Research Article **Open Access**

Effects of *Lacticaseibacillus paracasei* 327 intake on the intestinal environment in healthy adult Japanese: a randomized, double-blind, placebo-controlled, parallel-group trial

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Submission Date: January 17th, 2024; **Acceptance Date:** March 13th, 2024; **Publication Date:** March 19th, 2024

Please cite this article as: Senda-Sugimoto Y., Mihara T., Higuchi Y., Uchiyama K., Takara. T., Takahashi H. Effects of Lacticaseibacillus paracasei 327 intake on the intestinal environment in healthy adult Japanese: a randomized, double-blind, placebo-controlled, parallel-group. Functional Foods in Health and Disease 2024; 14(3):184-206. DOI: https://www.doi.org/10.31989/ffhd.v14i3.1336

ABSTRACT

Background: *Lacticaseibacillus paracasei* 327 (*L. paracasei* 327) is a lactic acid bacteria isolated from brown rice. It has been reported that sterilized *L. paracasei* 327 improves bowel movement. Given the reported relationship between intestinal microbiota and bowel movement, we hypothesized that *L. paracasei* 327 also improves the intestinal environment through its involvement in fluctuations in the composition of the intestinal microbiota.

Objective: The purpose of this study was to verify the effects of the consumption of food containing sterilized *L. paracasei* 327 on the intestinal environment in healthy Japanese adults.

Methods: A randomized, double-blind, placebo-controlled, parallel-group trial was conducted in 110 healthy adults aged 20 to 64 years with a defecation frequency of three to five times per week. Participants were divided into two groups, one receiving 25 mg (approximately $5x10^{10}$ bacteria) of sterile *L. paracasei* 327 once daily for two weeks (55 participants) and other receiving placebo for two weeks (55 participants), to test its effect on the intestinal environment.

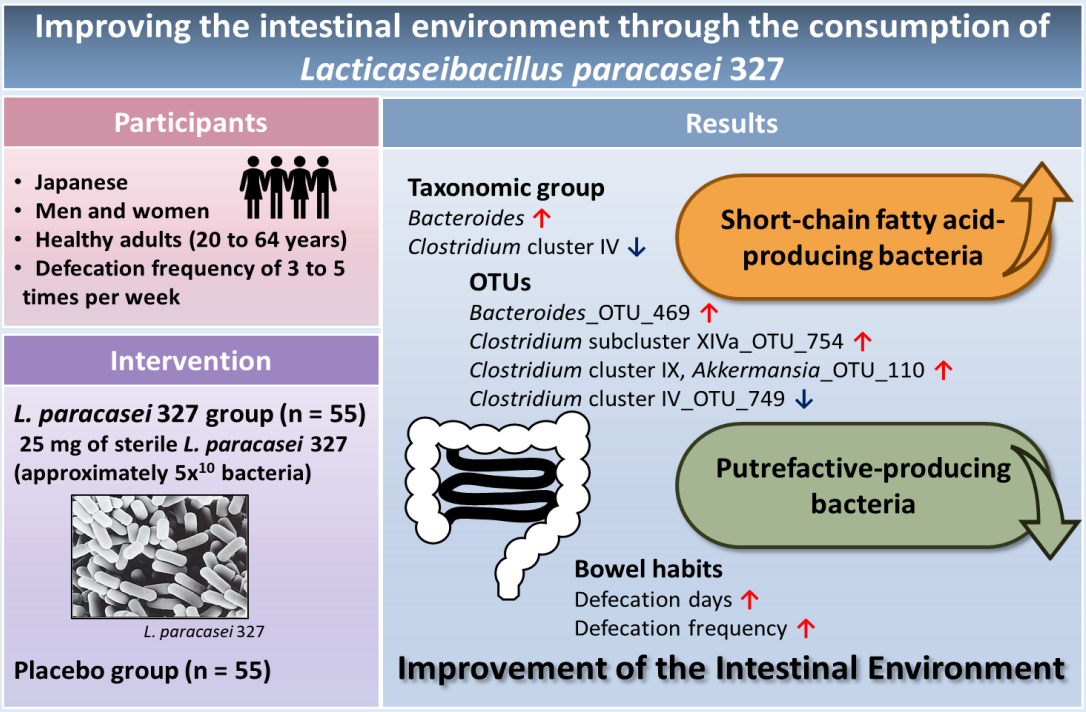
Results: Intestinal microbiota analysis showed that *L. paracasei* 327 ingestion resulted in a trend towards an increase in *Bacteroides* and a significant decrease in *Clostridium* cluster IV in each taxonomic group compared to the placebo group. In addition, for each operational taxonomic units (OTUs), *Bacteroides*_OTU_469 and *Clostridium* subcluster XIVa_OTU_754 were significantly increased, and *Clostridium* cluster IX, *Akkermansia*_OTU_110 tended to increase, while

Clostridium cluster IV_OTU_749 significantly decreased. Furthermore, defecation days and defecation frequency increased significantly.

Conclusion: The presumptive species of bacteria in the taxonomic group and OTUs increased by *L. paracasei* 327 ingestion included short-chain fatty acid-producing bacteria. In addition, *Clostridium* cluster IV_OTU_749, which was reduced by *L. paracasei* 327 ingestion, may also be involved in the production of putrefactive products in the intestine. Therefore, these findings suggest that *L. paracasei* 327 ingestion improves the intestinal environment by increasing short-chain fatty acid-producing bacteria, suppressing putrefactive-producing bacteria, and improving defecation.

Trial registration: UMIN000049859 (UMIN-CTR)

Keywords: Lactic acid bacteria; Postbiotics; *Lacticaseibacillus paracasei 327*; Intestinal microbiota; Defecation; Clinical trial



INTRODUCTION

The human intestinal tract is home to 10 to 100 trillion microorganisms and more than 1,000 species of bacteria, archaea, eukaryotes, and viruses, collectively referred to as the intestinal microbiota. The intestinal microbiota has co-evolved with the host over thousands of years, establishing a mutually beneficial relationship with each other and the intestinal environment. The majority of microorganisms in the intestinal tract are bacteria, which play important roles in health, such as maintaining the integrity of the mucosal barrier, supplying nutrients such as vitamins and essential amino acids, protecting against pathogens, and maintaining proper immune function [1-2]. Lactic acid bacteria and Bifidobacteria are well known beneficial bacteria in the intestinal microflora [3-4], but recently *Bacteroides* and *Clostridium* cluster have also attracted attention as newly recognized beneficial bacteria. *Bacteroides* is the most abundant bacteria in the

human intestine and is rich in polysaccharide-degrading enzymes and has been reported to degrade polysaccharides to produce short-chain fatty acids such as acetic acid, propionic acid, and butyric acid, and to contribute to the production of useful metabolic products by providing nutrients to other intestinal bacteria [5]. Similarly, *Clostridium* clusters contain many species of bacteria involved in the production of short-chain fatty acids from polysaccharides [6].

The intestinal microbiota communicates with the host via metabolites such as short-chain fatty acids and bacterial structures such as secreted proteins, peptidoglycans, polysaccharides, teichoic acid, lipoteichoic acid, and DNA, and is intricately and closely involved in maintaining host homeostasis [7]. Therefore, disturbances in the intestinal microbiota affect immune function, energy regulation, and intestinal hormone regulation, and may promote inflammation, leading to obesity, metabolic syndrome, and lifestyle-related diseases [8-11]. In particular, defecation disorders such as constipation and diarrhea are known to be closely related to the composition of the intestinal microbiota [12-13]. In order to prevent the decline in health-related quality of life (HRQoL) and labor productivity [14-15] caused by defecation disorders, it is useful to maintain a well-balanced intestinal environment, including the intestinal microbiota.

Although genetic factors are involved in the formation of the intestinal environment, an appropriate diet, prebiotics such as dietary fiber, probiotics such as lactic acid bacteria and synbiotics, which are a combination of both, are useful in creating an appropriate intestinal environment. For a long time, probiotics were thought to regulate the intestinal environment by delivering live microorganisms to the intestinal tract, but recent research has revealed that even bacteria that have been sterilized or otherwise treated can have beneficial effects. For example, sterilized *Lactobacillus gasseri* CP2305 regulates intestinal function and increases *Clostridium* cluster IV and *Bifidobacterium* [16-17], and sterilized *Enterococcus faecalis* EC-12 increases *Bifidobacterium* [18], sterilized *Enterococcus faecalis* KH2 has been reported to regulate intestinal function and increase *Clostridium* cluster IV [19], and sterilized *Lactobacillus kefiri* GKL2 has been reported to regulate intestinal function, increase *Lactobacillales*, and decrease putrefactive products [20]. Accordingly, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined postbiotics in 2021 as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [21]. Postbiotic microorganisms are stable, safe foods that do not cause systemic infections or other side effects in the human body because they are not viable organisms, and there is no risk of acquiring or transmitting antibiotic-resistant genes [22]. Therefore, the intake of postbiotics are expected to safely maintain intestinal balance. More recently, foods that provide humans with more than the basic nutrition described above have been classified as functional foods (FF) and are defined by the Functional Food Centre (FFC) as “Natural or processed foods that contain biologically-active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms” [23-25].

Lacticaseibacillus paracasei 327 (formerly designated as *Lactobacillus casei* subsp. *casei* 327: *L. paracasei* 327) is a lactic acid bacteria isolated from brown rice [26] and it has been reported that intake of live bacteria has a probiotic-like action that improves defecation frequency in volunteers with a tendency towards constipation [27]. In addition to live bacteria, sterilized *L. paracasei* 327 has also been reported to have beneficial postbiotic-like effects on the body. For instance, clinical trials have shown that 50 mg (approximately 1 x 10^{11} bacteria) of sterilized *L. paracasei*

327 inhibits water evaporation from the skin [28-29], and clinical trials have shown that *L. paracasei* 327 at 25 mg (approximately 5 x 10^{10} bacteria) improves defecation frequency and the number of defecation days [30]. As a relationship between defecation frequency and intestinal microbiota has been reported [12], it was hypothesized that *L. paracasei* 327 could also improve the intestinal environment through its involvement in fluctuations in the intestinal microbiota. Therefore, the purpose of this study was to evaluate the effects on the intestinal environment of sterilized *L. paracasei* 327 in a randomized, double-blind, placebo-controlled, parallel-group comparison trial when healthy Japanese men and women aged 20 to 64 years with a defecation frequency of three to five times per week ingested 25 mg of sterilized *L. paracasei* 327 (approximately 5 x 10^{10} bacteria) once daily for two weeks.

MATERIALS AND METHODS

Study design: This study was designed as a randomized, double-blind, placebo-controlled, parallel-group trial with participants allocated in 1: 1 ratio by a block randomization method. The study protocol was approved by the institutional review board of Takara Clinic (Tokyo, Japan) on December 14, 2022 (approval number: 2212-00173-0027-11-TC), and the study summary was registered in the public database UMIN clinical trial registration system (UMIN-CTRID : UMIN000049859). This study was conducted under the supervision of a physician in accordance with the principles of the "Declaration of Helsinki" (amended in October 2013) and the "Ethical Guidelines for Life Sciences and Medical Research Involving Human Subjects" (March 23, 2021 / partially amended on March 10, 2022), always taking the protection of the human rights of participants into consideration. The protocol was not changed from its initial form during the study period.

Study participants: Participants for the study were recruited through an online website (https://www.go106.jp/) operated by ORTHOMEDICO Inc. (Tokyo, Japan). The study details were disclosed to participants before their enrollment, and investigators obtained their informed consent. Participants who met the eligibility criteria and did not violate the exclusion criteria were selected from those who provided written consent. The eligibility and exclusion criteria are listed below.

Eligibility Criteria

1. Japanese; 2. Men and women; 3. Participants aged between 20 and 64 years; 4. Healthy participants; 5. Participants whose defecation frequency is three to five times per week; 6. Participants whose occupancy rate of *Bifidobacterium* in feces is relatively low

Exclusion Criteria

1. Participants who are undergoing medical treatment or have a medical history of malignant tumor, heart failure, and myocardial infarction; 2. Participants who have a pacemaker or an implantable cardioverter defibrillator (ICD); 3. Participants who are currently undergoing treatment for chronic disease such as arrhythmia, hepatopathy, nephropathy, cerebrovascular disorder, rheumatism, diabetes, dyslipidemia, or hypertension; 4. Participants who have chronic constipation; 5. Participants who have other diseases that may significantly affect bowel movement, or who have a medical history of such diseases; 6. Participants who have asthma, or may develop asthma during this trial; 7. Participants whose occupancy rate of the order *Lactobacillales* or *Bifidobacterium* at screening (before consumption; Scr) is 0%; 8. Participants who habitually ingest foods rich in lactic acid bacteria, or lactic acid bacteria fortified health foods or medicines; 9. Participants who habitually ingest foods rich in *Bacillus subtilis* var. *natto* (*B. natto*), or *B. natto* fortified health foods or medicines; 10. Participants who routinely take medicines, health foods, "Foods for Specified Health Uses (FOSHU)," or "Foods with Functional Claims (FFC)" that may affect bowel movement / take ingredients that affect bowel movement (such as dietary fiber) in fortified foods; 11. Participants who routinely use or take other FOSHU

or FFC; 12. Participants who are currently taking medications (including herbal medicines) and supplements; 13. Participants who are allergic to medicines and/or study food-related products; 14. Participants who are day- and night- shift workers / work nights frequently during this trial; 15. Participants who plan to go abroad during this trial; 16. Participants whose anthropometric measurements, physical examination values or laboratory values markedly deviate from the reference values at the pre-consumption test; 17. Participants who are pregnant, lactating, or planning to become pregnant during this trial; 18. Participants with COVID-19; 19. Participants who have been enrolled in other clinical trials within the preceding 28 days before the agreement to participate in this trial or plan to participate another trial during this trial; 20. Participants judged by the physician as ineligible to participate in this study

The recruitment period for study participants was from December 2022 to January 2023, and the implementation period was from January 2023 to March 2023, at Takara Clinic (Tokyo, Japan). In order to confirm the health status of the participants, participants kept a diary after the date of consent, interviews were conducted during visits to the clinic, and a dietary survey was conducted using a calorie and nutrition diary (CAND) [31]. In addition, the participants were asked to strictly adhere to the following compliance requirements throughout the study period.

Compliance

1. Consume the study food in accordance with the prescribed dosage and administration. The daily dose should be taken on the same day and not carried over to the next day or later; 2. Consume the study food to achieve a minimum 80% intake rate; 3. From the date of consent until the final examination, avoid binge eating and drinking and make no changes in lifestyle; 4. From the date of consent until the final examination, the participants are prohibited from consuming in excess or initiating a habitual intake of any food or drug that may affect the intestinal environment; 5. From the date of obtaining study consent until the final examination, in so far as possible, the participants should refrain from consuming any foods or medicines (other than those permitted in the study) that may affect the intestinal environment. If such foods or medicines are ingested, record them in the diary; 6. Do not drink alcohol or exercise excessively from the day before each examination until the end of the examination on that day; 7. Do not eat or drink anything after 21:00 on the day before each examination. Consumption of stury foods is also prohibited. However, water only may be consumed. Functional water and tea are not allowed; 8. If any change in physical condition occurs during the study period, immediately contact the contracted clinical research organization for further instructions; 9. Foods for specified health use, foods with functional claims, and other foods/beverages with possible functionality should be avoided as much as possible during the study period; 10. Avoid vaccination against the novel coronavirus as much as possible during the study period. If vaccination is unavoidable, it should be recorded on the drug use confirmation form and the contracted clinical research organization should be notified; 11. During the study period, take comprehensive measures to prevent infection by coronaviruses, etc. (thorough hand washing and disinfection, wearing masks, etc.), and if there is any suspicion of infection, immediately contact the contract clinical research organization and ask for instructions on further measures to be taken.

Intervention: The intervention food was a tablet containing 25 mg (approximately $5x10^{10}$) of sterile *L. paracasei* 327 per tablet (250 mg) and the placebo was a tablet identical in appearance and flavor to the *L. paracasei* 327 containing food (KAMEDA SEIKA CO., LTD., Niigata, Japan). The daily dose was one tablet, and participants consumed one of the designated tablets once a day with water or lukewarm water for two weeks. The compositions of the study foods are shown in Table 1.

Table 1. The composition and nutritional content of the *L. paracasei* 327 containing food and placebo

Ingredients		**Placebo**	***L. paracasei* 327 containing food**
		Glucose, starch, calcium stearate, fine granular silica, hydroxypropylcellulose	*L. paracasei* 327[a], glucose, starch, calcium stearate, fine granular silica, hydroxypropylcellulose
Nutritional facts (value for daily dose, 250 mg)			
Energy	(kcal)	0.90	0.90
Protein	(g)	0.00	0.02
Lipid	(g)	0.00	0.01
Carbohydrate	(g)	0.22	0.20
Sodium	(mg)	0.05	0.16

[a]The *L. paracasei* 327 content was 25 mg (approximately 5 x 10^{10} bacteria) per daily dose (250 mg; one tablet).

Table 2. The study schedule

		Screening					**Intake period**	
		Information session	Scr -1w	Scr	Enrollment	Allocation	0w	2w
Registration	Selection based on eligibility criteria	X			X			
	Informed consent	X						
	Other procedures	X						
	Allocation					X		
Interventions	*L. paracasei* 327						◆---------◆	
	Placebo						◆---------◆	
Assessments	Primary outcome			X				X
	Secondary outcome			X				X
	Body measurement			X				
	Physical examination			X				
	Urine examination			X				
	Peripheral blood examination			X				
	Questionnaire			X				X
	Bowel diary		◆---------◆				◆---------◆	
	Daily diary	◆---------◆						

Scr-1w, 1 week prior to screening and pre-intake examinations; Scr, screening and pre-intake examinations; 0w, Date of intake start; 2w, Examination two weeks after intake.

Outcome: The study schedule and measurement points for each endpoint are shown in Table 2.

Primary outcome

The primary outcome was the intestinal microbiota at two weeks post intake. Fecal samples were collected from study participants and analyzed by Terminal Restriction Fragment Length Polymorphism (T-RFLP) [32] to evaluate phylogenetic bacterial groups and operational taxonomic units (OTUs). The analysis was contracted to TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan).

Secondary outcomes

Secondary outcomes were intestinal putrefactive products and organic acids, bowel diary, Japanese version of the Constipation Assessment Scale (CAS-MT), and Oguri-Shirakawa-Azumi Sleep Inventory MA version (OSA-MA). Fecal samples were collected from study participants, and putrefactive products and organic acids were analyzed at Kyoto Institute of Nutrition & Pathology, Inc. (Kyoto, Japan).

Participants recorded defecation days, defecation frequency, volume, shape, color, odor, and feeling after defecation in a bowel diary seven days prior to the screening and pre-intake examinations and every day from the day of intake until the day before the final examination. The seven days prior to the screening and pre-intake examination were designated as Period 1, the seven days from the start of intake to the seventh day of intake as Period 2, and the eighth day to the day before the two-week post-intake examination as Period 3. Defecation frequency, number of defecation days, and defecation volume were evaluated as the sum of each seven-day period. Shape, color, odor, and feeling after defecation were evaluated every seven days by calculating the average of each survey item and converting it to a whole number, rounded to one decimal place.

The Japanese version of the Constipation Assessment Scale (CAS-MT) [33] was used to evaluate constipation at screening and the pre-intake examination and at the two-week post-intake examination. The CAS score comprises the total score of the eight CAS-MT items.

The OSA Sleep Inventory MA version [34] was used for sleep quality assessment and was conducted at the study participant's home upon awakening on the day of screening and pre-intake examination and at the two-week post-intake examination.

Safety assessment

The safety evaluation was based on the incidence of adverse events.

Sample size: No study has ever evaluated the occupancy of intestinal microbiota in Japanese people after consumption of the *L. paracasei* 327 for two weeks. We assumed that the difference between the *L. paracasei* 327 group and the placebo group would be large (d = 0.80) in terms of the occupancy of the intestinal flora at the time of examination after two weeks of consumption. The number of participants was calculated using a statistical significance level (α) of 5% and a statistical power (1-β) of 90%, resulting in a requirement for 68 participants (34 in each group). In order to maximize the statistical power (1-β) as much as possible, the target number of participants was set at 100 (50 in each group). The statistical power (1-β) was recalculated to be 97.7%. In addition, the number of participants to be examined was set at 110 (55 in each group) with an expected dropout rate and/or noncompliance with the protocol of approximately 10% during the study period.

Randomization and blinding: Study participants who met eligibility criteria at the time of screening and pre-intake examination were assigned to either the *L. paracasei* 327 group or the placebo group according to a computer-generated allocation table created by an allocation manager, who was a third party not directly involved in the study. The allocation ratio for each group was 1:1. The allocation table was generated using SPSS Statistics version 23.0, and the algorithm employed stratified block random assignment. Stratification factors were defecation frequency per week (three to four or five times), gender (male or female), and age (over 40 or under 40 years old.

The allocation manager generated the allocation order based on the identification number of the study food and prepared the allocation list and emergency key. The emergency key for each study participant was sealed in an envelope. The envelopes were marked with the date of sealing and sealed with a tamper-proof seal. The allocation list and the emergency key were kept strictly confidential by the allocation manager until the study was completed and the data were fixed.

After the study was completed and the data were fixed, the allocation manager verified that the allocation list and emergency key had not been opened. At the time of opening, the date of opening was written down and affixed his/her name on the allocation list, and then the identification number of the study food was revealed.

The study participants, the principal investigator, the sub investigators, the institution conducting the study, the contract research organization (responsible for conducting, monitor, data manager, statistical analyst), the clinical laboratories, the members of the institutional review board, and the sponsor were all blinded to allocation.

Statistical method: Between-group comparisons of intestinal microbiota composition were made using ANCOVA with baseline values as covariate. The number of participants in which the composition ratio changed after the intervention was compared between groups using the chi-square test. Between-group comparisons of intestinal putrefactive products and organic acids were made using ANCOVA with baseline values as the covariate, and intra-group comparisons were made using a corresponding t-test. CAS-MT scores and OSA Sleep Inventory MA version scores were compared between groups using ANCOVA with baseline values as the covariate. Defecation days, defecation frequency, and defecation volume were compared between groups at each time point (time periods 2 and 3) using linear models that included baseline values and the interaction between baseline values and time points as covariates or mixed models that included study participants as factors. For shape, color, odor, and post-defecation feel, between-group comparisons were made using Fisher's exact probability test for each response. For analysis using synthetic variables, Fisher's exact probability test or chi-square test were used for between-group comparisons. Safety evaluations were summarized for each participant in the study for adverse events that occurred. The incidence of adverse events was also summarized by group, and the 95% confidence intervals of the incidence rates by group and the differences in incidence rates between groups were calculated. The incidence of adverse events in each group was compared by Fisher's exact probability test. All statistical analyses were performed with two-tailed tests, and the significance level was set at 5%. The software used was SPSS Statistics version 23.0. Multiplicity due to multiple items and multiple time points was not considered, and the significance level was not adjusted.

RESULTS

Participant flow: Figure 1 shows a flowchart of the study participants. Of the 211 individuals who agreed to participate in the study, 110 who met the eligibility criteria were included in the study and 55 each were assigned to the *L. paracasei* 327 and placebo groups.

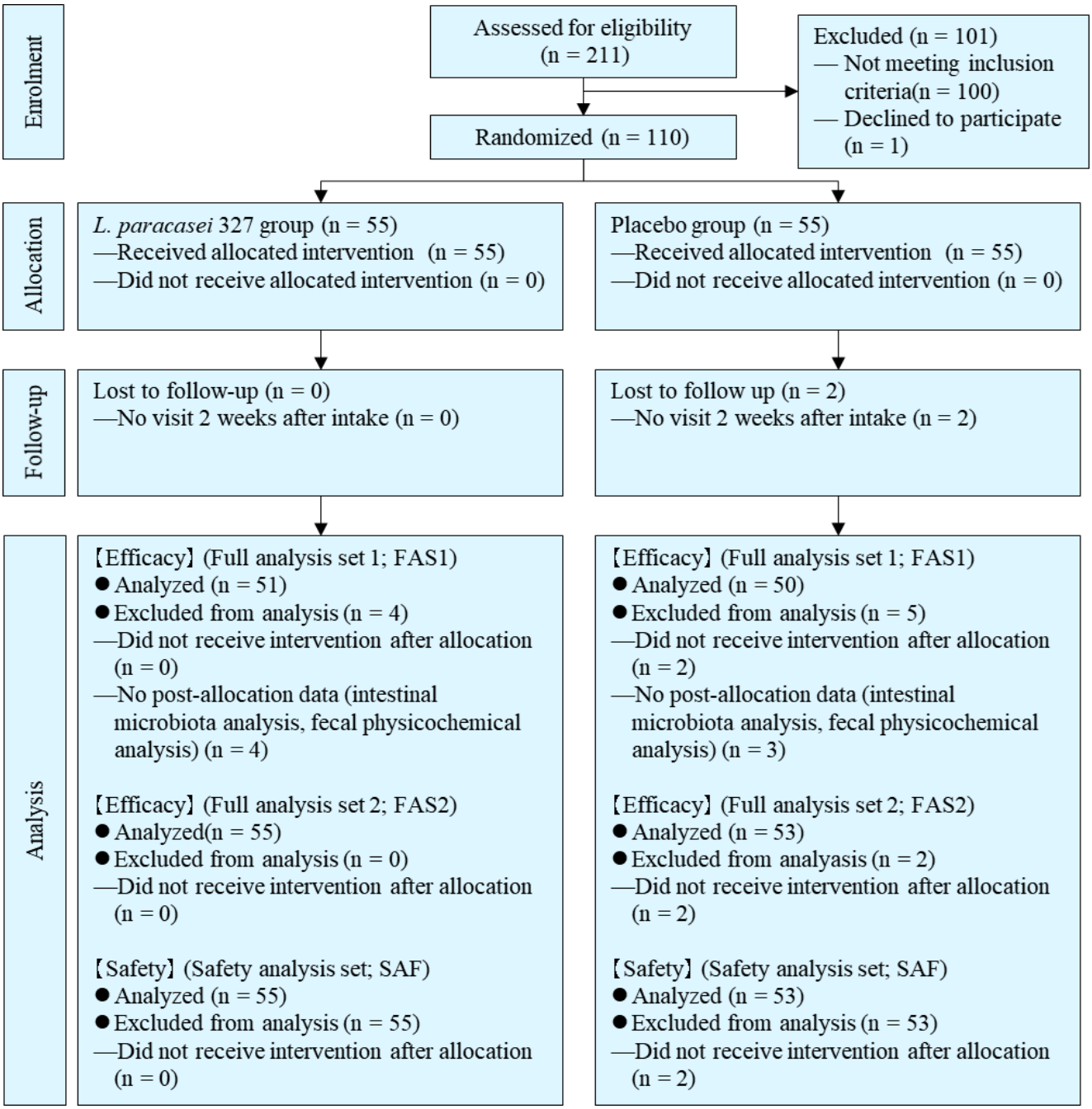


Figure 1. Flow diagram of participants in the study.

All participants received the allocated intervention, but two participants (placebo group) did not present for the scheduled examination two weeks after intake began. The data sets analyzed for the efficacy endpoints were the full analysis set (FAS) 1 and FAS2. In FAS1, 101 participants (51 in the *L. paracasei* 327 group and 50 in the placebo group) were included, excluding two participants in the placebo group who did not receive the

placebo after allocation and seven participants (four in the *L. paracasei* 327 group and three in the placebo group) for whom fecal collection was not obtained after allocation and for whom no data on intestinal microbiota analysis and fecal physicochemical analysis were available. In FAS2, 108 participants (55 in the *L. paracasei* 327 group and 53 in the placebo group) were included, excluding two participants in the placebo group who did not receive the placebo after allocation. The data set for the safety analysis was the safety analysis set (SAF) and included the same participants as those included in FAS2.

The background of the study participants in each analysis data set, including the intention-to-treat (ITT) set, is shown in Table 3.

Table 3. Background of the study participants in each analysis data set

Item	Unit		ITT		FAS1		FAS2, SAF	
			L. paracasei 327	Placebo	*L. paracasei* 327	Placebo	*L. paracasei* 327	Placebo
Number of participants	-	n	55	55	51	50	55	53
Gender	-	male	27 (49.1%)	27 (49.1%)	25 (49.0%)	25 (50.0%)	27 (49.1%)	26 (49.1%)
		female	28 (50.9%)	28 (50.9%)	26 (51.0%)	25 (50.0%)	28 (50.9%)	27 (50.9%)
Age	years	Mean (SD)	40.9 (11.5)	40.8 (11.2)	41.4 (11.8)	41.3 (11.3)	40.9 (11.5)	41.2 (11.1)
Height	cm	Mean (SD)	166.0 (7.4)	164.9 (7.8)	165.8 (6.9)	164.9 (7.6)	166.0 (7.4)	164.7 (7.6)
Body weight	kg	Mean (SD)	62.5 (12.9)	62.0 (12.5)	61.4 (11.4)	61.8 (12.8)	62.5 (12.9)	61.7 (12.5)
BMI	kg/m^2	Mean (SD)	22.5 (3.4)	22.6 (3.4)	22.2 (3.1)	22.6 (3.5)	22.5 (3.4)	22.6 (3.4)
Systolic blood pressure	mmHg	Mean (SD)	113.6 (16.3)	113.8 (11.6)	113.1 (16.2)	113.9 (11.6)	113.6 (16.3)	113.6 (11.6)
Diastolic blood pressure	mmHg	Mean (SD)	74.9 (11.3)	73.5 (8.9)	74.8 (11.6)	73.5 (9.1)	74.9 (11.3)	73.6 (9.0)

n, Number of participants; Mean, Mean value; SD, Standard deviation; ITT, Intention to treat; FAS, Full analysis set; SAF, Safety analysis set.

Primary outcome (The analysis data set was FAS1): The results of the analysis of the composition of the intestinal microbiota are shown in Table 4. The proportion of *Clostridium* cluster IV was significantly lower ($P = 0.024$) in the *L. paracasei* 327 group compared to the placebo group in the post-intake examination. The proportion of *Bacteroides* tended to be higher in the *L. paracasei* 327 group in the post-intake examination ($P = 0.068$).

Table 4. The composition of the intestinal microbiota

Item	unit	point	*L. paracasei* 327					Placebo					Between-group comparison				Effect size		
			n	Mean	SD	95% CI-	95% CI+	n	Mean	SD	95% CI-	95% CI+	Δ	95% CI-	95% CI+	*P*	d	95% CI-	95% CI+
Bifidobacterium	%	Pre-intake	51	3.18	2.97	-	-	50	3.00	2.98	-	-	0.18	-1.00	1.35	0.767	0.06	0.02	0.10
		Post-intake	51	3.92	4.38	2.67	5.13	50	4.12	4.50	2.89	5.37	-0.23	-1.98	1.51	0.790	0.05	0.01	0.09
Lactobacillales	%	Pre-intake	51	1.56	2.02	-	-	50	1.47	2.16	-	-	0.09	-0.74	0.91	0.833	0.04	0.00	0.08
		Post-intake	51	1.82	3.55	1.06	2.53	50	1.65	2.27	0.94	2.42	0.12	-0.92	1.16	0.824	0.04	0.01	0.08
Bacteroides	%	Pre-intake	51	32.69	10.11	-	-	50	33.41	11.05	-	-	-0.73	-4.91	3.46	0.732	0.07	0.03	0.11
		Post-intake	51	34.89	10.50	32.46	37.63	50	31.80	10.29	29.03	34.25	3.41	-0.26	7.09	0.068	0.37	0.33	0.41
***Bacteroides*_OTU_469**	%	Pre-intake	51	25.85	10.37	-	-	50	25.30	12.20	-	-	0.54	-3.93	5.02	0.810	0.05	0.01	0.09
		Post-intake	51	28.08	11.34	25.36	30.47	50	23.42	11.69	21.01	26.17	4.32	0.69	7.96	0.020*	0.48	0.44	0.52
Prevotella	%	Pre-intake	51	2.09	6.23	-	-	50	3.70	9.32	-	-	-1.61	-4.75	1.53	0.311	0.22	0.18	0.26
		Post-intake	51	3.80	8.10	2.50	6.27	50	5.16	9.65	2.65	6.47	-0.17	-2.86	2.52	0.899	0.03	-0.01	0.06
***Clostridium* cluster IV**	%	Pre-intake	51	6.87	5.00	-	-	50	5.22	3.83	-	-	1.65	-0.11	3.40	0.066	0.38	0.34	0.42
		Post-intake	51	3.79	2.95	2.73	4.22	50	4.38	3.35	3.96	5.46	-1.23	-2.29	-0.17	0.024*	0.46	0.42	0.50
***Clostridium* cluster IV_OTU_749**	%	Pre-intake	51	5.41	5.06	-	-	50	3.69	3.11	-	-	1.72	0.07	3.38	0.042*	0.45	0.41	0.49
		Post-intake	51	2.67	2.78	1.63	3.04	50	3.50	3.23	3.13	4.56	-1.51	-2.53	-0.50	0.004*	0.60	0.56	0.64
***Clostridium* subcluster XIVa**	%	Pre-intake	51	33.56	8.74	-	-	50	32.72	8.20	-	-	0.84	-2.50	4.19	0.619	0.10	0.06	0.14
		Post-intake	51	30.39	7.86	28.18	32.28	50	32.43	8.20	30.52	34.66	-2.36	-5.28	0.56	0.112	0.32	0.28	0.36
***Clostridium* cluster IX**	%	Pre-intake	51	3.38	4.06	-	-	50	4.18	3.84	-	-	-0.81	-2.37	0.75	0.306	0.21	0.17	0.25
		Post-intake	51	4.37	3.90	3.71	5.40	50	4.32	3.19	3.27	4.99	0.43	-0.78	1.63	0.485	0.14	0.10	0.18
***Clostridium* cluster XI**	%	Pre-intake	51	0.62	0.43	-	-	50	0.72	0.61	-	-	-0.09	-0.30	0.12	0.378	0.19	0.15	0.23
		Post-intake	51	0.50	0.44	0.32	0.70	50	0.58	0.85	0.39	0.77	-0.07	-0.34	0.20	0.603	0.11	0.07	0.14
***Clostridium* cluster XVIII**	%	Pre-intake	51	0.18	0.57	-	-	50	0.24	0.86	-	-	-0.06	-0.35	0.23	0.680	0.09	0.05	0.13
		Post-intake	51	0.23	0.65	0.14	0.36	50	0.26	0.77	0.12	0.34	0.02	-0.14	0.17	0.819	0.05	0.01	0.09
others	%	Pre-intake	51	14.13	4.56	-	-	50	13.61	4.77	-	-	0.52	-1.32	2.37	0.575	0.11	0.07	0.15
		Post-intake	51	14.71	5.37	13.49	15.67	50	13.73	3.65	12.77	14.97	0.71	-0.85	2.26	0.368	0.18	0.14	0.22

n, Number of participants; Mean, Mean value; SD, Standard deviation; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; d, Effect size; *Significantly different ($P < 0.05$)

Among OTUs, the proportion of *Bacteroides*_OTU_469 was significantly higher in the *L. paracasei* 327 group compared to the placebo group in the post-intake examination ($P = 0.020$), the proportion of *Clostridium* cluster IV_OTU_749 was significantly higher in the *L. paracasei* 327 group compared to the placebo group in the pre-intake examination ($P = 0.042$) and significantly lower in the *L. paracasei* 327 group compared to the placebo group in the post-intake examination ($P = 0.004$).

The OTUs for which the number of participants increased after the intervention due to the consumption of the *L. paracasei* 327 are shown in Table 5. The number of participants with an increased proportion of *Clostridium* subcluster XIVa_OTU_754 was significantly higher in the *L. paracasei* 327 group ($P = 0.030$). The number of participants with an increased level of *Clostridium* cluster IX, *Akkermansia*_OTU_110, tended to be higher in the *L. paracasei* 327 group ($P = 0.051$).

Table 5. Participants with increased OTUs after intervention

Items	*L. paracasei* 327			Placebo			Between-group comparison				
	n	Number of applicable participants	Percentage of applicable participants (%)	n	Number of applicable participants	Percentage of applicable participants (%)	Δ (%)	95% CI-	95% CI+	χ^2	*P*
***Clostridium* cluster IX, *Akkermansia*_OTU_110**	51	38	74.5	50	28	56.0	18.5	-0.1	37.1	3.820	0.051
***Clostridium* subcluster XIVa_OTU_754**	51	25	49.0	50	14	28.0	21.0	2.0	40.0	4.706	0.030*

n, Number of participants; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; *Significantly different ($P < 0.05$)

SECONDARY OUTCOMES

Intestinal putrefactive products and organic acids (The analysis data set was FAS1): The results of the analysis of intestinal putrefactive product and organic acids are shown in Table 6.

We found no items that showed significant differences in the between-group comparisons. On the other hand, intra-group comparisons revealed higher levels of acetic acid ($P = 0.038$), propionic acid ($P = 0.029$), iso-butyric acid ($P = 0.011$), iso-valeric acid ($P = 0.010$), and n-valeric acid ($P = 0.026$) in the *L. paracasei* 327 group after intervention. In the placebo group, propionic acid ($P = 0.034$) and n-butyric acid ($P = 0.012$) were higher after intervention Table 7.

Table 6. Intestinal putrefactive products and organic acids

Items	unit	point	*L. paracasei* 327					Placebo					Between-group comparison				Effect size		
			n	Mean	SD	95% CI-	95% CI+	n	Mean	SD	95% CI-	95% CI+	Δ	95% CI-	95% CI+	*P*	d	95% CI-	95% CI+
Indole	μmol/kg	Pre-intake	51	281.03	228.37	-	-	50	325.13	278.09	-	-	-44.10	-144.73	56.53	0.386	0.18	0.14	0.22
		Post-intake	51	369.95	313.85	299.48	461.63	50	387.20	316.06	294.50	458.27	4.18	-111.28	119.63	0.943	0.01	-0.02	0.05
Skatol	μmol/kg	Pre-intake	51	124.72	482.79	-	-	50	66.11	142.55	-	-	58.62	-82.56	199.79	0.409	0.22	0.18	0.26
		Post-intake	51	77.00	165.36	21.79	126.83	50	77.82	213.33	27.52	133.60	-6.25	-81.02	68.52	0.869	0.03	-0.01	0.07
p-Clesol	μmol/kg	Pre-intake	51	563.30	512.00	-	-	50	722.76	690.57	-	-	-159.46	-400.13	81.22	0.191	0.28	0.24	0.32
		Post-intake	51	495.21	445.92	382.96	635.81	50	466.03	481.11	323.89	579.27	57.80	-122.67	238.27	0.527	0.13	0.09	0.17
Phenol	μmol/kg	Pre-intake	51	55.93	114.00	-	-	50	58.17	108.17	-	-	-2.24	-46.12	41.63	0.919	0.02	-0.02	0.06
		Post-intake	51	75.54	151.82	47.55	104.67	50	49.32	65.38	19.89	77.58	27.37	-13.22	67.97	0.184	0.27	0.23	0.31
4-Ethylphenol	μmol/kg	Pre-intake	51	14.05	31.91	-	-	50	12.51	31.74	-	-	1.55	-11.02	14.12	0.807	0.05	0.01	0.09
		Post-intake	51	9.88	21.65	4.56	15.18	50	6.91	15.91	1.56	12.29	2.94	-4.61	10.49	0.442	0.16	0.12	0.20
Acetic acid	mmol/kg	Pre-intake	51	43.80	18.24	-	-	50	44.19	21.35	-	-	-0.39	-8.24	7.46	0.922	0.02	-0.02	0.06
		Post-intake	51	52.04	24.76	45.97	58.20	50	51.24	19.85	45.01	57.36	0.90	-7.79	9.59	0.838	0.04	0.00	0.08
Propionic acid	mmol/kg	Pre-intake	51	14.84	8.05	-	-	50	15.75	7.64	-	-	-0.90	-4.00	2.19	0.564	0.12	0.08	0.16
		Post-intake	51	19.62	13.66	16.40	22.93	50	19.41	9.30	16.06	22.66	0.30	-4.34	4.95	0.897	0.03	-0.01	0.07
iso-Butyric acid	mmol/kg	Pre-intake	51	0.05	0.34	-	-	50	0.10	0.52	-	-	-0.05	-0.23	0.12	0.544	0.13	0.09	0.17
		Post-intake	51	0.48	1.27	0.17	0.80	50	0.27	1.00	-0.06	0.58	0.23	-0.22	0.67	0.319	0.20	0.16	0.24
n-Butyric acid	mmol/kg	Pre-intake	51	7.04	5.59	-	-	50	6.57	3.65	-	-	0.47	-1.39	2.33	0.618	0.11	0.07	0.15
		Post-intake	51	7.93	5.43	6.54	9.14	50	8.54	4.48	7.32	9.94	-0.79	-2.64	1.06	0.399	0.17	0.13	0.21
iso-Valeric acid	mmol/kg	Pre-intake	51	0.06	0.42	-	-	50	0.10	0.51	-	-	-0.04	-0.23	0.14	0.641	0.10	0.06	0.14
		Post-intake	51	0.60	1.62	0.24	1.00	50	0.32	1.18	-0.08	0.70	0.31	-0.23	0.86	0.258	0.23	0.19	0.27
n-Valeric acid	mmol/kg	Pre-intake	51	0.04	0.27	-	-	50	0.00	0.00	-	-	0.04	-0.04	0.11	0.322	0.28	0.24	0.32
		Post-intake	51	0.45	1.31	0.14	0.72	50	0.13	0.66	-0.14	0.44	0.28	-0.13	0.69	0.177	0.27	0.24	0.31
Lactic acid	mmol/kg	Pre-intake	51	0.30	1.16	-	-	50	0.21	0.93	-	-	0.09	-0.32	0.51	0.657	0.09	0.05	0.13
		Post-intake	51	0.00	0.00	-0.07	0.07	50	0.05	0.37	-0.02	0.13	-0.05	-0.16	0.05	0.322	0.20	0.16	0.24
Succinic acid	mmol/kg	Pre-intake	51	1.37	1.66	-	-	50	3.44	7.49	-	-	-2.07	-4.25	0.11	0.062	0.52	0.48	0.56
		Post-intake	51	1.77	4.38	1.57	3.98	50	2.83	8.63	0.58	3.02	0.97	-0.76	2.70	0.267	0.23	0.19	0.26
Formic acid	mmol/kg	Pre-intake	51	0.00	0.00	-	-	50	0.03	0.23	-	-	-0.03	-0.10	0.03	0.322	0.29	0.25	0.33
		Post-intake	51	0.00	0.00	-0.06	0.06	50	0.04	0.31	-0.02	0.11	-0.04	-0.13	0.04	0.310	0.21	0.17	0.25

n, Number of participants; Mean, Mean value; SD, Standard deviation; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; d, Effect size

Table 7. Intestinal organic acids

Items	unit	Intervention	Pre-intake			Post-intake			Intra-group comparison			
			n	Mean	SD	n	Mean	SD	Δ	95% CI-	95% CI+	*P*
Acetic acid	mmol/kg	*L. paracasei* 327	51	43.80	18.24	51	52.04	24.76	8.24	0.49	15.98	0.038*
		Placebo	50	44.19	21.35	50	51.24	19.85	7.05	-0.12	14.22	0.054
Propionic acid	mmol/kg	*L. paracasei* 327	51	14.84	8.05	51	19.62	13.66	4.77	0.52	9.02	0.029*
		Placebo	50	15.75	7.64	50	19.41	9.30	3.66	0.29	7.04	0.034*
iso-Butyric acid	mmol/kg	*L. paracasei* 327	51	0.05	0.34	51	0.48	1.27	0.43	0.10	0.76	0.011*
		Placebo	50	0.10	0.52	50	0.27	1.00	0.17	-0.15	0.50	0.295
n-Butyric acid	mmol/kg	*L. paracasei* 327	51	7.04	5.59	51	7.93	5.43	0.89	-0.71	2.49	0.270
		Placebo	50	6.57	3.65	50	8.54	4.48	1.97	0.46	3.49	0.012*
iso-Valeric acid	mmol/kg	*L. paracasei* 327	51	0.06	0.42	51	0.60	1.62	0.55	0.14	0.95	0.010*
		Placebo	50	0.10	0.51	50	0.32	1.18	0.22	-0.15	0.59	0.238
n-Valeric acid	mmol/kg	*L. paracasei* 327	51	0.04	0.27	51	0.45	1.31	0.41	0.05	0.77	0.026*
		Placebo	50	0.00	0.00	50	0.13	0.66	0.13	-0.06	0.32	0.162
Lactic acid	mmol/kg	*L. paracasei* 327	51	0.30	1.16	51	0.00	0.00	-0.30	-0.63	0.03	0.072
		Placebo	50	0.21	0.93	50	0.05	0.37	-0.15	-0.44	0.13	0.289
Succinic acid	mmol/kg	*L. paracasei* 327	51	1.37	1.66	51	1.77	4.38	0.40	-0.91	1.71	0.546
		Placebo	50	3.44	7.49	50	2.83	8.63	-0.61	-1.71	0.48	0.268
Formic acid	mmol/kg	*L. paracasei* 327	51	0.00	0.00	51	0.00	0.00	NA	NA	NA	NA
		Placebo	50	0.03	0.23	50	0.04	0.31	0.01	-0.10	0.12	0.841

n, Number of participants; Mean, Mean value; SD, Standard deviation; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; *Significantly different (*P*<0.05)

Effects on defecation days, defecation frequency, defecation volume, and fecal conditions (The analysis data set was FAS2): The results of the analysis of defecation days, defecation frequency, and defecation volume are shown in Table 8. Defecation days was significantly higher (*P* = 0.020) in the *L. paracasei* 327 group compared to the placebo group during Period 3.

Defecation frequency was significantly higher (*P* = 0.010, *P* = 0.001) in the *L. paracasei* 327 group compared to the placebo group during periods 2 and 3. No significant improvement in shape, color, odor, or feeling after defecation was observed with consumption of the *L. paracasei* 327 (data not shown).

Table 8. Defecation days, defecation frequency, defecation volume and CAS-MT score

Item	unit	point	*L. paracasei* 327					Placebo					Between-group comparison				Effect size		
			n	Mean	SD	95% CI-	95% CI+	n	Mean	SD	95% CI-	95% CI+	Δ	95% CI-	95% CI+	P	d	95% CI-	95% CI+
Defecation days	days	Period 1	55	4.0	0.9	-	-	53	4.0	0.8	-	-	0.0	-0.3	0.4	0.817	0.05	0.01	0.08
		Period 2	55	5.1	1.5	4.7	5.5	53	4.7	1.7	4.3	5.1	0.4	-0.2	1.0	0.164	0.23	0.19	0.27
		Period 3	55	5.5	1.2	5.1	5.9	53	4.8	1.5	4.4	5.2	0.7	0.1	1.3	0.020*	0.39	0.35	0.42
Defecation frequency	times	Period 1	55	4.1	0.9	-	-	53	4.2	0.9	-	-	-0.1	-0.4	0.3	0.642	0.09	0.05	0.13
		Period 2	55	6.1	3.0	5.5	6.8	53	5.0	2.0	4.4	5.6	1.2	0.3	2.0	0.010*	0.43	0.39	0.47
		Period 3	55	6.7	2.1	6.1	7.3	53	5.2	1.9	4.6	5.8	1.5	0.6	2.3	0.001*	0.54	0.51	0.58
Defecation volume	pieces	Period 1	55	27.6	25.9	-	-	53	21.4	20.3	-	-	6.2	-2.7	15.1	0.168	0.28	0.24	0.31
		Period 2	55	37.0	34.2	27.2	43.0	53	28.6	31.6	22.5	38.6	4.5	-6.8	15.8	0.433	0.14	0.11	0.18
		Period 3	55	43.2	32.9	33.7	49.5	53	30.5	30.2	24.1	40.2	9.4	-1.9	20.8	0.101	0.30	0.27	0.34
CAS-MT score	point	Pre-intake	55	4.8	2.7	-	-	53	4.7	2.6	-	-	0.1	-0.9	1.1	0.900	0.02	-0.01	0.06
		Post-intake	55	3.3	2.5	2.7	3.9	53	4.2	2.9	3.7	4.9	-1.0	-1.8	-0.1	0.029*	0.43	0.40	0.47

n, Number of participants; Mean, Mean value; SD, Standard deviation; 95% CI, 95% Confidence Interval; Δ, Difference between groups; d, Effect size; P, Probability value; *Significantly different (P<0.05)

The Japanese version of the Constipation Assessment Scale (CAS-MT) (The analysis data set was FAS2): The results of the analysis of the CAS-MT score are shown in Table 8. The CAS-MT score was significantly lower (P = 0.029) in the *L. paracasei* 327 group compared to the placebo group in the post-intake examination.

The OSA Sleep Inventory MA version (The analysis data set was FAS2): No significant improvement in the OSA Sleep Inventory MA version was observed with consumption of the *L. paracasei* 327 (data not shown).

Supplementary analysis using synthetic variables (The analysis data set was FAS1): To evaluate the relationship between changes in intestinal microbiota and putrefactive products, we conducted an analysis using synthetic variables (Table 9.). The *L. paracasei* 327 group showed significantly lower values than the placebo group for "Increased *Clostridium* cluster IV_OTU_749 and increased Scatol (P = 0.031)" and "Increased *Clostridium* cluster IV_OTU_749 and increased phenol (P < 0.001)".

Table 9. Analysis of intestinal microbiota and putrefactive products

Items	*L. paracasei* 327			Placebo			Between-group comparison			
	n	Number of applicable participants	Percentage of applicable participants (%)	n	Number of applicable participants	Percentage of applicable participants (%)	Δ (%)	95% CI-	95% CI+	*P*
Increased *Clostridium* cluster IV_OTU_749 and increased Scatol	51	4	7.8	50	12	24.0	-16.2	-30.4	-1.9	0.031*
Increased *Clostridium* cluster IV_OTU_749 and increased phenol	51	3	5.9	50	17	34.0	-28.1	-43.7	-12.6	0.000*

n, Number of participants; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; *Significantly different (*P*<0.05)

To evaluate the relationship between changes in intestinal microbiota and defecation frequency, we conducted an analysis using synthetic variables (Table 10.). The *L. paracasei* 327 group showed significantly higher values than the placebo group for "increased *Bacteroides*, decreased *Clostridium* cluster IV and higher defecation frequency in period 3 (P = 0.038)", "increased *Clostridium* subcluster XIVa_OTU_754 and higher defecation frequency in period 3 (P =0.050)" and "increased *Clostridium* cluster IX, *Akkermansia*_OTU_110 and higher defecation frequency in period 3 (P = 0.005)"

Table 10. Analysis of intestinal microbiota and defecation frequency

Items	*L. paracasei* 327			Placebo			Between-group comparison			
	n	Number of applicable participants	Percentage of applicable participants (%)	n	Number of applicable participants	Percentage of applicable participants (%)	Δ (%)	95% CI-	95% CI+	*P*
Increased *Bacteroides*, decreased *Clostridium* cluster IV and increased defecation frequency in period 3	51	21	41.2	50	11	22.0	19.2	1.0	37.3	0.038*
Increased *Clostridium* subcluster XIVa_OTU_754 and increased defecation frequency in period 3	51	20	39.2	50	10	20.0	19.2	1.4	37.0	0.050*
Increased *Clostridium* cluster IX, *Akkermansia*_OTU_110 and increased defecation frequency in period 3	51	31	60.8	50	16	32.0	28.8	9.3	48.2	0.005*

n, Number of participants; 95% CI, 95% Confidence Interval; Δ, Difference between groups; *P*, Probability value; *Significantly different (*P*<0.05)

Safety assessment (The analysis data set was SAF): Three participants in the *L. paracasei* 327 group and three participants in the placebo group had adverse events, but recovered following administration of medication. The principal investigator determined that none of the adverse events were causally related to the *L. paracasei* 327 or placebo. Therefore, there were no adverse events attributable to the *L. paracasei* 327 or placebo.

DISCUSSION

In this study, healthy Japanese men and women between the ages of 20 and 64 with a defecation frequency of three to five times per week were asked to consume 25 mg (approximately $5x10^{10}$) of sterile *L. paracasei* 327 once a day for two weeks to verify its effect on the intestinal environment.

In the intestinal microbiota analysis by taxonomic group at the two-week post-intake examination, the proportion of *Clostridium* cluster IV was significantly lower and *Bacteroides* was higher in the *L. paracasei* 327 group than in the placebo group (Table 4.). By OTUs, while proportions of *Bacteroides*_OTU_469 and *Clostridium* subcluster XIVa_OTU_754 were significantly higher, the proportion of *Clostridium* cluster IV_OTU_749 was significantly lower in the *L. paracasei* 327 group than in the placebo group. In addition, the proportion of *Clostridium* cluster IX, *Akkermansia*_OTU_110, tended to be higher, in the *L. paracasei* 327 group than in the placebo group (Table 4., 5., 11.). These results suggest that the consumption of the *L. paracasei* 327 altered the composition of the intestinal microbiota (Table 5., 11.).

Table 11. Summary of intestinal microbiota variation

Taxonomic group	Operational Taxonomic Unit (OTU)	Variation	*P*	Presumed bacterial species
Bacteroides		↑	$P < 0.100$	
	*Bacteroides*_OTU_469	↑	$P < 0.050$	*Phocaeicola vulgatus*
				Bacteroides faecis
				Bacteroides uniformis
				Bacteroides fragilis
				Bacteroides rodentium
				Bacteroides faecichinchillae
				Bacteroides ovatus
				Bacteroides nordii
				Bacteroides intestinalis
				Bacteroides caccae
				Bacteroides stercoris
				Phocaeicola dorei
				Parabacteroides distasonis
				Parabacteroides merdae
				Phocaeicola vulgatus
***Clostridium* cluster IV**		↓	$P < 0.050$	
	Clostridium cluster IV_OTU_749	↓	$P < 0.050$	*Faecalibacterium prausnitzii*
				Subdoligranulum variabile
				Sutterella stercoricanis
Clostridium* cluster IX, *Akkermansia		-	-	
	Clostridium cluster IX, *Akkermansia*_OTU_110	↑	$P < 0.100$	*Megamonas funiformis*
				Megasphaera hexanoica
				Megasphaera elsdenii
				Veillonella dispar
				Veillonella ratti
				Akkermansia muciniphila
				Bifidobacterium animalis
***Clostridium* subcluster XIVa**		-	-	
	Clostridium subcluster XIVa_OTU_754	↑	$P < 0.050$	*Roseburia hominis*
				Ruminococcus gnavus
				Pseudobutyrivibrio ruminis / Pseudobutyrivibrio xylanivorans
				Clostridium nexile
				Anaerostipes hadrus
				Eubacterium hallii

Bacterial species presumed to belong to *Bacteroides*_OTU_469, *Clostridium* subcluster XIVa_OTU_754 and *Clostridium* cluster IX, *Akkermansia*_OTU_110 are considered to be associated with the production of short-chain fatty acids. In *Bacteroides*_OTU_469, in vitro studies have reported that *Bacteroides fragilis* produces acetic acid [35], and *Bacteroides caccae* [36], *Bacteroides ovatus* [37] and *Phocaeicola* (*Bacteroides*) *vulgatus* [38] produce acetic acid and propionic acid. In vivo studies have reported that production of acetic acid and propionic acid has been observed when *Bacteroides ovatus* was established alone in germ-free mice [37]. In *B. uniformis*–monoassociated mice, short-chain fatty acids (acetic acid, propionic acid, iso-butyric acid, and iso-valeric acid) in feces were found to be increased compared to germ-free mice [39].

Furthermore, *Bacteroides stercoris* and *Bacteroides fragilis* have also been postulated as "keystone species" affecting the structure of the human intestinal microbiota, including the growth of butyrate-producing bacteria [40-41]. *Roseburia hominis* and *Eubacterium hallii* presumed to belong to *Clostridium* subcluster XIVa_OTU_754*,* have been identified as butyrate-producing bacteria [42]. In *Clostridium* cluster IX *Akkermansia*_OTU_110, *Megasphaera elsdenii* has been identified as a propionic acid-producing bacterium, *Akkermansia* muciniphila as an acetic acid- and propionic acid-producing bacterium[43-44]. Therefore, it is possible that variations in the composition ratio of these bacteria may affect the production of short-chain fatty acids, as confirmed in this study.

Intestinal organic acid measurements were compared pre- and post- ingestion, and two items, propionic acid and n-butyric acid, were significantly higher in the placebo group, whereas five items, acetic acid, propionic acid, iso-butyric acid, iso-valeric acid, and n-valeric acid, were significantly higher in the *L. paracasei* 327 group (Table 7.). The significant increase in more items in the *L. paracasei* 327 group compared to the placebo group, where pre- and post-intervention changes in short-chain fatty acids were not uniform, suggests that the composition of the intestinal microbiota, which varied in the *L. paracasei* 327 group, may be involved in the production of short-chain fatty acids. It is possible that the participants in this study were not fed a restricted or constant diet, so that significant differences in intestinal putrefactive products and organic acid measurements could not be identified in between-group comparisons [45].

Bacterial species presumed to belong to *Clostridium* cluster IV_OTU_749 are known to be involved in the production of amino acid metabolites such as phenol and skatole in the intestine [46]. Although the study was unable to confirm significant differences between groups for each of the putrefactive parameters (Table 6.), the percentage of participants with “increased *Clostridium* cluster IV_OTU_749 and increased levels of scatole or phenol” was significantly lower in the *L. paracasei* 327 group than in the placebo group (Table 9.). From this result, it was inferred that the consumption of the *L. paracasei* 327 suppressed the accumulation of putrefactive products associated with the growth of *Clostridium* cluster IV_OTU_749, especially *Faecalibacterium prausnitzii* and *Subdoligranulum variabile*. Considering these results and reports, it is possible that consumption of the *L. paracasei* 327 increased the bacterial species involved in short-chain fatty acid production and suppressed those involved in putrefactive production.

In the bowel diary, defecation days during the second week of intake (from day 8 to the day before the two-week post-ingestion examination), the defecation frequency during the first week of intake (from the start of intake to day 7), and the second week of intake were all significantly higher in the *L. paracasei* 327 group than in the placebo group (Table 8.). These results were consistent with a previous study in which approximately 5×10^{10} bacteria/day of sterilized *L. paracasei* 327 was

ingested by constipation-prone healthy participants and showed improvement in defecation frequency, number of defecation days, and volume of defecation [30]. In addition to bowel diaries, the study also showed that CAS scores on the CAS-MT were significantly lower in the *L. paracasei* 327 group than in the placebo group after intake for two weeks (Table 8.). The CAS-MT uses eight questions to evaluate defecation on a 3-point scale from 0 to 2. A lower score for each question indicates no problem with the corresponding question, and the scores for each question are added together for the overall CAS-MT score. It has been reported that CAS-MT scores are higher in constipated participants than in healthy participants [33]. Since no significant differences between the two groups were found after the intervention based on the dietary survey performed in this study (data not shown), the results of this study were not considered to be due to group differences in the dietary habits of the study participants. Therefore, the results of this study suggest that the *L. paracasei* 327 may be beneficial for improving defecation in individuals who are not constipated but have a lower defecation frequency than the general Japanese population.

Interestingly, Tanabe et al. in a survey study of healthy Japanese participants reported that *Bacteroides* occupancy was significantly lower, and the *Clostridium* cluster IV composition ratio was significantly higher in those who were determined to be constipated than in those who were not [47]. A study comparing the intestinal microbiota of healthy Italians and participants with functional constipation found that the percentage of *Bacteroides* was significantly lower in participants with functional constipation than in healthy Italians [48]. Therefore, there could be a relationship between the analysis of intestinal microbiota in this study and the results of bowel diaries.

In order to investigate the relationship between the intestinal microbiota analysis and the results of the bowel diary, a between-group comparison was conducted on the number of participants in which the defecation frequency changed along with the composition of the intestinal microbiota before and after the intervention. The results showed that the percentage of participants with "increased *Bacteroides*, decreased *Clostridium* cluster IV, and increased defecation frequency in period 3" was significantly higher in the *L. paracasei* 327 group than in the placebo group (Table 10.). In addition, the percentage of participants with "increased *Clostridium* subcluster XIVa_OTU_754 and increased defecation frequency in period 3" and with "increased *Clostridium* cluster IX, *Akkermansia*_OTU_110 and increased defecation frequency in period 3" was significantly higher in the *L. paracasei* 327 group than in the placebo group (Table 10.). Therefore, in addition to *Bacteroides* and *Clostridium* cluster IV, an increase in the proportion of *Clostridium* subcluster XIVa_OTU_754 and *Clostridium* cluster IX, *Akkermansia* genus_OTU_110 could also be associated with an increase in defecation frequency. In addition, short-chain fatty acids produced by intestinal bacteria are known to promote peristalsis of the intestinal tract via the production and/or release of serotonin [49-51]. In fact, it has been reported that oral administration of *L. paracasei* 327 to mice increases serotonin-positive cells and promotes peristalsis of the intestinal tract [52]. In this study, the increase in short-chain fatty acid-producing bacteria was observed after consumption of the *L. paracasei* 327, suggesting that the short-chain fatty acids produced by the intestinal bacteria may promote the production and/or release of serotonin, which in turn may promote intestinal peristalsis and improve defecation frequency.

In summary, the results suggest that the consumption of the *L. paracasei* 327 affected the intestinal microbiota of healthy Japanese men and women aged 20 to 64 years old with a defecation frequency of three to five times per week, and improved the defecation by promoting the growth of bacteria related to the production of short-chain fatty acids and

inhibiting the growth of intestinal putrefactive product-producing bacteria, thereby improving the intestinal environment.

One limitation of this study is the possibility that the study population may affect the results. *Bacteroides* and *Clostridium* cluster IV, which were found to be affected by the intake of the *L. paracasei* 327 containing food in this study, may have been affected by defecation status [47-48,53], and variations in the proportion of these bacteria could have involved both a direct effect of the intake of the *L. paracasei* 327 containing food and an indirect effect of the variation in defecation frequency. In addition, the participants were not restricted in their diet, which may have affected the composition of the intestinal microbiota and the production of organic acids. In the future, it will be important to verify the effects of the intake of the *L. paracasei* 327 on the intestinal environment by constructing subgroups with defecation frequency as a factor and by designing a study that does not limit the participants by defecation frequency.

Finally, in the safety investigation, adverse events were identified in some participants in this study. However, in all participants, based on the criteria established at the time of study design, the principal investigator concluded that there was no causal relationship with the study food, confirming that the intake of the study food was safe under the conditions of this study.

CONCLUSION

We conducted this study to verify the effects on the intestinal environment of the consumption of food containing sterilized *L. paracasei* 327 in healthy Japanese men and women aged 20 to 64 years who had a defecation frequency of three to five times per week. Intestinal microbiota analysis after consumption for two weeks showed an increase in *Bacteroides* and a decrease in *Clostridium* cluster IV in each taxonomic group. In addition, for each OTU, *Bacteroides*_OTU_469, *Clostridium* cluster IX, *Akkermansia*_OTU_110, and *Clostridium* subcluster XIVa_OTU_754 increased, while *Clostridium* cluster IV_OTU_749 decreased. The presumptive species of bacteria in the taxonomic group and OTUs increased by *L. paracasei* 327 ingestion included short-chain fatty acid-producing bacteria. In fact, intra-group comparisons before and after ingestion showed that the number of short-chain fatty acid items that were significantly increased after the intervention was greater in the *L. paracasei* 327 intake group than in the placebo group. Analysis using synthetic variables also suggested that Clostridium cluster IV_OTU_749, which was reduced by *L. paracasei* 327 ingestion, is involved in the production of putrefactive products in the intestine. Furthermore, bowel diaries and CAS-MT showed an improvement in constipation tendency with the intake of *L. paracasei* 327. The results of this study suggest that consumption of *L. paracasei* 327 improves the intestinal environment of healthy adult Japanese by promoting the growth of short-chain fatty acid-producing bacteria, inhibiting the growth of intestinal putrefactive-producing bacteria, and improving defecation. In addition, consumption of the study foods was safe under the conditions of this study.

List of abbreviations: *L. paracasei* 327: *Lacticaseibacillus paracasei* 327, OTUs: operational taxonomic units, UMIN: University Hospital Medical Information Network, HRQoL: health-related quality of life, ISAPP: International Scientific Association for Probiotics and Prebiotics, FFC: the Functional Food Centre, FF: functional foods, ICD: implantable cardioverter defibrillator, Scr: screening, FOSHU: Foods for Specified Health Uses, CAND: calorie and nutrition diary, T-RFLP: Terminal Restriction Fragment Length Polymorphism, CAS: Constipation Assessment Scale, OSA-MA: Oguri-Shirakawa-Azumi Sleep Inventory MA version, FAS: full analysis set, ITT: intention-to-treat, SAF: safety analysis set, 95% CI: 95% Confidence Interval

Acknowledgements/Funding: We appreciate ORTHOMEDICO Inc. for supporting the work of this study as the contract research organization as well as all participants and staff who cooperated in this study. There is no funding to disclose in this study.

Competing interests: The study was sponsored by KAMEDA SEIKA CO., LTD., entrusted the study was conducted by ORTHOMEDICO Inc. Yoshie Senda-Sugimoto, Toshihiro Mihara, Yuki Higuchi, Kimiko Uchiyama, and Hajime Takahashi are employees of KAMEDA SEIKA CO., LTD.

Authors' contributions: Yoshie Senda-Sugimoto: conceptualization, formal analysis, methodology and writing-original draft; Toshihiro Mihara: conceptualization, methodology, writing-review and editing; Yuki Higuchi: conceptualization, methodology, project administration, writing-review and editing; Kimiko Uchiyama: conceptualization, methodology, writing-review and editing; Tsuyoshi Takara: investigation, methodology, writing-review and editing; Hajime Takahashi: project administration, supervision, writing-review and editing. All authors have read and agree to the published version of the manuscript.

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