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Sakulora™ (*Lacticaseibacillus paracasei* strain Shidare) improves bowel function: A randomized, double-blind, placebo-controlled trial

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ABSTRACT

Introduction and objective: Heat-killed lactic acid bacteria with postbiotic effects have emerged as promising functional food ingredients for promoting intestinal health. Sakulora™, which consists of heat-killed *Lacticaseibacillus paracasei* strain Shidare (strain Shidare) isolated from weeping cherry blossom, may exert postbiotic effects on the gut microbiota. However, its impact on the intestinal environment and intestinal mobility remains unclear. Therefore, the present study investigated the efficacy of Sakulora™ on intestinal serotonin and fecal IgA concentrations in mice and bowel function in healthy adults through a randomized, double-blind, placebo-controlled trial.

Methods: In the pre-clinical phase, mice were administered heat-killed strain Shidare, and intestinal serotonin and fecal IgA concentrations were assessed. Forty-four healthy adults with mild constipation were then randomly assigned to

receive either Sakulora™ (containing 50 billion cells of the heat-killed strain Shidare) or a placebo daily for 2 weeks. The frequency of bowel movements in one week was used as the primary outcome.

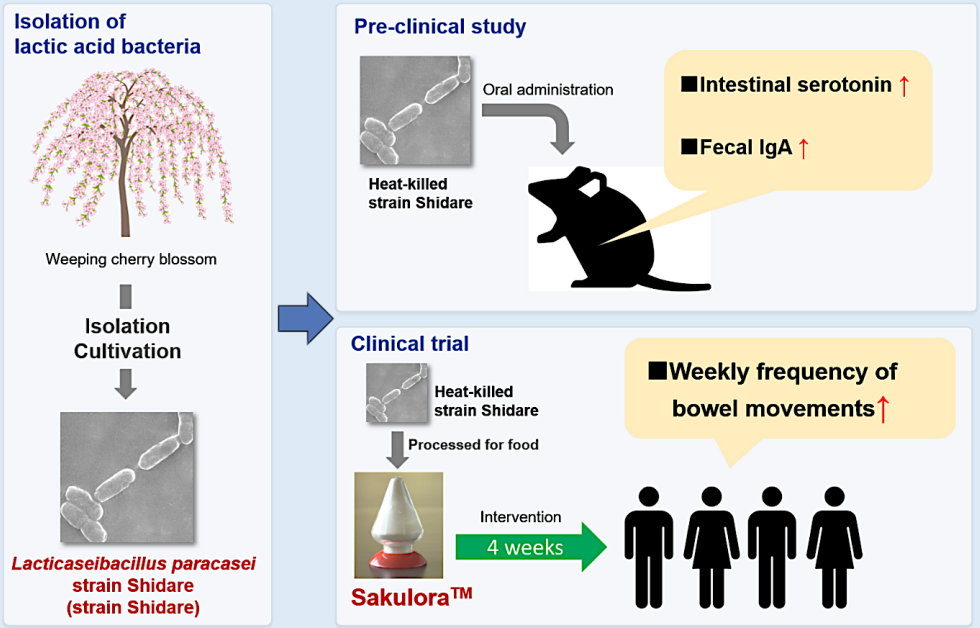
Results: In murine models, the administration of heat-killed strain Shidare significantly increased intestinal serotonin and fecal IgA concentrations. In the clinical trial, the weekly frequency of bowel movements was significantly higher in the Sakulora™ group than in the placebo group. No significant improvements were observed in the secondary endpoints, including the gut microbiota and fecal short-chain fatty acids. No serious adverse events developed throughout the study period.

Novelty of the Study: The bowel movement-improving effect of the heat-killed strain Shidare was demonstrated for the first time, and its mechanism was suggested to be an increase in intestinal serotonin and IgA levels.

Conclusions: Sakulora™ containing heat-killed strain Shidare effectively improved the bowel function, supporting its potential as a safe functional food ingredient for maintaining digestive health in healthy individuals.

Trial Registration: UMIN-CTR: UMIN000054746

Keywords: *Lacticaseibacillus paracasei*; postbiotics; bowel function; intestinal environment



Graphical Abstract: Sakulora™ (*Lacticaseibacillus paracasei* strain Shidare) improves bowel function

INTRODUCTION

Constipation is primarily attributed to stress, irregular lifestyle patterns, and insufficient physical exercise. Prolonged constipation leads to the accumulation of putrefactive and fermentation products in the colon, ultimately resulting in abdominal distension, abdominal pain, and reduced quality of life (QOL) due to stress. Previous studies reported that the intestinal flora in

patients with chronic constipation or irritable bowel syndrome (IBS) with constipation exhibited significant abnormalities (dysbiosis) from that in healthy individuals [1, 2]. Another study demonstrated that excessive gas production due to abnormal intestinal fermentation was a major cause of abdominal bloating and pain [3]. In addition, stress-related constipation reduces QOL [4, 5], and the impact of constipation on QOL may be equivalent to that of other common chronic diseases, such as allergies, musculoskeletal disorders, and inflammatory bowel disease [6]. The prevalence of functional constipation globally is estimated to be approximately 10-15% depending on symptom-based criteria (Rome I-IV) and markedly varies across different countries and regions. While this geographic heterogeneity suggests contributions from environmental, cultural, dietary, and genetic factors, functional constipation is a highly prevalent chronic condition that may affect any individual, and of clinical significance [7]. The attenuation of constipation by improving bowel movements not only normalizes bowel frequency and consistency but may also contribute to the recovery of the intestinal environment, including the balance of intestinal fermentation and gas dynamics, as well as overall QOL.

Improving the intestinal environment with probiotics or prebiotics is an effective strategy for the amelioration of constipation. Probiotics are defined as "live microorganisms that confer a beneficial effect on the host when consumed in adequate amounts" [8]. Lactic acid bacteria (LAB) and bifidobacteria are the most used probiotics. Previous studies demonstrated the beneficial effects of probiotic supplementation on bowel function. Yamakawa et al. [9] showed that 2-week administration of *Lactiplantibacillus plantarum* strain SN13T significantly increased defecation frequency. Similarly, Iwasaki et al. [10] found that consuming a beverage containing *Lacticaseibacillus casei* for 2 weeks improved the intestinal environment and facilitated bowel movements. Prebiotics, defined as "substrates that are selectively utilized by host microorganisms, conferring a health benefit" [11], represent another approach to modulating gut function, and inulin and fructooligosaccharides are well-characterized examples. The continuous intake of chicory inulin for 4 weeks improved bowel movements, while 8-week administration of inulin to IBS patients reduced symptom severity and frequency [12, 13]. Fructooligosaccharides increased the frequency of bowel movements when administered to healthy individuals for 2 weeks and to older adults with functional constipation for 4 weeks [14, 15]. In addition, polydextrose and Mulukhiyah (*Corchorus olitorius*)-derived dietary fiber have also been reported to improve bowel movements [16, 17]. On the other hand, recent evidence suggests that heat-killed LAB alleviate constipation as a postbiotic, which is defined as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [18]. Previous studies reported that heat-inactivated strains, including *Bifidobacterium longum* strain CLA8013, *L. paracasei* strain 327, and *Enterococcus faecalis* strain KH2, modulated gut microbiota composition and increased the frequency of bowel movements [19-21]. Collectively, these findings indicate that LAB act not only as live probiotics in regulating intestinal function, but also as dead bacteria in a postbiotic manner.

We successfully isolated a novel LAB from the petals of weeping cherry blossom (*Prunus pendula*) in Saitama Prefecture, Japan, and identified *L. paracasei* through molecular taxonomic analysis. This strain was subsequently designated *L. paracasei* strain Shidare (strain Shidare). *L. paracasei* strains have been shown to exert various beneficial effects, including improvements in bowel movements [22], enhanced resistance to common cold infections [23], and the amelioration of skin conditions [24]. Based on the established beneficial effects of *L. paracasei* species, we hypothesized that strain Shidare may possess similar health-promoting

properties. However, the functional activities of this strain have yet to be characterized. Therefore, we herein investigated the effects of strain Shidare on the intestinal environment and evaluated the efficacy of Sakulora™, a food-grade processed powder containing this strain, in improving bowel movements.

MATERIALS AND METHODS

L. paracasei strain Shidare: *L. paracasei* strain Shidare was isolated from weeping cherry blossom (*P. pendula*) petals growing in Saitama Prefecture, Japan, and was identified in a 16S rRNA gene sequence analysis (GenBank accession number: LC801630). The strain was deposited at the National Institute of Technology and Evaluation under the deposit number BP-04138.

Reagents: Radioimmunoprecipitation assay (RIPA) lysis buffer, a protease and phosphatase inhibitor cocktail, BCA protein assay kit, and IgA Mouse Uncoated ELISA kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). A Serotonin ELISA Kit was obtained from Abnova Corporation (Taipei, Taiwan).

Animals: Seven-week-old male BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were acclimated for 7 days under 22 ± 2℃ and 50% ± 5% R.H. and were fed a standard non-purified diet (CE-2, Clea Japan Inc., Shizuoka, Japan). Animal experiments were performed in accordance with the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987). All animal experiments were approved by the Ethics Committee of Oryza Oil & Fat Chemical Co., Ltd (Aichi, Japan).

Animal experiments: Heat-killed strain Shidare (5, 50, or 100 billion cells/kg) suspended in water was orally administered to mice once daily for 14 days, while water was given to mice as the control group. Feces were collected for 24 hr, from the 13th to the 14th day of administration, dried at room temperature for 24 hr and weighed. Feces were then immersed in RIPA lysis buffer for the quantification of IgA concentrations. On day 14, the colon was collected under isoflurane anesthesia and immersed in RIPA buffer for the quantification of serotonin concentrations. Feces and colons immersed in RIPA lysis buffer were stored at -80°C for later analyses.

Quantitative analysis of fecal IgA and intestinal serotonin concentrations: The collected feces and colons were homogenized using RIPA lysis buffer and TissueRuptor (Qiagen, Hilden, Germany). The concentrations of IgA in feces and serotonin in the colon were measured using the IgA Mouse Uncoated ELISA Kit and Serotonin ELISA Kit, respectively. The concentration of intestinal serotonin was corrected by the total protein content.

Clinical examination of bowel movements and the intestinal environment: Sakulora™: Sakulora™-P, a food-grade powder containing the heat-killed strain Shidare, was manufactured by Bio-Lab Co., Ltd. (Saitama, Japan) and YAEGAKI Biotechnology Inc. (Hyogo, Japan). The product composition was 33% heat-killed strain Shidare and 67% maltodextrin. Heat-killed strain Shidare was washed, and culture fluid was removed to manufacture powder. High-pressure homogenizer processing was performed to prevent bacterial aggregation and enhance dispersibility and absorbance [25]. Powder processing was performed by the spray drying method. The bacterial cell count was certified as 1.0×10^{12} cells/g by direct microscopy count.

Participants and grouping: All participants were recruited through the Go106 website (https://www.go106.jp/) operated by ORTHOMEDICO Co., Ltd. (Tokyo, Japan) between June 19 and June 26, 2024. Inclusion criteria were healthy Japanese adults of both sexes with 3 to 5 bowel movements during the 7 days prior to screening.

Exclusion criteria were as follows:

1) Currently undergoing treatment for or having a history of malignant tumors, heart failure, or myocardial infarction.

2) An implanted pacemaker or implantable cardioverter defibrillator.

3) Currently receiving treatment for the following chronic diseases:

arrhythmia, liver dysfunction, chronic kidney disease, cerebrovascular disease, rheumatic disease, diabetes, dyslipidemia, hypertension, or other chronic diseases.

4) Consuming foods for specified health uses and foods with functional claims.

5) Consuming yogurt, LAB beverages, natto, or kimchi at least four times a week.

6) Taking/using pharmaceuticals (including herbal medicines) or supplements.

7) Participants with allergies to pharmaceuticals, test products, or related foods.

8) Pregnant, breastfeeding, or intending to become pregnant during the trial period.

9) Participated in other clinical trials during the 28 days prior to obtaining consent or plans to participate during the trial period.

10) Other individuals deemed inappropriate for this trial by the principal investigator.

Participant screening was conducted based on daily bowel movement records in a stool diary during the week prior to the screening test. Selected test participants were asked to avoid excessive eating and drinking, maintain their usual lifestyle habits, and refrain from consuming LAB beverages and fermented foods (such as cheese, yogurt, kimchi, and natto) as much as possible from the date of consent to the final examination.

Test samples and allocation: The test samples were indistinguishable brown capsules containing Sakulora™ or a placebo, provided by Oryza Oil & Fat Chemical Co., Ltd. as hard capsules. The active capsules contained 50 mg of Sakulora™-P (containing 50 billion cells of strain Shidare) and 100 mg of maltodextrin. Placebo capsules contained 150 mg of maltodextrin.

Participants who met the inclusion criteria during the screening examination were assigned to either the active or placebo group according to the computer-generated allocation table by the allocation controller at ORTHOMEDICO Inc. The allocation ratio for each group was 1:1. The allocation table was generated using R, and the algorithm adopted block random allocation with random block sizes.

Oryza Oil & Fat Chemical Co., Ltd. provided the test samples with yellow or green markings on the packages. They strictly kept the sample information until the study period was over. ORTHOMEDICO Inc. created an allocation table and emergency keys based on the identification markers of the test samples. The emergency keys were placed in envelopes for each participant, sealed and stamped with the date of sealing. The allocation table and emergency keys were strictly managed by ORTHOMEDICO Inc. and were not disclosed to the organizer until the end of the test. After the trial ended and data had been finalized, ORTHOMEDICO Inc. confirmed that the allocation table and emergency keys were unopened, recorded the date of opening, and signed the allocation table. At this point, test sample identification numbers were revealed. It was necessary to allocate participants to ensure that there were no significant differences in the mean and standard deviation (SD) of the number of bowel movements in the 7 days prior to the screening test or age between the groups.

Study protocol and intervention: This randomized, placebo-controlled, double-blind, parallel-group study was conducted at Medical Corporation Seishinkai, Takara Clinic (Tokyo, Japan), and Nerima Medical Association Minami-machi Clinic (Tokyo, Japan), with statistical analyses being performed by ORTHOMEDICO Inc. The study protocol was registered in the University Hospital

Medical Information Network Clinical Trials Registry (UMIN000054746, https://center6.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000062555). The number of participants was set at the maximum number possible within our budget. Participants were instructed to consume one capsule daily following breakfast for a period of 2 weeks. In the event of a missed dose, participants were advised to take it upon remembering, ensuring the complete daily dosage was consumed within the same day, rather than delaying it to the next day.

The following items were examined during the 7 days prior to the screening test (period 1), from the start of the intervention to the 7th day (period 2), and from the 8th to the 14th day (period 3). Participants were asked to keep a daily bowel movement diary, recording the frequency of bowel movements, days with bowel movements, the amount of stool, stool shape, stool smell, and a feeling of relief after bowel movements. Among these, the actual frequency of bowel movements during period 3 was used as the primary outcome, while the other items were used as secondary outcomes. The frequency of bowel movements and days with bowel movements were evaluated based on the number of times recorded in the bowel movement diary. The amount of stool was evaluated based on the size of a No. 7 film case container (distributor: Roppon-Ashi Entomological Books, Tokyo, Japan) with the following size, capacity: 20 mL / Diameter: 26 mm / Case height: 54.5 mm / Total height: 57.0 mm, and results were expressed in Film Case Equivalent (FCE) units. Stool shape was recorded according to the Bristol Scale [26]. Stool smell was evaluated on a 5-point scale: “the smell has become quite strong”, “the smell has become slightly stronger”, “no change from usual”, “the smell has become slightly weaker”, and “the smell has become quite weak”. A feeling of relief after bowel movements was evaluated on a 3-point scale: “feeling of refreshment”, “normal”, and “feeling of residual stool”.

Regarding the other secondary outcomes, the following items were evaluated at the time of screening and 2 weeks after the intervention. In the evaluation of the intestinal microbiota, the following were measured and assessed: *Bifidobacterium*, *Lacticaseibacillus*, *Streptococcus*, *Enterococcus*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, Shannon’s diversity index, observed OTUs, Faith’s Phylogenetic Diversity, and Chao1. The measurement of the intestinal microbiota was entrusted to Cykinso, Inc. (Tokyo, Japan). In short-chain fatty acid (SCFA) measurements, acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, *n*-valeric acid, lactic acid, succinic acid, and formic acid in stool were quantified. The quantification of organic acids was entrusted to the Kyoto Institute of Nutrition & Pathology, Inc. (Kyoto, Japan). We also evaluated subjective symptoms using the Japanese Constipation Assessment Scale (CAS-MT).

Laboratory tests: Height, body weight, and body mass index (BMI) were measured in the screening examination, and body weight and BMI were also assessed 2 weeks after the intervention. Blood pressure was measured at the time of screening and 2 weeks after the intervention. Blood and urine were analyzed by LSI Medience Corporation (Tokyo, Japan), and all items were examined at baseline and two weeks after the intervention. A venous blood sample was collected from an arm vein, and the following tests were performed for a safety assessment.

The hematological and biochemical components examined were as follows: the white blood cell count, red blood cell count, hemoglobin, hematocrit, platelet count, AST, ALT, γ-GTP, total bilirubin, total protein, blood urea nitrogen, creatinine, uric acid, sodium, potassium, chloride, serum amylase, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, and hemoglobin A1c (NGSP). In addition, urine samples were collected for a

qualitative evaluation, including urinalysis of protein, glucose, pH, and occult blood.

Ethics, adherence, and compliance: The present study was conducted in accordance with the Declaration of Helsinki (2013) and the Ethical Guidelines for Life Science and Medical Research Involving Human Subjects. Prior to the study, the protocol and methodology were approved by the Ethics Committee of Takara Clinic on June 13, 2024 (Approval ID: 2406-00023-0178-11-TC). Any significant deviations from the protocol required prior authorization from the committee. All participants received a comprehensive explanation of the study protocol and objectives before providing their informed consent. None of the participants had affiliations with the sponsoring or funding organizations.

Statistical analysis: An intention-to-treat (ITT) was selected as the analysis dataset for the primary and secondary outcomes. The results obtained are shown as means and SD. In statistical analyses of the primary outcomes, data were analyzed using mixed-effects models with group, period, and their interaction as fixed effects, the baseline value and its interaction with period as covariates, and subject as a random effect. In statistical analyses of the secondary outcomes, actual values for bowel movement frequencies, days with bowel movements, and the amount of stool were analyzed using the same mixed-effects model as the primary outcome. Improvements in stool shape, stool smell, and a feeling of relief after bowel movements, and CAS-MT questionnaire responses were evaluated using χ^2 tests. Intestinal microbiota, SCFA, and CAS scores were analyzed using ANCOVA with baseline values as covariates. All statistical analyses were performed using two-sided tests, with the significance level set at 5%. SPSS Statistics version 23.0 (IBM Japan) was used as the software. This trial was analyzed with a focus on the primary outcome, and the multiplicity arising from the secondary outcome set hypothetically was not taken into consideration.

RESULTS

Effects of L. paracasei strain Shidare on the intestinal environment of mice: Figure 1 shows the effects of strain Shidare on the intestinal environment in BALB/c mice following a 14-day oral administration period. The 24-hour fecal weight generally increased at a dose of 100 billion cells/kg (Figure 1A). On the other hand, intestinal serotonin concentration increased in a dose-dependent manner, with a significant elevation being observed at 100 billion/kg (Figure 1B). Furthermore, the concentration of IgA in feces significantly increased with the administration of 50 billion cells/kg of strain Shidare (Figure 1C).

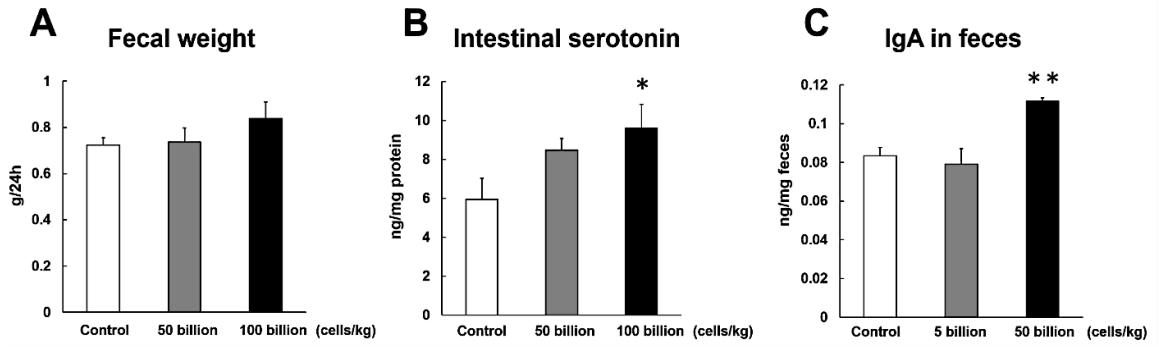


Figure 1. Effects of *L. paracasei* strain Shidare on fecal weight, intestinal serotonin, and fecal IgA in mice. *(A) Feces were dried for 24 hr to remove moisture, and the dry weight was then measured. (B) Intestinal serotonin was measured by ELISA. (C) Fecal IgA was assessed by ELISA. Each column represents the mean and S.E. (n=3-5). Asterisks denote significant differences from the control at *p < 0.05, **p < 0.01.*

Effects of L. paracasei strain Shidare in the clinical trial

Study performance: The clinical trial was conducted between August 24 and September 18, 2024. Figure 2 shows the participant recruitment and selection processes. Of the 63 individuals initially screened, 44 met the eligibility criteria and were subsequently enrolled in the study. These participants were randomly allocated to two intervention groups, with 22 participants in each group. No dropouts were observed throughout the trial period, and the efficacy and safety analysis datasets were ITT and a safety analysis population (SAF), respectively, with all trial participants included in the analysis. Table 1 shows the background of the study participants in each analysis dataset.

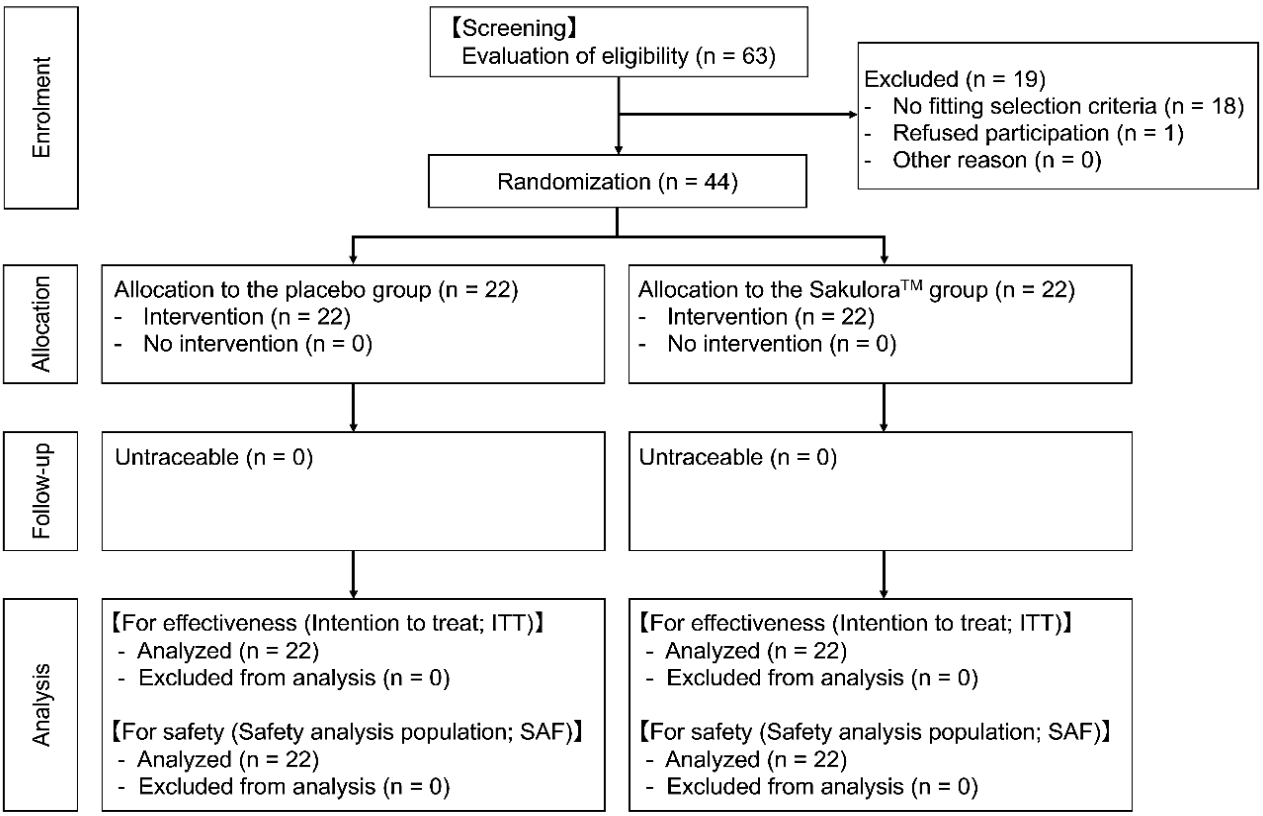


Figure 2. Flowchart for tracking test participants.

Table 1. Subject profiles.

	ITT, SAF	
	Sakulora™ (n = 22)	Placebo (n = 22)
Sex	Male: 9 (40.9%) Female: 13 (59.1%)	Male: 8 (36.4%) Female: 14 (63.6%)
Age	49.6±11.6	50.5±11.4
Height (cm)	163.7±8.3	162.2±7.4
Body weight (kg)	60.0±14.4	58.4±13.1
BMI (kg/m^2)	22.2±4.4	22.1±4.0
Systolic blood pressure (mmHg)	115.6±18.9	113.2±16.6
Diastolic blood pressure (mmHg)	77.6±13.3	73.5±11.9
Frequency of bowel movements (times/week)	4.0±0.9	4.0±0.8

Each value, except sex, represents the mean and S.D. No significant differences were observed between the Sakulora™ and placebo groups.

Bowel movement diary parameters: The results obtained for the frequency of bowel movements, days with bowel movements, and the amount of bowel movements in the bowel movement diary are shown in Table 2. The frequency of bowel movements during period 3, which was the primary outcome, was significantly higher in the Sakulora™ group (6.1±1.9 times) than in the placebo group (5.1±1.6 times). Furthermore, the amount of bowel movements was higher in the Sakulora™ group (33.3±20.5 FCE) than in the placebo group (15.9±9.3 FCE) during period 3. However, the Sakulora™ group also had significantly higher values (19.8±15.7 FCE) than the placebo group (12.3±5.5 FCE) during period 1, which was prior to the administration of the test product. No significant differences in the number of bowel movements per day were observed between the groups. Key results are additionally summarized in Supplementary Figure S1.

The results for stool shape, stool smell, and a feeling of relief after bowel movements in the bowel movement diary are shown in Table 3. No significant differences in stool shape or smell were noted in either group. On the other hand, regarding a feeling of relief after bowel movements, the number of cases showing improvement in Period 2 was significantly lower in the Sakulora™ group (2 cases) than in the placebo group (8 cases).

Table 2. Bowel movement diary parameters.

	Period 1 (Baseline)			Period 2 (1 week)		Period 3 (2 weeks)	
	Sakulora™	**Placebo**		**Sakulora™**	**Placebo**	**Sakulora™**	**Placebo**
Days of bowel movements (days/week)	3.7±0.8	3.7±0.8		4.8±1.5	4.3±1.2	5.2±1.3	4.8±1.3
Frequency of bowel movements (times/week)	4.0±0.9	4.0±0.8		5.4±2.0	4.7±1.5	6.1±1.9*	5.1±1.6
Amount of bowel movements (FCE)	19.8±15.7#	12.3±5.5		25.0±14.0	15.5±12.2	33.3±20.5**	15.9±9.3

Each value represents the mean and S.D. The number sign and asterisks denote significant differences between the Sakulora™ and placebo groups at #p< 0.05 (Welch's t*-test) and *p < 0.05 and **p < 0.01 (Linear mixed model with baseline values as covariates, and the time point, group, interaction between the time point and group, interaction between the baseline and time point, and study participants as factors), respectively.*

Table 3. Stool shape, stool smell, and a feeling of relief after bowel movements in the bowel movement diary.

	Period 2 (1 week)		Period 3 (2 weeks)	
	Sakulora™	**Placebo**	**Sakulora™**	**Placebo**
Number of cases in which "stool shape" after the intervention improved from that in period 1 (cases)	4	10	6	9
Number of cases in which "stool smell" after the intervention improved from that in period 1 (cases)	5	7	4	7
Number of cases in which "a feeling of relief after bowel movements" after the intervention improved from that in period 1 (cases)	2 *	8	5	7

*Each value represents the mean and S.D. The asterisk denotes a significant difference between the Sakulora™ and placebo groups at *p < 0.05 (χ^2 tests).*

The intestinal microbiota and SCFA in feces: Tables 4 and 5 present the results obtained on the intestinal microbiota and SCFA in feces, respectively. No significant changes were observed in any parameters for the intestinal microbiota or SCFA following the intake of Sakulora™. Regarding propionic acid, the Sakulora™ group showed a significantly lower value than the placebo group prior to intake, while no significant difference was noted two weeks after intake.

Table 4. Intestinal microbiota.

	Baseline		2 weeks	
	Sakulora™	Placebo	Sakulora™	Placebo
***Bifidobacterium* (%)**	0.029±0.039	0.036±0.034	0.032±0.036	0.038±0.032
***Lacticaseibacillus* (%)**	0.003±0.006	0.005±0.018	0.000±0.001	0.008±0.027
***Streptococcus* (%)**	0.007±0.006	0.007±0.012	0.007±0.010	0.006±0.009
***Enterococcus* (%)**	0.000±0.001	0.000±0.000	0.000±0.000	0.001±0.002
***Firmicutes* (%)**	0.480±0.069	0.450±0.068	0.456±0.112	0.465±0.067
***Bacteroidetes* (%)**	0.416±0.075	0.433±0.062	0.430±0.115	0.417±0.071
***Actinobacteria* (%)**	0.058±0.058	0.059±0.040	0.058±0.045	0.063±0.038
***Proteobacteria* (%)**	0.036±0.031	0.047±0.041	0.039±0.031	0.043±0.047
Shannon's diversity index	6.5±0.5	6.2±0.6	6.2±0.8	6.2±0.6
Observed OTUs	264.8±64.1	227.3±71.0	241.9±83.5	221.2±68.5
Faith's Phylogenetic Diversity	30.0±6.7	26.5±7.2	28.2±8.4	25.5±6.9
Chao1	274.4±68.2	234.5±74.9	249.6±88.3	228.2±71.9

Each value represents the mean and S.D.

No significant differences were observed between the Sakulora™ and placebo groups.

Table 5. SCFA in feces.

	Baseline		2 weeks	
	Sakulora™	Placebo	Sakulora™	Placebo
Succinic acid (mmol/kg wet feces)	0.5±0.5	2.3±7.4	0.4±0.4	2.3±7.0
Lactic acid (mmol/kg wet feces)	0.0±0.0	0.1±0.3	0.0±0.0	0.4±1.2
Formic acid (mmol/kg wet feces)	0.0±0.1	0.1±0.5	0.0±0.2	0.0±0.0
Acetic acid (mmol/kg wet feces)	42.4±21.5	51.8±26.3	49.8±26.0	64.1±34.1
Propionic acid (mmol/kg wet feces)	15.3±7.3 *	21.2±10.2	17.8±8.0	22.0±11.7
Isobutyric acid (mmol/kg wet feces)	1.6±1.8	1.7±1.8	1.0±1.6	1.5±1.6
***n*-Butyric acid (mmol/kg wet feces)**	10.0±6.7	10.8±9.2	11.1±8.7	14.7±11.8
Isocaproic acid (mmol/kg wet feces)	1.9±2.1	1.7±1.8	1.2±1.9	1.8±1.9
***n*-Caproic acid (mmol/kg wet feces)**	0.8±1.2	1.1±1.4	0.8±1.3	1.7±2.0

Each value represents the mean and S.D.

*The asterisk denotes a significant difference between the Sakulora™ and placebo groups at *p < 0.05 (Welch's* t*-test).*

CAS-MT scores: Table 6 presents the results on CAS scores and individual CAS-MT questionnaire items. CAS scores showed no significant intergroup differences. Among the individual CAS-MT questionnaire items, the number of cases showing improvement in "pain in the anus during bowel movements" was significantly lower in the Sakulora™ group (0 cases) than in the placebo group (4 cases).

Table 6. CAS scores.

	Baseline		2 weeks	
	Sakulora™	Placebo	Sakulora™	Placebo
CAS scores (points)	4.7±2.5	4.6±1.9	3.3±2.8	2.8±2.1
CAS-MT questionnaire				
Number of cases in which a "Feeling of bloating (feeling of swelling)" after the intervention improved from that at baseline (cases)	—	—	7	9
Number of cases in which "low flatulence" after the intervention improved from that at baseline (cases)	—	—	6	5
Number of cases in which "infrequent bowel movements" after the intervention improved from that at baseline (cases)	—	—	7	8

	Baseline		2 weeks	
	Sakulora™	Placebo	Sakulora™	Placebo
Number of cases in which a "Feeling of fullness in the rectum" after the intervention improved from that at baseline (cases)	—	—	7	7
Number of cases in which "Pain in the anus during bowel movements" after the intervention improved from that at baseline (cases)	—	—	0 *	4
Number of cases in which "low stool volume" after the intervention improved from that at baseline (cases)	—	—	6	7
Number of cases in which "Hard stools that are difficult to pass" after the intervention improved from that at baseline (cases)	—	—	7	7
Number of cases in which "watery stool" after the intervention improved from that at baseline (cases)	—	—	5	4

*Each value represents the mean and S.D. The asterisk denotes a significant difference between the Sakulora™ and placebo groups at *p < 0.05 (χ^2 tests).*

DISCUSSION

The present study investigated the effects of the oral administration of strain Shidare on the intestinal environment in mice. As shown in Figure 1, intestinal serotonin concentrations were significantly higher after the oral administration of 100 billion cells/kg of strain Shidare, while only a slight increase was noted in 24-hour fecal weight. Serotonin (5-hydroxytryptamine) in the gastrointestinal tract acts as a neurotransmitter in the enteric nervous system, with the majority stored in enteric chromaffin (EC) cells of epithelial cells [27, 28]. Serotonin released from EC cells activates 5-HT_3 and 5-HT_4 receptors initiate the peristaltic reflex, thereby promoting propulsive movement in the intestines and shortening the colonic transit time [29]. Therefore, strain Shidare may improve bowel movements by promoting peristalsis increasing serotonin secretion in the intestines. The administration of 50 billion cells/kg of the strain Shidare also significantly increased fecal IgA concentration, as an indicator reflecting intestinal IgA levels [30]. Intestinal IgA functions as a primary defense factor that protects the intestinal epithelium from intestinal toxins and pathogenic microorganisms. It also contributes to improving the intestinal environment by enhancing mucosal immunity and maintaining intestinal homeostasis [31]. In this experiment, strain Shidare increased the concentration of IgA in mouse feces, suggesting that it promoted intestinal IgA secretion and improved the intestinal environment.

Based on its efficacy in animal studies, a double-blind, placebo-controlled, parallel-group trial was conducted to evaluate the effects of Sakulora™, a processed powder containing strain Shidare, on bowel movements in healthy Japanese participants. This trial involved 44 healthy Japanese adults (men and women) with 3 to 5 bowel movements during the 7 days prior to screening. The mean frequency of bowel movements during Period 1 was 4.0 in both the placebo group and the Sakulora™ group (Table 1). In an internet-based questionnaire survey of 15,000 Japanese men and women (aged 20–79), an average of 6.9 bowel movements per week was reported by 5,155 respondents (2,542 men, 2,613 women) [32]. This finding suggests that the participants in the present study were constipated.

A meta-analysis of the efficacy of probiotics for constipation demonstrated that probiotic supplementation increased the weekly frequency of bowel movements by 0.98 to 1.29 times in patients with IBS, functional constipation, and Parkinson's disease [33-35]. This improvement, representing at least one

additional bowel movement per week, is similar to the efficacy of established therapeutic interventions. The analysis of the primary outcome, the frequency of bowel movements during period 3, revealed a significantly higher frequency in the Sakulora™ group (6.1 times/week) than in the placebo group (5.1 times/week) (Table 2). The weekly frequency of bowel movements in the Sakulora™ group was within the normal range for healthy Japanese adults, and the difference between groups was greater than one bowel movement per week. Therefore, the ingestion of Sakulora™ was considered to exert medically significant effects to promote the frequency of bowel movements.

While the increased frequency of bowel movements suggested the modulation of the gut microbiota and enhanced SCFA production, neither parameter showed significant between-group differences, except for a small change in propionic acid (Tables 4 and 5). In gut microbiota and fecal SCFA analyses, several individuals had values below the detection limit (Data not shown). Therefore, changes in gut microbiota and SCFA attributed to Sakulora™ supplementation may not have been detectable in these participants. Further investigations into the effects of Sakulora™ on the gut microbiota and SCFA are needed to incorporate baseline detectability as an inclusion criterion. Other studies on *L. paracasei* demonstrated that administrating strain CNCM I-1572 (24 billion CFU/day) for 4 weeks to IBS patients reduced *Ruminococcus* enriched in IBS, and this was accompanied by increases in both acetate and butyrate production [36]. Nevertheless, direct comparisons between the present results and these findings require caution because the aforementioned study differed in two key aspects: the enrolment of IBS patients rather than healthy individuals and the use of viable rather than heat-killed LAB. Conversely, in healthy adult populations, the daily administration of strain LC01 at 10^{10} CFU/day for 4 weeks resulted in significant increases in the relative abundance of *Lacticaseibacillus*, *Bifidobacterium*, and *Roseburia intestinalis*. In contrast, significant decreases in *Escherichia coli* and significant increases in acetic acid and butyric acid among fecal SCFA were observed [37]. However, caution is again needed when making direct comparisons with the present study because viable LAB were used in that study, whereas heat-killed LAB were employed in our investigation.

The CAS-MT used for measurements in the present study was originally developed as a scale for patients with constipation as a side effect of morphine. On the other hand, the Japanese version of CAS has been validated in healthy individuals not receiving morphine. It has also been applied in recent clinical research to identify and grade constipation severity [38]. As shown in Table 6, an analysis of the CAS-MT questionnaire revealed that the number of participants reporting improvement in “pain in the anus during bowel movements” from baseline was significantly lower in the Sakulora™ group than in the placebo group. This result was attributed to an increase in the number of cases with improvement in the placebo group, compared to 0 cases in the Sakulora™ group. Therefore, changes in stool shape may have reduced anal pain during defecation. As shown in Table 3, the number of participants with an improved stool shape following ingestion was higher in the placebo group than in the Sakulora™ group during period 2 (10 vs. 4, respectively) and period 3 (9 vs. 6, respectively). Hard and loose stools are both known to increase anal irritation [39]. Therefore, the improved stool shape in the placebo group may have contributed to a reduction in anal pain during defecation. The lack of a change in this parameter despite an increased defecation frequency in the Sakulora™ group may be explained by the findings of a meta-analysis by Bielefeldt et al. [40], which suggested that while an increased frequency of bowel movements ameliorates abdominal pain, anal pain may be mediated

by distinct mechanisms.

Regarding the mechanisms underlying the increased frequency of bowel movements in this clinical trial, intestinal serotonin and fecal IgA concentrations, which were evaluated in animal studies, were not measured because they require highly invasive procedures; therefore, we prioritized outcome measures directly related to the clinical endpoint of improved bowel movements, such as the gut microbiota and SCFA. Despite the absence of observable changes in the gut microbiota and SCFA, it remains plausible that Sakulora™ affected intestinal serotonin and IgA concentrations similarly in humans. On the other hand, Sakulora™ was manufactured using a high-pressure homogenizer during the powdering process to suppress bacterial cell aggregation. It has been reported that powder form of lactic acid bacteria produced by this manufacturing method exhibits improved dispersibility, absorption into the Peyer's patches and its efficacy [25]. Therefore, in this study, it is conceivable that the enhanced dispersibility and absorbency achieved through the manufacturing method may have contributed to improving the intestinal environment.

The safety assessment of Sakulora™ showed good tolerability, with no adverse events reported or observed throughout the ingestion period under the study conditions.

Scientific Innovation and Practical Implications: We report the first discovery of *L. paracasei* strain Shidare from weeping cherry trees, demonstrating efficacy in improving bowel movements with a favorable safety profile for functional food applications.

Here, we discuss the alignment between this study and the Functional Food Center (FFC) 17-Step Model [41]. According to the FFC 17-Step Functional Food Product Development Model, Step 8 focuses on providing preclinical evidence of efficacy and safety, whereas Step 9 requires clinical trials addressing dosage, efficacy, and safety. In the present study, we provide Step 8-level evidence by animal models demonstrated that strain Shidare improves the intestinal environment. In addition, our findings contribute to Step 9 by a clinical trial involving healthy individuals with mild constipation demonstrated that a specific dose of strain Shidare safely improved bowel movements. Therefore, this manuscript primarily supports Steps 8–9 of the FFC 17-Step Functional Food Product Development Model and helps bridge preclinical evidence toward clinical validation for functional food development.

CONCLUSION

The present study demonstrated that the oral administration of heat-killed *L. paracasei* strain Shidare-containing Sakulora™ (50 billion cells/day) significantly improved the frequency of bowel movements in healthy Japanese adults with mild constipation. In preclinical animal studies, the administration of strain Shidare (100 billion cells/kg) significantly increased intestinal serotonin concentrations and slightly increased fecal weight, while fecal IgA concentrations were significantly elevated by a dose of 50 billion cells/kg. These results suggest that Sakulora™ improves bowel movements through multiple mechanisms, including enhanced intestinal peristalsis via serotonin secretion and improved intestinal immunity through IgA production.

List of Abbreviations: QOL, quality of life; IBS, irritable bowel syndrome; LAB, lactic acid bacteria; strain Shidare, *L. paracasei* strain Shidare; RIPA, radioimmunoprecipitation assay; SD, standard deviation; FCE, Film Case Equivalent; SCFA, short-chain fatty acid; CAS-MT, Japanese Constipation Assessment Scale; BMI, body mass index; NGSP, hemoglobin A1c; ITT, intention to treat; SAF, safety analysis population; EC, enteric chromaffin;

Competing Interests: The authors declare no conflicts of interest associated with this manuscript.

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