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Effects of heat-killed *Lactobacillus casei* subsp. *casei* 327 intake on defecation in healthy volunteers: a randomized, double-blind, placebo-controlled, parallel-group study

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***Lactobacillus casei* subsp. *casei* 327 (*L. casei* 327) was isolated from brown rice. A preliminary study showed that intake of 50 mg of heat-killed *L. casei* 327 is effective in improving defecation. In this study, we conducted a randomized, double-blind, placebo-controlled, parallel-group trial to investigate the effect of intake of heat-killed *L. casei* 327 (25 mg: approximately 5×10^{10} bacteria) on defecation in healthy volunteers with relatively low defecation frequencies. We selected 104 healthy Japanese adults with relatively low defecation frequencies (approximately 3–5 times a week) by screening and pretrial tests. Subjects (n=52 in each group) were randomly given a tablet containing *L. casei* 327 (group A) or a placebo tablet (group P) daily for 2 weeks. After eliminating data for 9 subjects who met the exclusion criteria for efficacy analysis, data for 95 subjects were analyzed. The defecation frequency and number of defecation days during the intake period and their changes from the pretrial period were significantly higher in group A than in group P. The fecal volume during the intake period was higher in group A than group P, but the difference was not statistically significant. However, the change from the pretrial period was significantly higher in group A than in group P. There were no significant differences between groups in the values of fecal shape, color, odor, and feeling after defecation. These results suggested that intake of *L. casei* 327 improves defecation in healthy adults who have relatively low defecation frequencies.**

Key words: clinical study, defecation, healthy volunteers, lactic acid bacteria, *Lactobacillus casei*

INTRODUCTION

Foods including probiotics are familiar parts of our diet. Probiotics are defined as "living microorganisms that benefit the health of the host when ingested in a sufficient quantity" [1], and lactobacilli and bifidobacteria are most common in processed foods [2, 3]. Growing evidence suggests that these microorganisms have potential health benefits [4, 5]. In particular, lactobacilli and fermented milk using lactobacilli have intestine function conditioning [6], cholesterol lowering [7], blood pressure reduction [8], and immunomodulatory [9] effects. Moreover, the health-promoting activities of lactic acid bacteria are strain specific [10].

On the other hand, a variety of health benefits of dead, frequently heat-killed, microorganisms have been reported in studies of animals and humans [11]. Several studies have demonstrated that some heat-killed lactobacilli enhance immunity [12, 13], alleviate symptoms of atopic dermatitis [14, 15], reduce body fat [16], and improve skin hydration condition [17]. Daily consumption of beverages containing nonviable *Lactobacillus gasseri* CP2305 ameliorated the intestinal environment and intestinal functions of healthy participants [18]. Heat-killed cocci in lactic acid bacteria are also reported to improve the fecal microbiota [19] and improve skin condition [20]. Consumption of heat-killed *Enterococcus faecalis* EC-12 improved the fecal microbiota and intestinal environment of healthy volunteers [19]. However, to date, the beneficial effects of nonviable lactic acid bacteria on defecation have not been fully investigated.

Lactobacillus casei subsp. *casei* 327 (*L. casei* 327) was isolated from brown rice [21]. Orally ingested *L. casei* 327 reached the intestinal tract, and *L. casei* 327-supplemented fermented milk improved the intestinal environment [22] and defecation [21] and suppressed urinary and fecal mutagen levels that had been induced by burned beef consumption [23]. Intake of heat-killed *L. casei* 327 improved skin condition in healthy female volunteers [24, 25]. In addition, a preliminary study we performed suggested the benefit of heat-killed *L. casei* 327 (50 mg) on defecation in healthy volunteers with relatively low defecation frequencies (our unpublished results). In this study, we conducted a randomized, double-blind, placebo-controlled, parallel-group trial to investigate the beneficial effects of low-dose (25 mg) intake of heat-killed *L. casei* 327 on defecation in healthy volunteers with relatively low defecation frequencies.

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Table 1. Composition of the test tablets

	Placebo tablet	Active tablet
Ingredients	Glucose, starch, calcium stearate, fine granular silica, hydroxypropylcellulose	*L. casei* subsp. *casei* 327[a], glucose, starch, calcium stearate, fine granular silica, hydroxypropylcellulose
Nutritional facts (value for daily dose, 0.25 g)		
Energy (kcal)	0.9	0.9
Protein (g)	0.00	0.02
Fat (g)	0.00	0.01
Carbohydrate (g)	0.22	0.20
Sodium (mg)	0.05	0.16

[a]The content of *L. casei* subsp. *casei* 327 was 25 mg (approximately 5×10^{10} bacteria) per daily dose (0.25 g; one tablet).

MATERIALS AND METHODS

Test foods

To prepare the heat-killed *L. casei* 327, cultured *L. casei* 327 was washed, heated at 120°C for 10 s with a plate heater, and dried with a spray dryer. Nonviability was confirmed with BCP plate count agar. The dietary supplements used in this study were *L. casei* 327-containing tablets and placebo tablets (Kameda Seika Co., Ltd., Niigata, Japan). The compositions of the tablets are shown in Table 1. The content of *L. casei* 327 was 25 mg (approximately 5×10^{10} bacteria) per daily dose (0.25 g: one tablet). Subjects consumed one tablet once a day with a drink for 2 weeks.

Subjects and study design

This study was a randomized, doubled-blind, parallel-group, placebo-controlled study. It was conducted according to the Principles of the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects issued by the Ministry of Health, Labour and Welfare, Japan. The study protocol was approved by the institutional review board of Ueno Clinic (Tokyo, Japan) on May 11, 2017 (Approval Number: 170511-1), and a change in the number of subjects from 100 to 104 was approved by the institutional review board of Ueno Clinic on July 8, 2017 (Approval Number: 170713-6). The study lasted from May 2017 to September 2017 and was registered as UMIN000027398 in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry.

The study details were disclosed to subjects before their enrollment, and investigators obtained their informed consent. Then, subjects underwent a screening test (lifestyle questionnaire, medical interview, somatometry, and physical examination). Healthy adults aged 20–64 years who had relatively low defecation frequencies (approximately 3–5 times a week) were recruited for the study. The exclusion criteria were as follows: (i) regular ingestion of any food or medicine rich in lactic acid bacteria (such as yogurt, beverages, health foods, supplements, or pharmaceuticals that contained lactic acid bacteria); (ii) regular ingestion of any food or medicine rich in natto bacteria (such as natto, health foods, supplements, or pharmaceuticals that contained natto bacteria); (iii) regular ingestion of any food, medicine, or dietary fiber that affects bowel movement; (iv) use of any laxative at the time of the screening test or regular use of such medication; (v) possible allergy to the test food; (vi) any gastrointestinal disease or a history of gastrointestinal surgery, except appendectomy; (vii) any bowel disease that affects bowel movement such as irritable bowel syndrome and ulcerative colitis; (viii) suffering from asthma or expectation of an asthma attack during the study; (ix) performing shift work or planning to work the night shift during the study; (x) plan to travel abroad during the study; (xi) serious diseases (such as diabetes mellitus or liver, kidney, or heart failure) or a case history of such; (xii) being under treatment for any disease except eyestrain; (xiii) having a history of values that are outside screening test reference ranges or abnormality of cardiopulmonary function and judged unsuitable for participation in the trial; (xiv) values that are considerably outside screening test reference ranges; (xv) participation in any other clinical trial or plan to do so after providing informed consent to participate in the study; (xvi) plan to get pregnant or nurse a baby during the study; (xvii) judgment of unsuitability based on lifestyle questionnaire responses; and (xviii) judgment of unsuitability by the principal investigator.

Eligible subjects kept a log of their stool status, physical conditions, and usage of medicine starting 2 weeks before pretrial testing (medical interview, somatometry, physical examination, and laboratory tests). Based on the results of the pretrial testing and analysis of log data, 104 of the eligible subjects were randomly and sequentially assigned using random number tables to one of two masked product groups: those receiving *L. casei* 327-containing tablets (group A; n=52) and those receiving placebo tablets (group P; n=52). The required number of subjects was calculated based on the findings of a preliminary clinical trial investigating the effect of *L. casei* 327 50 mg on defecation. TTC Co., Ltd. (Tokyo, Japan), performed the allocation, sealed the envelope containing the allocation table, and eventually opened the envelope; thus the subjects, those who recruited the subjects, and the investigators remained blind to all allocation information during the trial. After randomization,

it was confirmed that the two groups had similar defecation frequency distributions.

The subjects ingested the test tablets daily for 2 weeks and recorded their physical conditions and usage of medicine. Clinical surveys (medical interview, somatometry, and physical examination) were administered after the intake period of the test tablets. During the trial, the principal investigator and assistants instructed the participants (i) to avoid lifestyle changes such as to food, drinking, exercise, and sleeping; (ii) to avoid overexercising, undereating, and overeating; (iii) to not consume health foods (foods for specified health uses, foods with function claims, and dietary supplements); (iv) to not change their habits regarding dietary intake of foods that contain dietary fiber, lactic acid bacteria, oligosaccharides, or natto bacteria; (v) to refrain from using medicine; (vi) to go to bed by 12:00 a.m. and sleep well on the day before the clinical survey. In addition, the subjects were prohibited from (i) drinking alcohol from the day before testing until the completion of testing and (ii) eating and drinking (except water) from 10:00 p.m. on the day before testing until the completion of testing.

The primary outcome measures were defecation frequency and fecal volume, and the secondary outcome measures were defecation days and fecal condition (fecal shape, fecal color, fecal odor, and feeling after defecation). Efficacy analysis was performed on data from subjects who completed the study. However, we excluded from final analysis (i) those who took less than 80% of the stated number of tablets, (ii) those who could not keep accurately a log of their defecation status due to diarrhea derived from anything except the test foods, (iii) those who did not keep an adequate log or whose behavior cast doubt on the reliability of their clinical data, (iv) those who met the exclusion criteria after enrollment or who did not follow the required restrictions, and (v) those for whom there were justifiable reasons for exclusion. To analyze the safety of consuming the test tablets, we monitored the development of adverse events in subjects who consumed the test tablet at least once.

Measurements of defecation frequency, fecal volume, defecation days, and fecal condition

During the study, the number of defecations, fecal volume, shape, color, odor, and the feeling after defecation were recorded daily in journals. Subjects estimated the fecal volume as the number of cylindrical columns (diameter 2.5 cm × length 5 cm). Fecal shape was scored was as follows: 1, hard or separate lumps; 2, hardish; 3, banana-like; 4, soft or coil-like; and 5, pulpy or watery. Fecal color was scored as follows: 1, yellow; 2, ocher; 3, brown; 4, dark brown; and 5, black. Fecal odor was scored as follows: 1, odorless; 2, a little odor; 3, normal; 4, strong; and 5, very strong. Feeling after defecation was scored as follows: 1, refreshed; 2, mostly refreshed; 3, no feeling; 4, feeling of somewhat incomplete defecation; and 5, feeling of incomplete defecation.

Statistical analysis

All measured values are expressed as the mean ± standard deviation (SD) or standard error (SE). Regarding defecation frequency, fecal volume, and defecation days, the values during the pretrial and intake periods and the changes from the pretrial values were compared statistically between group A and group P by using the unpaired Student's t-test; for fecal conditions, the values during the pretrial and intake periods were compared statistically between groups A and P by using the Mann-Whitney *U* test. Values with $p<0.05$ were considered statistically significant. The data for defecation frequency, fecal volume, and defecation days were analyzed with the Microsoft Excel 2013 software (Microsoft, Redmond, WA, USA). The data for fecal condition were analyzed with the IBM SPSS Statistics 23 software (IBM, Armonk, NY, USA).

RESULTS

Subjects

A study outline is shown in Fig. 1. From among the applicants who provided written informed consent (n=397) and completed screening and pretrial tests, we selected the 104 subjects who had relatively low defecation frequencies (approximately 3–5 times a week) and allocated them to either the active (*L. casei* 327-containing tablet; group A, n=52) or placebo (n=52) treatment. All subjects completed the study. Nine subjects (2 in group A and 7 in group P) were eliminated from the efficacy analysis because they met the exclusion criteria for efficacy analysis. Consequently, the data for 95 subjects (50 in group A and 45 in group P) were analyzed. The safety analysis set included all participants (n=52 per group). The background characteristics for each group are shown in Table 2. There were no significant differences between group A and P in age, height, body weight, body mass index, systolic blood pressure, diastolic blood pressure, and pulse rate.

Effects on defecation frequency, defecation days, fecal volume, and fecal conditions

Defecation frequency, defecation days, and fecal volume during the pretrial and intake periods and their changes from the pretrial values are summarized in Table 3. Defecation frequency during the intake period was significantly higher in group A than in group P ($p=0.020$), and its change from the pretrial values was also significantly higher in group A than in group P ($p=0.025$). Defecation days during the intake period was significantly higher in group A than in group P ($p=0.024$), and its change from the pretrial values was also significantly higher in group A than in group P ($p=0.028$). Fecal volume during the intake period was higher in group A than group P, but there were no significant differences between group A and P. However, its change from the pretrial value was significantly higher in group A than in group P ($p=0.033$).

Fecal condition during the pretrial and intake periods is summarized in Table 4. Fecal shape scores of both groups were higher during the intake period compared with those during the pretrial period. Fecal color and feeling after defecation

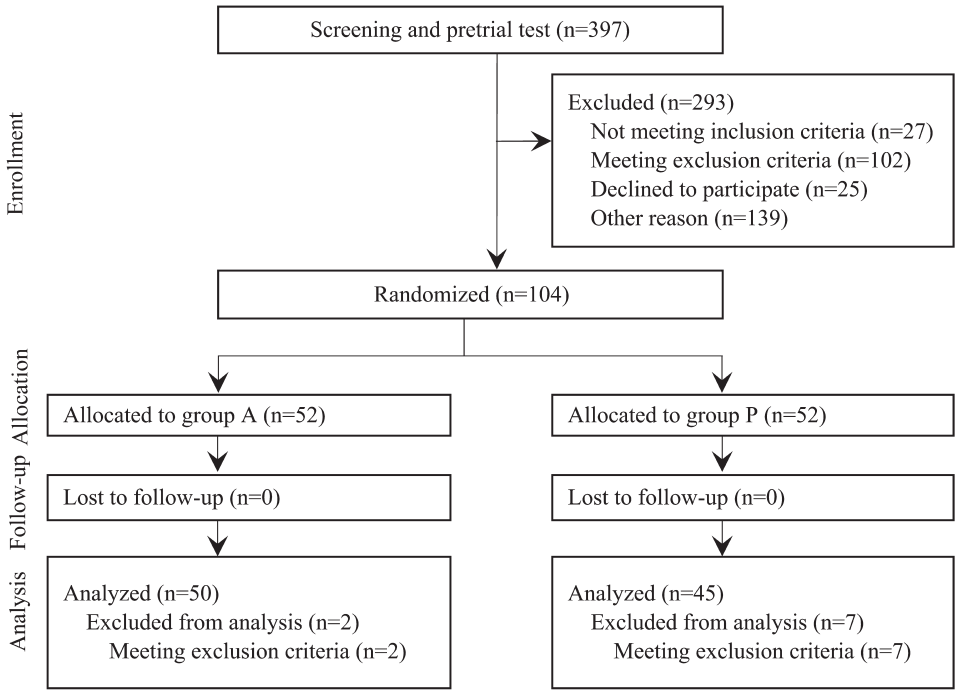

Fig. 1. Outline of the study.

Subjects in group A consumed tablets containing *L. casei* 327, and subjects in group P consumed placebo tablets. Exclusion before randomization due to judgment by the investigator (n=139) was mainly based on defecation frequency, fecal volume, and fecal condition.

Table 2. Background characteristics of the subjects analyzed for efficacy

Item	Group	Observed value	p value
Number of subjects	A	50	0.838
	P	45	
Age (years)	A	46.2 ± 9.9	0.711
	P	45.4 ± 10.4	
Height (cm)	A	164.24 ± 7.49	0.760
	P	163.76 ± 7.76	
Body weight (kg)	A	61.00 ± 10.50	0.473
	P	59.49 ± 9.84	
Body mass index (kg/m^2)	A	22.54 ± 3.02	0.423
	P	22.08 ± 2.51	
Systolic blood pressure (mmHg)	A	118.1 ± 14.5	0.968
	P	118.3 ± 16.0	
Diastolic blood pressure (mmHg)	A	71.7 ± 11.0	0.949
	P	71.6 ± 11.0	
Pulse rate (beats/min)	A	73.7 ± 10.2	0.843
	P	74.2 ± 12.2	

Each value is expressed as the mean ± SD. The p values were determined by unpaired Student's t-tests.

scores of both groups were lower during the intake period compared with those during the pretrial period. Fecal odor scores of both groups during the intake period hardly changed compared with those during the pretrial period. There were no significant differences between groups in the scores for fecal shape, color, odor, and feeling after defecation.

Adverse events

A total of 15 mild adverse events (9 in group A and 6 in group P) occurred in the study period. The principal investigator judged that none of the mild adverse events were related to intake of either tablet.

DISCUSSION

In this study, we demonstrated that daily consumption of a tablet containing 25 mg of heat-killed *L. casei* 327 for 2 weeks improved the defecation frequency, defecation days, and fecal volume of healthy subjects who had relatively low defecation frequencies.

It has been reported that a placebo response is observed in clinical trials for irritable bowel syndrome [26]. As shown in Table 3, the defecation frequency, defecation days, and fecal volume not only in the active group but also in the placebo group seemed to be higher during the intake period than during the pretrial period. A placebo response may also have

Table 3. Defecation frequency, defecation days, and fecal volume during pretrial and intake periods

Item	Group	Pretrial	Intake	Change[a]
Defecation frequency (times/week)	A	3.7 ± 0.1	5.0 ± 0.2*	1.3 ± 0.2*
	P	3.6 ± 0.1	4.3 ± 0.2	0.7 ± 0.2
Defecation days (days/week)	A	3.7 ± 0.1	4.6 ± 0.2*	0.9 ± 0.2*
	P	3.6 ± 0.1	4.1 ± 0.2	0.5 ± 0.1
Fecal volume (pieces/week)	A	9.77 ± 0.69	15.81 ± 1.36	6.04 ± 1.04*
	P	9.93 ± 0.63	13.09 ± 1.14	3.16 ± 0.79

Each value represents the mean ± SE. [a]Changes from pretrial values. Comparisons between group A and P by unpaired Student's t-test: *p<0.05.

Table 4. Scores for fecal conditions during the pretrial and intake periods

Item	Group	Pretrial	Intake
Fecal shape	A	1.9 ± 0.1	2.5 ± 0.1
	P	1.8 ± 0.1	2.5 ± 0.1
Fecal color	A	3.4 ± 0.1	3.2 ± 0.1
	P	3.5 ± 0.1	3.1 ± 0.1
Fecal odor	A	3.2 ± 0.1	3.0 ± 0.1
	P	3.2 ± 0.1	3.1 ± 0.1
Feeling after defecation	A	3.4 ± 0.1	3.0 ± 0.1
	P	3.2 ± 0.1	2.9 ± 0.1

Each value represents the mean ± SE. Refer to the Materials and Methods section for scores. The average scores for the pretrial and intake periods were respectively calculated. No significant difference was found on comparison of the values for group A and P using the Mann-Whitney *U* test.

occurred in this study. Defecation frequency, defecation days, and fecal volume during intake period and/or their changes from the pretrial period in the active group were significantly higher than those in the placebo group, and this was important for evaluation of the beneficial effect of *L. casei* 327 on defecation.

Major gastrointestinal (GI) functions are controlled by the enteric nervous system (ENS), and myenteric neurons, one of the components of the ENS, are mainly involved in the control of GI motility [27]. Various intraluminal factors, such as glucose, butyrate, and acetate, or intramucosal factors of immune, glial, or epithelial origin can modulate neuronal functions. For example, butyrate (luminal origin) [28], serotonin (5-hydroxytryptamine, 5-HT; mucosal origin) [29], or interleukin-1β or interleukin-6 (immune origin) [30] can increase neuronal excitability. Soret *et al.* suggested that short-chain fatty acids (SCFAs) could play an important role in controlling neuromediator gene expression in the ENS and concomitantly GI function such as motility [31].

Bifidobacterium in the intestinal tract produces succinate and lactate, as well as SCFAs, such as acetate and propionate. SCFAs affect GI and colonic motility [32]. There have been a few reports of the beneficial effects of nonviable lactobacilli and enterococci on intestinal function and microbiota. Daily consumption of beverages containing nonviable *Lactobacillus gasseri* CP2305 for 3 weeks improved the number of daily bowel movements and increased the intestinal *Bifidobacterium* content of healthy participants with a tendency toward constipation or frequent bowel movements [18]. Consumption of heat-killed *Enterococcus faecalis* EC-12 by healthy volunteers for 14 days increased the level of bifidobacteria and the amount of fecal SCFAs [19]. The influence of heat-killed *L. casei* 327 on intestinal microbiota is being investigated. The mechanism by which the dead cells of lactic acid bacteria increase bifidobacteia in intestinal microbiota is still unclear.

Approximately 90% of 5-HT in the body can be found in the GI tract, where it regulates GI functions including motility and secretions, the majority of which are synthesized and stored in enterochromaffin (EC) cells [33, 34]. The SCFAs generated by bacterial fermentation in the GI tract and/or physical stimulation of stool transit activate 5-HT secretion [35–38]. The secreted 5-HT stimulates the submucosal sensory branch of the ENS and induces acetylcholine release from motor neurons in the muscularis externa [39, 40]. The acetylcholine binds to muscarinic receptors on smooth muscle, induces muscular contraction processes such as plasma membrane depolarization and Ca^{2+} influx, and activates GI motility [41, 42]. *Ex vivo* studies exploiting isolated guinea pig intestine preparations have shown that luminal application of 5-HT initiates the peristaltic reflexes [43]. Similar effects have been observed with 5-HT4 receptor agonists [44]. In fact, the 5-HT4 receptor agonist, tegaserod, has proved effective in therapy for patients with constipation-predominant irritable bowel syndrome [45]. Heat-killed *Lactobacillus brevis* SBC8803 is reported to induce 5-HT release from intestinal cells [46] and to promote peristalsis via 5-HT3 receptors in rats [47]. Our unpublished data suggested that heat-killed *L. casei* 327 led to an increase in expression of *Tph1*, the rate-limiting enzyme of 5-HT synthesis, in colonic epithelial cells and an increase in 5-HT in colonic tissue in orally administrated BALB/c mice. EC cells are known to express Toll-like receptors [48], which play important roles in bacterial recognition. Heat-killed *L. casei* 327 may stimulate the bacterial recognition system in intestinal cells such as EC cells and activate 5-HT synthesis and amplification of colonic muscle contraction for improvement of defecation.

Since the number of fecal lactobacilli was increased by administration of fermented milk with viable *L. casei* 327 [22], viable *L. casei* 327 should be resistant to low pH and digestive enzymes in the human GI tract. Heat-killed *L. casei* 327 may resist digestive juices, reach the GI tract, and then affect the epithelial cells and/or intestinal microbiota. Further studies are needed in order to verify this hypothesis.

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