9 mixture. The enzyme mixture was prepared from rat liver homogenate, and it has been used to confirm the mutagenic potential of not only chemical substances but also their metabolites [40].

 

### *Subjects*

Through a series of advertisements, healthy volunteers between 23 and 71 years of age were recruited in Hiroshima, Japan, and its surrounding areas. The inclusion criteria for subjects were that they were healthy males or females aged 20–74 years with an average defecation frequency of less than 5 times per week. The exclusion criteria were as follows: subjects who (1) had allergic hypersensitivity to silk, apples, or dairy products; (2) took medicine for a chronic disease; (3) consumed foods or supplements containing silk protein; (4) were predicted to be sick after screening; (5) used supplements or functional foods that may affect defecation frequency and the fecal microbiota; (6) were pregnant or breastfeeding; or (7) had taken part in other clinical trials within 3 months of the commencement of the present trial. Before the start of the trial, informed written consent was obtained from each subject for the use of their clinical samples and data in research.

### *Test and placebo drinks*

The test drink used in this clinical study was a mixture of fermented S/F-M, taste additives (apple juice and oligosaccharides), and additives for flavoring, and the drink was prepared to contain at least 142 mg and 511 mg of sericin and fibroin, respectively, and approximately $2 \times 10^{10}$ live cells of the BM32-1 strain (in 100 mL). The sericin and fibroin mixture was prepared from green cocoons of a new silkworm race called "Ryokken 2 gou", which produces green cocoons with higher flavonoid levels, reared by an aseptic rearing system using an artificial diet [41].

The fermented S/F-M was prepared by BM32-1 cultivation of the sericin and fibroin mixture supplemented with pineapple juice, yeast extract, and skim milk, as follows: for pre-seed cultivation, a portion of a frozen stock of the BM32-1 strain was inoculated into an MRS broth and incubated without agitation at 28°C for 12 hr. The cells were collected by centrifugation and washed with phosphate-buffered saline (PBS; pH 7.4). The cell suspension was inoculated at 1% (v/v) into heat-treated (at 100°C for 10 min) 20% (w/v) pineapple juice supplemented with 1% (w/v) yeast extract and 5% (w/v) sodium hydrogen carbonate to prepare seed culture, followed by incubation at 28°C for 12 hr without agitation. For the preparation of fermented S/F-M, 50 L of S/F-M was prepared to contain 0.5% (w/v) sericin and 1.8% (w/v) fibroin supplemented with 1% (w/v) pineapple juice (5 times concentrated), 1% (w/v) yeast extract, and 1.1% (w/v) skim milk in a 100 L jar-fermenter. After heat treatment of the mixture at 100°C for 10 min, the above-mentioned seed culture was inoculated at 1% (v/v) into the S/F-M and incubated with agitation (42 rpm) at 28°C for 48 hr. The fermented S/F-M (final pH 3.2) was then mixed with heat-treated (at 100°C for 10 min) additives as shown in Supplementary Table 1. For the placebo drink preparation, a final concentration of 1.52% (w/v) maltodextrin solution was used instead of the sericin and fibroin mixture, and the BM32-1 strain was not inoculated for the placebo preparation. Both drinks were produced by Kimono Brain Co., Ltd. (Niigata, Japan).

### *Study design*

This clinical trial was conducted from June 2022 to October 2022 at Hiroshima University (Hiroshima, Japan) using a double-blind, randomized, placebo-controlled parallel-group study. Eligible subjects were enrolled and stratified by sex (male or female) and then assigned to the test or placebo group using the blocked randomization method. For assignment, a random allocation table was generated with the Microsoft Excel software in a 1:1 allocation ratio with a block size of 2–6. The randomization assignment was carried out by only non-clinical staff who were not involved in the analysis of the present trial; therefore, the subjects and analytical staff were blinded to the assignment information.

All through the 12-week study period, subjects were instructed to maintain their ordinary life and eating habits as much as possible and not to donate blood. They were also directed to consume a bottle (100 mL) of either the test or placebo drink daily at any time during the day for 12 weeks. Daily dated diary record forms were provided to the subjects throughout the trial period to record the contents of their meals (for 3 days before each examination/measurement), their intake of the drinks, their health conditions, and their medication. They were also asked to fill out questionnaires on defecation frequency; feces form, amount, and odor; and feeling of incomplete evacuation (Tables 1 and 2). After recruitment, the subjects were also directed to record the same information in the diary for 2 weeks before the start of the intake period, and then based on those records, the baselines for each questionnaire were calculated. The subjects were also instructed to visit Hiroshima University for a physical examination, biochemical measurements, and urinalysis every 4 weeks.

The present trial's protocol was approved by the Ethics Committee of Hiroshima University (approval no. C2022-0001; date of approval: May 19, 2022) prior to advertisement, and it was performed according to the guidelines of the Helsinki Declaration. This study was also registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR; ID, UMIN000047859; date of registration, May 25, 2022).

### *Analyses of questionnaire data regarding feces*

The change in average defecation frequency (times/week) was set as the primary outcome parameter. Changes in feces form, amount, and odor and daily feeling of incomplete evacuation

**Table 1.** Chart for the Bristol Stool Form Scale (modified from Lewis *et al.* [42])

| Score | Description |
|---|---|
| 1 | Separate hard lumps, like nuts |
| 2 | Sausage-shaped but lumpy |
| 3 | Like a sausage or snake but with cracks on its surface |
| 4 | Like a sausage or snake, smooth and soft |
| 5 | Soft blobs with clear-cut edges |
| 6 | Fluffy pieces with ragged edges, a mushy stool |
| 7 | Watery, no solid pieces |

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**Table 2.** Questionnaire items for self-assessment and scoring characteristics

| Score | Items | |
|---|---|---|
| | Amount of feces | Odor of feces |
| 1 | Extra large | Very weak |
| 2 | Large | Weak |
| 3 | Medium | Medium |
| 4 | Small | Strong |
| 5 | Extra small | Very strong |

were set as secondary outcomes. These data were analyzed based on the responses to the questionnaires included in the daily record form. Forms of feces were scored individually in accordance with the Bristol Stool Form Scale, which classifies feces into 7 types based on their degree of form and hardness (Table 1) [42, 43]. The self-assessment questionnaire asked about the amount and odor of feces, as shown in Table 2. Weekly averages of each score were individually calculated for the evaluation.

### *Analyses of physical examination and biochemical measurements*

As secondary outcomes, changes in scores for skin texture and moisture, body mass index, body fat percentage, total serum immunoglobulin E (IgE), blood glucose, and serum liver function indices, specifically aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transpeptidase (γ-GTP), were also analyzed. Blood and urine samples were obtained after at least 9 hr of fasting and sent to SRL Inc. (Tokyo, Japan) for clinical biochemical measurements and urinalysis, respectively. Skin texture and moisture were measured using a portable skin measurement device (MSA Lite +, MORITEX Corporation, Saitama, Japan), according to the manufacturer's instruction. Body fat percentage was measured using a body composition analyzer (BC-118E, TANITA, Tokyo, Japan). The BC-118E analyzer first measures body weight and bioelectrical impedance and then calculates the body fat percentage via multiple regression analysis based on the correlation between data obtained from bioelectrical impedance analysis (BIA) and dual energy X-ray absorptiometry (DXA) methods [44]. Blood pressure was measured using a fully automatic sphygmomanometer (HBP-9020, OMRON, Kyoto, Japan) according to the Japanese Society of Hypertension Guidelines for the Management of Hypertension 2019 (JSH 2019) [45]. Newly emerged or worsened adverse events after intervention were evaluated when those grades shifted higher, according to the Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v5.0).

### *Fecal microbiota analysis based on the 16S rRNA-encoding gene*

Fecal samples were collected within 3 days of the clinical visit (at weeks 0 and 12, respectively) before and after the intake period according to the manufacturer's instruction using a fecal collection tube (FS-0003, TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) filled with 4 mL of RNA*later* Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA). Fecal DNA extraction was performed according to the previously reported method using lytic enzymes, with some modifications [46, 47]. The collected feces were homogeneously suspended in the RNA*later* solution in the collection tube, and the suspension was filtered with a 100 μm mesh filter (Sysmex Partec GmbH, Goerlitz, Germany) to remove undigested food fragments. A 2 mL aliquot of the filtrate was centrifuged at 9,000 × g for 15 min at 4°C, and the pellet was resuspended and washed with 5 mL of PBS (pH 7.4). After additional washing with a TE10 buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) under the same conditions as above, the pellet was resuspended in 650 μL of a TE10 buffer containing 23 mg/mL lysozyme (from chicken egg white, Sigma-Aldrich, St. Louis, MO, USA), and the suspension was incubated at 37°C. After reaction for 1 hr, 40 μL of Achromopeptidase solution (FUJIFILM Wako Pure Chemical Corporation, 50 U/μL stock prepared in a TE10 buffer) was added and further incubated for 30 min. The cells were lysed by adding 100 μL of 10% (w/v) SDS solution and 10 μL of proteinase K solution (FUJIFILM Wako Pure Chemical Corporation, 400 U/mL, recombinant) and incubated at 55°C for 1 hr. Phenol/chloroform/isoamyl alcohol (25/24/1) extraction was performed to remove the denatured protein, and a pellet of DNA was obtained by ethanol precipitation. The obtained DNA sample was further purified by ribonuclease treatment (RNase A, Nippon Gene Co., Ltd., Tokyo, Japan) followed by ethanol precipitation.

The 16S rRNA-based microbiota analysis was done at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan) using the Illumina MiSeq sequence platform and MiSeq Reagent Kit v3 (Illumina Inc., San Diego, CA, USA) with a 300 bp read length paired-end method according to the protocol described previously [48]. Briefly, the V3–V4 region of 16S rRNA-encoding genes was amplified, and the resultant fragments were purified and further used as a template for the second PCR reaction with the primer pair 5′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-ACACTCTTTCCCTACACGACGC-3′ and 5′-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTGACTGGAGTTCAGACGTGTG-3′ under the following conditions (the Xs in each primer are index sequences designed to identify each sample in the analysis system). The amplified fragments were purified, followed by analysis by the analytical instrument. The taxonomic assignments of the obtained data were determined using the Quantitative Insights into Microbial Ecology (QIIME) 2.0 pipeline [49].

### *Statistical analyses*

In the present study, the sample size for each group required to detect a 30% difference with an estimated standard deviation (SD) of 3.4 times/week for the average defecation frequency was calculated to be 25 with a two-sample t-test using a power of 80% and a significance level of 0.05 (two-sided). The supposed difference and the SD for the sample size were calculated based on the data from our previous clinical trial [32]. When the obtained data, including values, were analyzed in accordance

 

with the intention-to-treat principle [50], twenty multiple-imputed data sets were generated. The resultant analyses were combined to compare each outcome. Welch's t-test [51] was applied to compare the baseline characteristics, and changes from the baseline in each outcome were examined by analysis of covariance (ANCOVA) using each baseline value as a covariate. Changes in the relative abundance of each item in the fecal microbiota were statistically analyzed using the Mann–Whitney *U* test and Wilcoxon's signed rank test for inter- and intra-group analyses, respectively. Fisher's exact test was used for adverse events to assess differences between the two groups. All statistical analyses were performed using IBM SPSS Statistics 17.0 J for Windows (IBM Japan, Tokyo, Japan).

## RESULTS

### *Identification of the plant-derived BM32-1 strain*

To screen LAB that can grow in the protein mixture of sericin and fibroin, some strains registered in our library were employed. Among them, a bacterium isolated from a flower of *Euonymus sieboldianus* var. *sieboldianus*, designated BM32-1, was confirmed to be usable for fermenting the protein mixture. Based on an entire 16S rRNA-encoding gene sequence analysis, the BM32-1 strain was identified as *L. lactis*.

### *Safety evaluations of the BM32-1 strain*

An acute oral toxicity test using the BM32-1 strain showed no significant changes in activity, behavior, health status, or intake-related illness or death in the tested animals. Furthermore, the *umu* test revealed that the BM32-1 strain does not have a mutagenicity risk regardless of the presence or absence of S9 mixture.

### *Recruitment and baseline characteristics of the subjects*

Figure 1 shows a flow diagram for the subjects in the present study. Among 133 applicants who expressed interest in participating in the trial, 50 eligible subjects (aged 23–71) satisfied the criteria for enrollment and were enrolled in the study after an explanatory meeting and screening. The subjects were randomly assigned to the test or the placebo group.

The baseline characteristics of the eligible subjects are summarized in Table 3. There were no significant differences in the listed items between the two groups. During the trial period, although some subjects were absent on the day of the clinical visit because they were ill (attendance rate was 97.0%), no subjects dropped out of the study; thus, all subjects completed the study. The minimum, maximum, average, and median confirmed compliance rates, including omissions in taking daily drinks, were 56.6, 100, 94.9, and 98.8% and 85.4, 100, 97.6, and 98.8% in the test and placebo groups, respectively. At the end of the period, each subject was asked which group (test or placebo) they

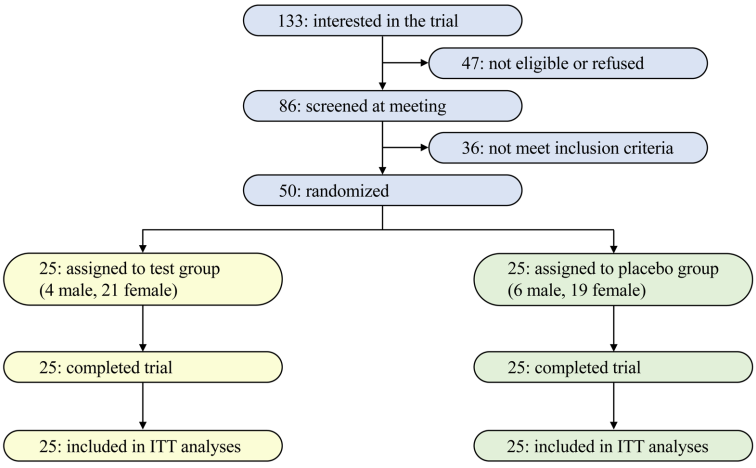


**Fig. 1.** Flow diagram of the subjects in the present trial.

**Table 3.** The baseline characteristics of the eligible subjects

| | Test (*n*=25) | Placebo (*n*=25) | p-value |
|---|---|---|---|
| Age (years) | 52.6 ± 13.9 | 54.3 ± 11.9 | 0.640 |
| Male | 68.0 ± 5.4 (*n*=4) | 60.7 ± 7.4 (*n*=6) | 0.108 |
| Female | 49.7 ± 13.1 (*n*=21) | 52.3 ± 12.5 (*n*=19) | 0.516 |
| Height (cm) | 159.4 ± 6.5 | 160.3 ± 8.6 | 0.666 |
| Body weight (kg) | 54.3 ± 10.5 | 57.6 ± 16.2 | 0.386 |
| BMI (kg/m$^2$) | 21.3 ± 4.1 | 22.2 ± 5.0 | 0.530 |
| Average defecation frequency (times/week) | 4.2 ± 1.5 | 3.6 ± 1.9 | 0.214 |
| Systolic blood pressure (mmHg) | 107.8 ± 13.4 | 106.6 ± 16.5 | 0.776 |
| Diastolic blood pressure (mmHg) | 64.6 ± 10.4 | 62.5 ± 9.3 | 0.462 |

Data are indicated as mean ± standard deviation.
p-values are calculated using the Welch's t-test. BMI: body mass index.

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thought they had been assigned to, and the results revealed that 54.0% of the subjects were correct, indicating that blinding was successfully achieved.

### *Effect of the fermented S/F-M drink on primary and secondary outcomes*

While there were no statistically significant secondary outcomes, changes in the average defecation frequency as the primary outcome during the 12-week trial period were compared between the two groups (Table 4). There was only a modest increment (1.1 times the frequency at baseline) in the placebo group; conversely, a remarkable increase, which averaged 1.4 times that at baseline, was observed in the test group. Thus, there was a significant difference between the groups in the changes in the average defecation frequency (p=0.017).

Other changes related to skin conditions; feces characteristics with regard to the daily form, amount, and odor of feces; and daily feeling of incomplete evacuation are summarized in Table 5 as secondary outcomes. Based on the average fecal form score, calculated using the Bristol Stool Form Scale at baseline, the subjects were divided into two groups according to the Bristol scores of <4 (harder stools) and 4< (softer stools) and then analyzed separately. Interestingly, the individual Bristol scores of the subjects tended to move toward the middle score (=4) regardless of the drink; thus, there was no statistical significance with regard to the scores (p=0.705 and 0.518, respectively). Additionally, the percentages of daily feeling of incomplete evacuation in both groups decreased similarly during the trial period (p=0.847). Including other secondary outcomes, no statistical differences were observed in any of the secondary outcomes.

### *Monitoring adverse effects*

For the purpose of detecting the adverse effects of test and placebo drink intake, blood pressure and hematological and other serum parameters were also analyzed. Based on the CTCAE v5.0 criteria, no significant differences between the two groups were observed in adverse events with a possible relation to the study design or treatments (Table 6). In addition, although some subjects suffered from transient diarrhea during the study period, there were no differences in the number of subjects between the two groups. Among the items monitored throughout the study, the changes in average serum total protein, lactate dehydrogenase, and amylase in each group showed either statistical significance (p<0.05) or tendencies (p<0.1), although the changes were not abnormal (Supplementary Table 2).

### *Changes in the fecal microbiota*

To perform a statistical analysis, the obtained sequences from the fecal microbiota were annotated as 12, 21, 31, 53, and 100 phyla, classes, orders, families, and genera, respectively. There were significant differences in the changes in relative abundance for 13 taxa (Enterobacteriales at the order level; Porphyromonadaceae, Streptococcaceae, Enterococcaceae, Enterobacteriaceae, Peptococcaceae, and Gemellaceae at the family level; and *Parabacteroides*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Klebsiella*, and *Anaerotruncus* at the genus level) between the two groups after treatment. Among those taxa, Peptococcaceae, Gemellaceae, and *Klebsiella* were omitted from the analysis because they were found in less than half of the subjects (Fig. 2A). Among the remaining 10 taxa, the relative abundances of only Enterococcaceae, *Enterococcus*, and *Clostridium* significantly changed in the test group based on the intra-group analyses, whereas that of *Anaerotruncus* was found to be significantly altered in the placebo group (Fig. 2B). The changes in relative abundance observed in Enterococcaceae and *Enterococcus*, especially, were notable; on the other hand, their abundances seemed to be strikingly increased by intake of the test drink (p<0.001).

Among the annotatable species in the present study, there were significant inter-group differences in *Bacteroides eggerthii*, *Veillonella dispar*, *Clostridium cocleatum*, and *Parabacteroides distasonis*. However, the detection frequencies were quite low (only less than one-quarter) for *B. eggerthii* and *V. dispar*; therefore, only the latter two species were analyzed. Further analyses revealed that the relative abundance of *C. cocleatum* was significantly increased (p<0.05) and that of *P. distasonis* tended to increase (p<0.1) in the test group after the intake period (Fig. 2A and 2B).

## DISCUSSION

Chronic constipation is a common symptom of bowel dysfunction that accompanies infrequent bowel habits and difficult stool passage. People with constipation have abdominal discomfort [52]. In general, the prevalence of constipation in adults is estimated to be approximately 15%. Constipation is often caused by eating a low-fiber diet with insufficient drinking water; thus, studies have considered its cause to be a failure in intestinal function. However, many studies have shown that the dysbiosis of the gut microbiota is of concern with constipation [53]. In particular, an increase of Bacteroidota (formerly Bacteroidetes) and decrease of bifidobacteria and lactobacilli are typical in people with constipation [54–56]. Because LAB have been reported to be effective in various disorders including

**Table 4.** Changes in average defecation frequency

| | Test (*n*=25) | Placebo (*n*=25) | p-value |
|---|---|---|---|
| Average defecation frequency (times/week) | | | |
| Baseline | 4.2 ± 0.3 | 3.6 ± 0.4 | |
| Change at 1–4 week | 0.87 ± 0.28 | 0.55 ± 0.29 | (0.242) |
| Change at 5–8 week | 1.2 ± 0.3 | 0.32 ± 0.29 | (0.011*) |
| Change at 9–12 week | 1.5 ± 0.4 | 0.37 ± 0.27 | 0.017* |

Data are indicated as mean ± standard error.
p-values are calculated by analysis of covariance (ANCOVA) using each baseline value as a covariate.

 

**Table 5.** Changes in secondary outcomes

| | Test (*n*=25) | Placebo (*n*=25) | p-value |
|---|---|---|---|
| Bristol score (<4 at baseline) | (n=9) | (n=11) | 0.705 |
| Baseline | 2.2 ± 0.3 | 2.4 ± 0.3 | |
| Change at 9–12 week | 0.90 ± 0.34 | 0.88 ± 0.21 | |
| Bristol score (4< at baseline) | (n=13) | (n=9) | 0.518 |
| Baseline | 4.8 ± 0.2 | 4.5 ± 0.1 | |
| Change at 9–12 week | −0.44 ± 0.16 | −0.21 ± 0.07 | |
| Daily feeling of incomplete evacuation (%) | | | 0.847 |
| Baseline | 37.9 ± 6.6 | 39.1 ± 8.6 | |
| Change at 9–12 week | −7.5 ± 6.2 | −9.4 ± 4.7 | |
| Amount of feces (score) | | | 0.231 |
| Baseline | 3.49 ± 0.10 | 3.63 ± 0.15 | |
| Change at 9–12 week | −0.08 ± 0.11 | −0.02 ± 0.13 | |
| Odor of feces (score) | | | 0.821 |
| Baseline | 3.04 ± 0.08 | 3.05 ± 0.12 | |
| Change at 9–12 week | −0.14 ± 0.07 | −0.19 ± 0.07 | |
| Skin texture (score) | | | 0.502 |
| Baseline | 26.4 ± 2.0 | 33.6 ± 2.4 | |
| Change at 12 week | 8.8 ± 2.2 | 5.1 ± 2.0 | |
| Skin moisture (score) | | | 0.622 |
| Baseline | 76.3 ± 1.0 | 76.5 ± 1.1 | |
| Change at 12 week | −17.6 ± 1.8 | −19.2 ± 1.8 | |
| Total serum IgE ($log_{10}$ IU/mL) | | | 0.557 |
| Baseline | 1.81 ± 0.14 | 1.84 ± 0.13 | |
| Change at 12 week | −0.011 ± 0.013 | 0.000013 ± 0.013 | |
| BMI ($kg/m^2$) | | | 0.150 |
| Baseline | 21.3 ± 0.8 | 22.2 ± 1.0 | |
| Change at 12 week | −0.03 ± 0.10 | −0.20 ± 0.08 | |
| Body fat percentage (%) | | | 0.879 |
| Baseline | 24.3 ± 1.8 | 23.8 ± 2.0 | |
| Change at 12 week | 1.08 ± 0.24 | 1.16 ± 0.31 | |
| Blood glucose (mg/dL) | | | 0.230 |
| Baseline | 96.2 ± 1.2 | 96.1 ± 2.0 | |
| Change at 12 week | 1.2 ± 0.8 | −0.8 ± 1.4 | |
| AST (U/L) | | | 0.155 |
| Baseline | 20.0 ± 0.9 | 22.4 ± 1.4 | |
| Change at 12 week | −0.79 ± 0.80 | −0.2 ± 1.0 | |
| ALT (U/L) | | | 0.794 |
| Baseline | 17.1 ± 1.0 | 19.7 ± 2.3 | |
| Change at 12 week | 0.2 ± 1.1 | −0.2 ± 1.6 | |
| γ-GTP (U/L) | | | 0.128 |
| Baseline | 22.0 ± 2.3 | 22.0 ± 2.9 | |
| Change at 12 week | 1.7 ± 1.2 | −0.8 ± 1.8 | |

Data are indicated as mean ± standard error.
p-values are calculated by analysis of covariance (ANCOVA) using each baseline value as a covariate.
BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase.

constipation, some LAB are expected to manage constipation by affecting the gut microbiota as probiotics [57]. In the present trial, it was revealed that the 12 weeks of intake of the fermented S/F-M drink containing the living plant-derived LAB *L. lactis* BM32-1 significantly improves constipation by modifying the fecal microbiota. Although there was also a certain effect on defecation frequency in the test group after 4 weeks during the intake period, no statistical significance was observed compared with the placebo group (p=0.242). The observed transient effect on the frequency in the placebo group may be due to the psychological placebo effect in a short period. In addition, Table 4 also shows changes in the defecation frequency at intervals of 4 weeks, indicating that the improvement in defecation with the test drink showed a temporal increase, whereas only a few increases were observed in the placebo group during each period. The slight increases were probably due to the apple juice and oligosaccharides added to enhance the taste, as they may act as dietary fiber and prebiotics [58], respectively. In contrast to defecation, changes in other questionnaire-related feces characteristics were similar in both groups, indicating that those items may have no connection to constipation improvement.

Although constipation is reported to be associated with skin conditions and obesity [59–61], no noticeable effects were observed on related measures as secondary outcomes (Table 5).

**Table 6.** Number and ratio of subjects showing adverse events possibly related to the study design or treatments

| | Test (*n*=25) | Placebo (*n*=25) | p-value |
|---|---|---|---|
| ALT increased | | | 1.000 |
| Grade 1 | 0 | 1 (4%) | |
| Anemia | | | 0.541 |
| Grade 1 | 2 (8%) | 4 (16%) | |
| Grade 2 | 1 (4%) | 0 | |
| Blood bilirubin increased | | | 1.000 |
| Grade 1 | 1 (4%) | 1 (4%) | |
| Blood lactate dehydrogenase increased | | | 1.000 |
| Grade 1 | 1 (4%) | 1 (4%) | |
| Cholesterol high | | | 0.561 |
| Grade 1 | 8 (32%) | 11 (44%) | |
| Creatinine increased | | | 1.000 |
| Grade 1 | 0 | 1 (4%) | |
| Diarrhea | | | 1.000 |
| Grade 1 | 3 (12%) | 3 (12%) | |
| Hemoglobin increased | | | 1.000 |
| Grade 1 | 1 (4%) | 1 (4%) | |
| Hyperglycemia | | | 1.000 |
| Grade 1 | 0 | 1 (4%) | |
| Hypertension | | | 1.000 |
| Grade 1 | 1 (4%) | 0 | |
| Hypertriglyceridemia | | | 1.000 |
| Grade 1 | 2 (8%) | 2 (8%) | |
| Hyperuricemia | | | 0.490 |
| Grade 1 | 0 | 2 (8%) | |
| Hypotension | | | 1.000 |
| Grade 1 | 10 (40%) | 9 (36%) | |
| Serum amylase increased | | | 0.490 |
| Grade 1 | 0 | 2 (8%) | |
| Grade 2 | 1 (4%) | 0 | |

p-values are calculated by Fisher's exact test. ALT: alanine aminotransferase.

There were also no statistical differences or tendencies regarding hepatic parameters and IgE, which were expected to be improved based on previous reports [29, 62]. It may take a longer treatment period to improve them, or this may reflect differences in strain-specific health-promoting effects.

Only the changes in the relative abundances of *Enterococcus* (same as Enterococcaceae), *Clostridium*, *P. distasonis*, and *C. cocleatum* showed statistical significance or tendencies before and after treatment in the test group (Fig. 2). It is unclear why the microbiota were altered by intake of the test drink. The following are two possibilities: (1) live BM32-1 cells enhance the growth of those bacteria through the establishment of symbiosis (the probiotic effect), or (2) the fermented S/F-M contains substances promoting the growth of bacteria (the prebiotic effect). There have been few reports about the effects of sericin and fibroin on the microbiota. Okazaki et al. reported that consumption of sericin as a resistant protein improves constipation in model rats without differences in the profile of cecal microflora between sericin-fed and non-fed groups [21]. Another report showed that intake of acid hydrolyzed sericin and fibroin peptides ameliorates hyperglycemia in type 2 diabetes model rats with dose-dependent changes in the intestinal microbiota [63]. However, the microbial alteration might be due to the alleviation of hyperglycemia by improving insulin sensitivity rather than the prebiotic effect of the peptides. In fact, anti-diabetic treatments have been reported to recover dysbiosis in the gut microbiome [63, 64]. Sericin can retain water as well as other dietary fibers, resulting in a raised fecal water content; thus, its moisture retention may be responsible for the improvement of constipation [20, 65]. Judging from those results, both of the silk-derived proteins contained in the test drink are surmised to affect defecation conditions without modifying the intestinal microbiota, which may be caused by the BM32-1 cells or their metabolites.

Taxonomically, enterococci are non-sporulating, catalase-negative, and facultative anaerobic Gram-positive cocci that are natural components of the human microbiota [66]. The genus *Enterococcus*, which was separately classified in 1984, represents one of the main genera belonging to LAB, and some *Enterococcus* species have traditionally been used in the treatment of not only intestinal disorders but also various diseases as probiotic therapies since at least the 1950s [67]. The purpose of using probiotic strains is to improve the composition of the gut microbiota [68, 69], and some enterococcal strains have been shown to improve constipation in an animal model and human subjects [70–72]. Although a number of potential virulence and antibiotic resistance traits of enterococci that are not negligible have been reported to date, selected beneficial strains are applied to therapies and health-promoting foods or supplements, obligating us to evaluate whether the strains are safe for the application. In particular, our result showed that the increment

 

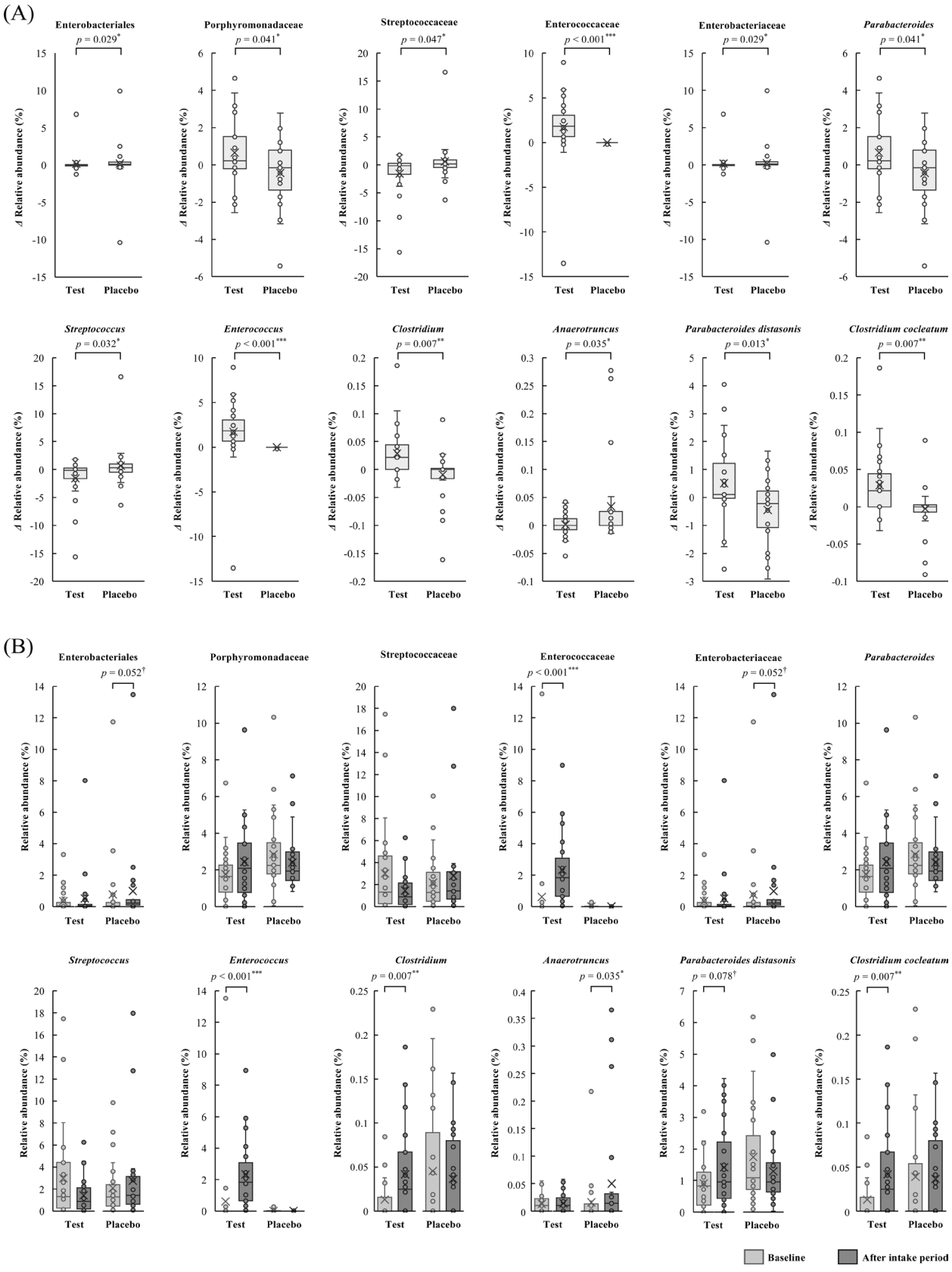


**Fig. 2.** Statistical analysis of the fecal microbiota before and after the intake period.

(A) Changes in the relative abundance of each item from baseline were compared between the test ($n$=25) and placebo ($n$=25) groups. (B) The relative abundances before and after the intake period in each group were compared individually. Data are shown as a box plot with medians (lines inside boxes), means (cross marks), 25–75 percentiles (limits of boxes), whiskers indicating the 95% data range, and outliers indicated by circles. The statistical analyses were performed using the Mann–Whitney $U$ test and Wilcoxon's signed rank test for inter- (A) and intra-group (B) analyses, respectively. $^{\dagger}$p<0.1, *p<0.05, **p<0.01, ***p<0.001.

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in the relative abundance of *Enterococcus* in the test group was extraordinary, strongly indicating that the change is a crucial factor in improving constipation.

Clostridia are obligate anaerobic Gram-positive bacteria that also present as normal flora in the intestinal tract of mammals [72]. The genus *Clostridium* consists of approximately 180 species, a few of which are pathogenic, producing very harmful toxins. However, *Clostridium* species are also used in biotechnological and therapeutic applications [73–77]. Many clostridia have been reported to produce butyric acid—a short-chain fatty acid (SCFA)—as a major fermentation product. Butyric acid produced by intestinal bacteria, including clostridia, is suggested to be capable of stimulating serotonin production in the gut, resulting in reduction of the intestinal transit time and relief from constipation [78, 79]. Furthermore, due to the inhibitory effect of SCFAs on proinflammatory mediator activities in the intestinal epithelium, butyric acid could potentially reduce difficulties in defecation through reduced intestinal inflammation [80]. Although no reports directly show the relationship between defecation and *P. distasonis* and *C. cocleatum*, which significantly increased in the test group, interestingly, each species has been reported to contribute to the improvement/prevention of inflammatory enteritis and *Clostridioides difficile* infection, respectively [81, 82]. Additionally, silk fibroin peptide has been shown to improve inflammation through suppression of elevated levels of inflammatory cytokines and enhancement of superoxide dismutase [83]. On the other hand, sericin protein, which is known as one of the resistant proteins, has also been reported to play a critical role in inflammatory diseases, such as hyperglycemia, hyperlipidemia, obesity, Alzheimer's diseases, and cancer, in addition to type-2 diabetes [63, 84]. Judging from those facts, our results suggest that the S/F-M fermented using the BM32-1 strain can be expected to help people with not only defecation troubles but also intestinal inflammation through the combination of probiotic, prebiotic, and postbiotic effects [85].

## CONFLICT OF INTEREST

None.

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