Randomized, double-blind, placebo-controlled, parallel-group study of the effect of *Lactobacillus paracasei* K71 intake on salivary release of secretory immunoglobulin A

Yuhi SAITO[1], Mikio FUJII[1], Toshiyuki WATANABE[1], Kentaro MARUYAMA[1], Yasuyuki KOWATARI[2], Hiromaru OGATA[3] and Takehisa KUMAGAI[1*]

[1]Kameda Seika Co., Ltd., 3-1-1 Kameda-Kogyodanchi, Konan-ku, Niigata-shi, Niigata 950-0198, Japan
[2]Aisei Hospital Ueno Clinic, 2-1-18 Higashiueno, Taito-ku, Tokyo 110-0015, Japan
[3]Sagamihara Royal Care Center, 380-1 Oshima, Midori-ku, Sagamihara-shi, Kanagawa 252-0135, Japan

Received September 12, 2016; Accepted December 7, 2016; Published online in J-STAGE December 28, 2016

***Lactobacillus paracasei* K71 was shown to be effective in alleviating the severity of atopic dermatitis in a randomized controlled trial, and a preliminary open-label trial suggested that strain K71 intake enhanced secretory immunoglobulin A (sIgA) release in the saliva. This study investigated the effect of K71 on sIgA release in a randomized, double-blind, placebo-controlled, parallel-group trial. The trial included 62 Japanese subjects aged 20–64 years with relatively low rates of salivary sIgA release. Subjects (n=31 in each group) were randomly given a tablet containing 100 mg (approximately 2×10^{11} bacteria) of K71 or a placebo tablet daily for 12 weeks. After eliminating data for eight subjects (four in each group) who met the exclusion criteria for efficacy analysis, data for 54 subjects were analyzed. The change in the rate of salivary sIgA release 8 weeks after initiation of the study compared with baseline was significantly higher in the K71 tablet group (105.5 ± 119.0 μg/min) than in the placebo group (52.7 ± 62.6 μg/min; p=0.047). There were no adverse events associated with intake of tablets containing K71. The safety of intake of *L. paracasei* K71 was also confirmed in an independent open-label trial with 20 healthy subjects who consumed excessive amounts of K71-containing food. *L. paracasei* K71 intake may therefore have some benefits in promoting mucosal immune function.**

Key words: lactic acid bacteria, *Lactobacillus paracasei*, secretory immunoglobulin A, immune function, clinical study

INTRODUCTION

Growing evidence suggests that lactic acid bacteria may have potential health benefits [1–3]. Lactic acid bacteria are Gram-positive, anaerobic, or facultative aerobic cocci or rods that produce lactic acid as a major metabolite [4] and include *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. The health-benefiting actions of lactic acid bacteria are species and strain specific [5]. Several species have been used as probiotics for more than 70 years [6], and their recognized beneficial activities include immune modulation [7] as well as anticancer [8] and hypocholesterolemic activities [5]. Regarding immune modulation, probiotic and commensal organisms have been shown to modulate the mucosal immune system through interaction with mucosal immune cells or epithelial cells [7]. Animal studies have suggested that living and heat-killed lactobacilli can elicit an immune response by binding to specific receptors on mucosal immune cells or epithelial cells to control cytokine/chemokine production, involving regulation of the function and generation of naive T cells, regulatory T cells, dendritic cells, and macrophages [7, 9–11].

Lactobacillus paracasei K71 is an isolate from sake lees, sake being the traditional Japanese alcoholic beverage made from polished rice [12]. We recently found that intake of a dietary supplement containing heat-killed *L. paracasei* K71 was effective in reducing the clinical severity of atopic dermatitis in a randomized controlled trial [13], suggesting that this bacterial strain had immunomodulatory activity. Furthermore, a preliminary clinical study suggested that intake of *L. paracasei* K71 enhanced secretory immunoglobulin A (sIgA) release in the saliva (unpublished observation). sIgA in the salivary glands is synthesized by plasma cells as dimeric IgA, constituting part of the first line of defense

*Corresponding author. Takehisa Kumagai, Kameda Seika Co., Ltd., 3-1-1 Kameda-Kogyodanchi, Konan-ku, Niigata-shi, Niigata 950-0198, Japan. E-mail: t_kumagai@kameda.co.jp

Table 1. Composition of the test tablets

	Placebo	Active
Ingredients	Crystalline cellulose, maltose, calcium stearate, fine granular silica	*L. paracasei* K71 [a], crystalline cellulose, maltose, calcium stearate, fine granular silica
Nutritional facts (value for daily dose, 0.5 g)		
Energy (kcal)	1.9	1.9
Protein (g)	0.00	0.06
Fat (g)	0.01	0.01
Carbohydrate (g)	0.46	0.39
Sodium (mg)	0.00	0.67

[a] The *L. paracasei* K71 content was 100 mg (approximately 2×10^{11} bacteria) per daily dose (0.5 g; two tablets).

against pathogen invasion [14]. The level of salivary sIgA release can reflect immune function in the intraoral and upper respiratory tract [15], and salivary sIgA has been suggested as a potential measure of mucosal and systemic immunity [16, 17].

Based on these results, we conducted a double-blind randomized controlled trial to investigate the effects of intake of a dietary supplement containing heat-killed *L. paracasei* K71 on salivary sIgA release. In addition, an open-label trial of excessive consumption of the supplement was performed to confirm its safety.

MATERIALS AND METHODS

Test foods

The foods used in this study were *L. paracasei* K71-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. paracasei* K71 was 100 mg (approximately 2×10^{11} bacteria) per daily dose (0.5 g; 2 tablets). In Trial 1 (efficacy examination), subjects consumed two of the designated tablets once a day with water, tea, or coffee for 12 weeks. The dose of *L. paracasei* K71 was based on our preliminary investigation, which demonstrated that consuming 100 mg/day of *L. paracasei* K71 for 12 weeks enhanced the rate of salivary sIgA release. The daily dose of 100 mg of *L. paracasei* K71 was also shown to be effective in alleviating the clinical severity of atopic dermatitis [13]. In Trial 2 (safety examination under excessive consumption), subjects consumed 1.5 g or 2.5 g of powdered formulation that respectively contained 300 mg (3-fold dose) or 500 mg (5-fold dose) of *L. paracasei* K71 once a day for 4 weeks.

Trial 1: efficacy examination

To explore the efficacy of intake of *L. paracasei* K71, we conducted a randomized, double-blind, parallel-group, placebo-controlled study at the incorporated medical institution Aisei Hospital Ueno Clinic (Tokyo, Japan), with the study being supported by funds from Kameda Seika Co., Ltd. The study protocol conformed 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 was approved by the institutional review board of Aisei Hospital Ueno Clinic on June 25, 2015. The study period was from June to December 2015. This study was registered under ID No. UMIN000018423 in the UMIN Clinical Trials Registry, Japan.

Subjects aged 20–64 years old were recruited for the study. The study details were disclosed to subjects before enrolment, and the investigators obtained informed consent from each subject. The inclusion criteria were as follows: male or female, age between 20 and 64 years, and relatively low rates of salivary sIgA release in a pretrial test (subject selection was based on our preliminary clinical test, in which subjects with relatively low sIgA release rate were enrolled). The exclusion criteria were as follows: prior use of health foods or medicines with high levels of lactic acid bacteria three or more times a week; (ii) use of health foods or dietary supplements that might enhance immune function; (iii) history of allergic disease such as seasonal rhinitis, perennial allergic rhinitis, asthma, atopic dermatitis, allergic conjunctivitis, food allergy, and metal allergy; (iv) receipt of therapy (such as hyposensitization therapy) that may affect the study results; (v) dental or intraoral treatment within 1 month before the screening test or plans for such treatment; (vi) dental or intraoral trouble accompanied by bleeding; (vii) shift-work or heavy physical labor; (viii) heavy exercise, such as long-distance running; (ix) disease requiring treatment; (x) serious disease such as diabetes mellitus, liver disease, kidney disease, heart disease, or a disease that affects corticosteroid secretion, or a history of such

disease; (xi) possible allergy to the test compound; (xii) subjects judged unsuitable based on screening-test values; (xiii) women who were pregnant, nursing, or planning to get pregnant or nurse during the study period; (xiv) subjects involved in other clinical trials within 1 month before giving informed consent or planning to join other clinical trials after giving informed consent; (xv) subjects judged unsuitable according to their responses to a lifestyle questionnaire; and (xvi) subjects judged unsuitable by the principle investigator. Sixty-two subjects who met the eligibility criteria were assigned sequentially based on random number tables to one of the two masked products: the *L. paracasei* K71-containing tablet (group A; n=31) and placebo tablet (group P; n=31). The number of subjects was calculated based on a preliminary clinical trial in which the effect of intake of *L. paracasei* K71 on the rate of salivary sIgA release was investigated. The allocation of subjects was performed by TTC Co., Ltd. (Tokyo, Japan), and an envelope that contained the allocation table was sealed off until breaking of the blinding. Thus the allocation information was concealed from the subjects, investigators, and researchers who recruited and assessed the participating subjects.

The subjects underwent clinical surveys in weeks 4, 8, and 12 following initial intake of the test food. The clinical survey and pretrial tests included the following: a lifestyle questionnaire (anamnesis, intake of pharmaceutical products and health foods, allergies, smoking, alcohol consumption, and life habits), medical interview (physical conditions and presence or absence of adverse events), somatometry (height, body weight, body mass index, blood pressure, and pulse), and saliva analysis (determination of sIgA concentration and rate of saliva secretion). Laboratory examinations (fasting levels of white and red blood cells, hemoglobin, hematocrit, platelets, total protein, albumin, total bilirubin, alkaline phosphatase, aspartate transaminase, alanine transaminase, lactate dehydrogenase, γ-glutamyltranspeptidase, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, urea nitrogen, creatinine, uric acid, Na, K, Cl, and glucose) and urinalysis (protein, glucose, and occult blood) were performed in the pretrial test and in week 12. From 2 weeks before supplement intake to completion of the study, subjects were asked to log details including their test-tablet ingestion, physical condition, and use of medications.

During the trial, the principal investigator and assistants instructed the participants as follows: (i) not to change their lifestyle, including diet, alcohol consumption, and sleep; (ii) to avoid over exercising, abstemious eating, or overeating; (iii) not to change their exercise habits; (iv) not to consume health foods, dietary supplements, lactic acid bacteria beverages, lactobacillus preparations, yogurt, or kimchi (Korean pickles); (v) to avoid vaccinations (e.g., for influenza) as much as possible; (vi) to log the amount and dose of pharmaceuticals used; (vii) to take the specified dose of the test food and log the intake time and dose every day; (viii) to record a lifestyle log every day; (ix) to avoid drinking alcohol the day before testing; (x) to avoid strenuous exercise on the day before and the day of testing; (xi) to finish eating their last meal of the day by around 10 p.m. and not consume anything thereafter other than water or warm water until the end of testing the following day; (xii) to go to bed by midnight and sleep well on the day before testing; (xiii) to brush their teeth without using toothpaste or mouthwash by 1 hr before saliva analysis on the day of testing; and (xiv) to avoid smoking until the end of testing on the day of testing.

The primary outcome measure was the rate of salivary sIgA release, and the secondary outcome measure was the concentration of salivary sIgA. Subjects who consumed less than 80% of the expected dose of the provided test food, who did not keep an adequate log or whose behavior cast doubt on the reliability of their clinical data, who met the exclusion criteria after enrollment or did not follow the required restrictions, or who met justifiable reasons for exclusion were excluded from efficacy analysis. To analyze the safety of the provided test food, we examined adverse events in subjects who consumed the test food at least once and explored measurement data for subjects who completed the study schedule.

Trial 2: safety examination under excessive consumption

To explore the safety of intake of *L. paracasei* K71, we conducted an open-label study at the incorporated medical institution Yuko-kai Meguro Medical Clinic (Tokyo, Japan), with the study being supported by funds from Kameda Seika Co., Ltd. The study protocol conformed 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 was approved by the institutional review board of Yuko-kai Akihabara Medical Clinic.

Healthy subjects aged 20–64 years old were recruited for the study. The study details were disclosed to subjects before enrolment, and the investigators obtained informed consent from each subject. Twenty subjects were assigned either to a "3-fold-dose" (300 mg *L. paracasei* K71 daily)

group (n=10; 5 males and 5 females) or "5-fold-dose" (500 mg *L. paracasei* K71 daily) group (n=10; 5 males and 5 females).

The subjects underwent clinical surveys before and during intake of the test food (on weeks 2 and 4), as well as 2 weeks after the end of the intake period. The clinical survey included the following: a lifestyle questionnaire, medical interview (physical conditions and presence or absence of adverse events), somatometry (height, body weight, body mass index, blood pressure, and pulse), laboratory examinations (fasting levels of white and red blood cells, hemoglobin, hematocrit, platelets, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total protein, albumin, total bilirubin, alkaline phosphatase, aspartate transaminase, alanine transaminase, lactate dehydrogenase, γ-glutamyltranspeptidase, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, urea nitrogen, creatinine, uric acid, Na, K, Cl, and glucose), and urinalysis (protein, glucose, and occult blood). During the study, subjects were asked to log details including their test food ingestion, physical condition, and use of medications.

During the trial, the principal investigator and assistants instructed the participants not to change their lifestyle, not to drink alcohol during two days before testing, and to finish eating their last meal of the day by around 10 p.m. on the day before testing.

Measurements of salivary sIgA

Subjects rinsed their mouth and gargled twice with 150 ml of water before testing. After sitting for 15 min, subjects underwent saliva sampling using a Salisoft® (Assist Co., Ltd., Tokyo, Japan). Subjects chewed the accessory plastic sponge for 2 min, and the sponge was then inserted into the accessory double-chamber plastic test tube. The tube was centrifuged at room temperature, and the obtained saliva sample was frozen until use. Saliva collection was performed in the morning (within 2 hr for each examination). The concentration of sIgA was determined according to the standard laboratory protocol at Daiichi Kishimoto Clinical Laboratories, Inc. (Sapporo, Japan). The rate of sIgA release (μg/min) was calculated by multiplying the concentration of sIgA (μg/dl) by the saliva flow rate per 1 min (dl/min).

Statistical analysis

All measured values are expressed as the mean ± standard deviation (SD), except for the error bars in Fig. 2, which represent the standard error. In trial 1, values were compared between groups A and P using χ^2 tests (for sex) or unpaired Student's t-tests (other parameters). In trial 2, values were compared with pretrial values using paired Student's t-tests. Values with $p<0.05$ were considered statistically significant.

RESULTS

Trial 1: efficacy examination

Subjects

The study outline is shown in Fig. 1. Applicants who provided written informed consent (n=187) underwent a pretrial test, and we selected 62 eligible subjects. All the subjects completed the study. Eight subjects (4 in group A and 4 in group P) were eliminated from the efficacy analysis because they met the exclusion criteria for efficacy analysis. Data obtained from 54 subjects (n=27 for each group) were therefore analyzed for efficacy. The background data for each group are shown in Table 2. There was no significant difference between the two groups in terms of age, height, body weight, body mass index (BMI), rate of salivary sIgA release, or sIgA concentration in saliva at the pretrial test.

Effects of L. paracasei K71 on rate of salivary sIgA release and saliva sIgA concentration

The effects of *L. paracasei* K71 on the rate of salivary sIgA release and saliva sIgA concentration are summarized in Table 3. The rate of salivary sIgA release was higher in group A than in group P throughout the study period. The change in rate of salivary sIgA release from baseline to week 8 (ΔsIgA release rate) was significantly higher in group A (105.5 ± 119.0 μg/min) than in group P (52.7 ± 62.6 μg/min; p=0.047) (Fig. 2A). There was no significant difference in sIgA concentrations between the two groups.

To explore the responses in more detail, we performed stratified analyses in subjects with pretrial baseline sIgA release rates below or above the average for the group as a whole. No significant difference in ΔsIgA release rate was observed in the subgroup with baseline sIgA release rates below the average (Fig. 2B), but the ΔsIgA release rate in group A (92.9 ± 92.9 μg/min) tended to be higher than that in group P (38.4 ± 65.1 μg/min, p=0.081) in the subgroup whose baseline sIgA release rates were above the average (Fig. 2C).

Adverse events

We observed a total of 28 mild adverse events (15 in group A and 13 in group P) and one moderate adverse event (in group P) during this study. The principle

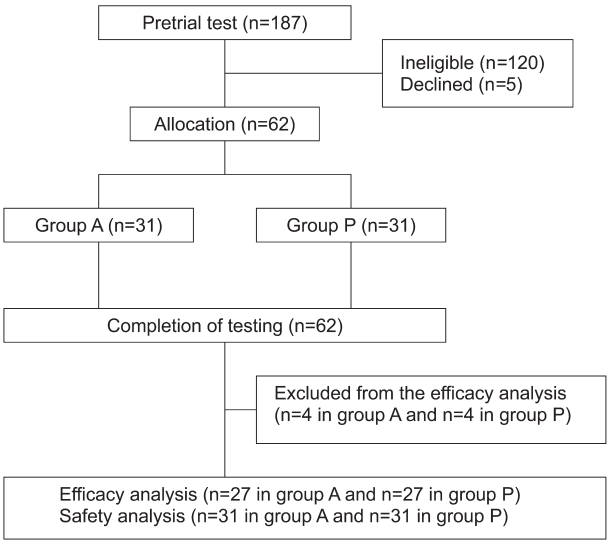

Fig. 1. Outline of the study. Subjects in group A consumed a dietary supplement containing *L. paracasei* K71, and subjects in group P consumed placebo tablets. Subjects were excluded from the efficacy analysis if they met the exclusion criteria.

Table 2. Background characteristics of the subjects analyzed for efficacy

Item	Group	Observed value	p value
Number of subjects (male/female)	A	27 (12/15)	0.79
	P	27 (13/14)	
Age (years)	A	43.4 ± 12.9	0.93
	P	43.7 ± 10.8	
Height (cm)	A	162.86 ± 8.59	0.22
	P	165.72 ± 8.16	
Body weight (kg)	A	57.19 ± 9.85	0.92
	P	57.48 ± 10.75	
BMI (kg/m^2)	A	21.46 ± 2.54	0.34
	P	20.79 ± 2.63	
Rate of salivary sIgA release (μg/min)	A	111.19 ± 35.31	0.98
	P	111.48 ± 35.19	
sIgA concentration in saliva (mg/dl)	A	32.97 ± 34.89	0.77
	P	30.51 ± 24.40	

Each value represents the mean ± SD, except for the number of subjects. p values were determined by χ^2 tests (sex) or unpaired Student's t-tests (other characteristics).

Table 3. Effects of *L. paracasei* K71 intake on rate of salivary sIgA release and sIgA concentration in saliva

Item	Group	n	Pretrial	Week 4	Week 8	Week 12
Analysis of all subjects						
Rate of sIgA release (μg/min)	A	27	111.2 ± 35.3	225.3 ± 135.6	216.7 ± 119.0	154.9 ± 87.5
	P	27	111.5 ± 35.2	176.8 ± 80.1	164.2 ± 66.8	143.8 ± 53.9
sIgA concentration (mg/dl)	A	27	33.0 ± 34.9	49.0 ± 40.7	39.5 ± 23.5	34.2 ± 29.7
	P	27	30.5 ± 24.4	44.2 ± 31.5	35.9 ± 19.6	32.0 ± 18.2
Stratified analysis: subjects with an sIgA release rate below the average at the pretrial test						
Rate of sIgA release (μg/min)	A	14	82.8 ± 17.9	190.1 ± 79.6	200.0 ± 142.6	142.3 ± 67.6
	P	12	81.5 ± 20.2	169.8 ± 90.7	152.1 ± 56.0	137.6 ± 65.4
sIgA concentration (mg/dl)	A	14	35.0 ± 47.7	51.0 ± 49.5	39.5 ± 27.4	36.1 ± 36.4
	P	12	32.4 ± 34.3	46.2 ± 31.6	37.5 ± 20.6	30.4 ± 17.5
Stratified analysis: subjects with an sIgA release rate above the average at the pretrial test						
Rate of sIgA release (μg/min)	A	13	141.8 ± 19.9	263.1 ± 173.1	234.7 ± 89.3	168.4 ± 106.1
	P	15	135.5 ± 24.2	182.4 ± 73.4	173.9 ± 74.8	148.8 ± 44.4
sIgA concentration (mg/dl)	A	13	30.7 ± 12.8	46.9 ± 30.5	39.4 ± 19.4	32.1 ± 21.6
	P	15	29.0 ± 13.3	42.6 ± 32.4	34.6 ± 19.3	33.2 ± 19.2

There was no intergroup difference by unpaired Student's t-test. Each value represents the mean ± SD.

investigator judged that none of the mild adverse events were related to intake of the test food. The moderate adverse event was an increase in serum triglycerides and decrease in high-density lipoprotein cholesterol levels. The principle investigator judged that this was related to the subject's diet and concluded that it was unlikely to be related to intake of the test compound. Regarding laboratory tests and somatometry results, slight deviations from the reference values were observed in 10 subjects in group A and 8 in group P, but the principle investigator judged that none of these deviations was of any clinical significance.

Trial 2: safety examination under excessive consumption

All of the enrolled subjects successfully completed the study, and all of the data obtained were subjected to analysis of safety. The background data of the subjects were as follows: (1) for the 3-fold-dose group, age, 42.5

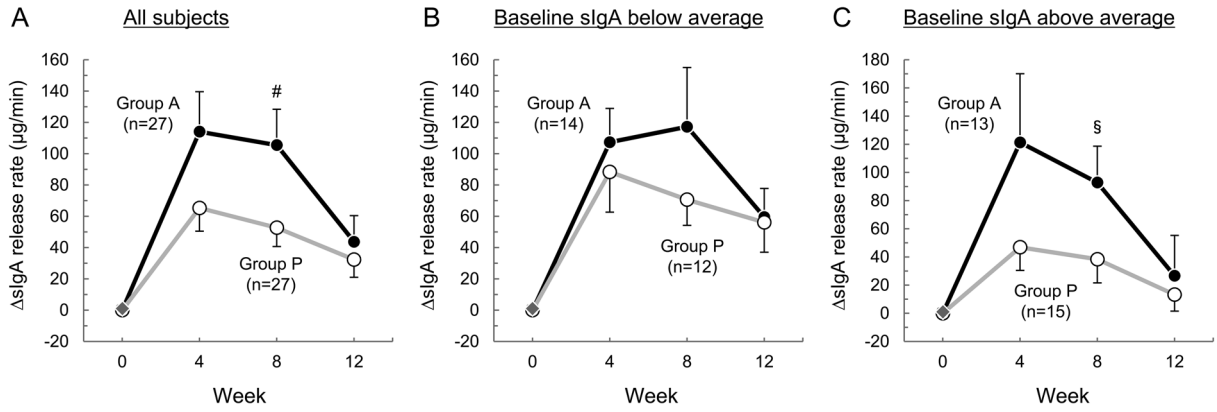

Fig. 2. Time course of the effect of *L. paracasei* K71 intake on the rate of salivary sIgA release. The changes in the rate of salivary sIgA release following initiation of test food intake (ΔsIgA release rate) in (A) all subjects, (B) subjects with sIgA release rates below average, and (C) subjects with sIgA release rates above average at the pretrial test are shown. Each value represents the mean ± SE. #p=0.047; §p=0.081 by unpaired Student's t-tests.

Table 4. Effects of excessive intake of *L. paracasei* K71 on parameters in hematology examination

Item	Group	Pretrial	Week 2	Week 4	Posttrial
White blood cell (number/μl)	L	5,853 ± 1,573	5,790 ± 1,225	5,731 ± 1,426	5,510 ± 1,321
	H	5,723 ± 1,375	5,512 ± 971	5,849 ± 1,375	5,707 ± 1,213
Red blood cell (number × 10^4 μl)	L	465.6 ± 56.4	462.9 ± 57.5	450.8 ± 58.5*	451.8 ± 60.3
	H	468.0 ± 40.1	462.3 ± 39.1	461.2 ± 38.7	466.1 ± 34.7
Hemoglobin (g/dl)	L	14.03 ± 1.56	13.76 ± 1.47	13.41 ± 1.56*	13.59 ± 1.59
	H	13.83 ± 1.10	13.82 ± 1.28	13.76 ± 1.21	13.99 ± 1.12
Hematocrit (%)	L	44.37 ± 4.52	43.82 ± 4.59	42.43 ± 5.09*	43.50 ± 4.78
	H	43.62 ± 2.90	43.48 ± 3.89	43.26 ± 3.73	44.10 ± 2.93
Platelet (number × 10^4 μl)	L	25.90 ± 3.91	25.22 ± 4.19	25.66 ± 4.34	25.57 ± 3.97
	H	24.73 ± 3.90	25.85 ± 4.29	23.90 ± 3.00	25.90 ± 4.69
MCV (fl)	L	95.6 ± 4.5	95.0 ± 5.8	94.3 ± 4.4	96.7 ± 4.2
	H	93.4 ± 3.8	94.1 ± 3.7	93.9 ± 4.4	94.7 ± 2.4
MCH (pg)	L	30.20 ± 1.42	29.85 ± 1.82	29.83 ± 1.36**	30.20 ± 1.65
	H	29.57 ± 1.08	29.89 ± 0.94*	29.86 ± 1.19	30.01 ± 0.88*
MCHC (%)	L	31.58 ± 0.56	31.41 ± 0.57	31.62 ± 0.72	31.25 ± 1.05
	H	31.71 ± 0.97	31.77 ± 0.69	31.83 ± 0.95	31.71 ± 0.68

Group L: 3-fold-dose group; group H: 5-fold-dose group, MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. Each value represents the mean ± SD. n=10 for both groups. *p<0.05; **p<0.01 by paired Student's t-test compared with the respective pretrial values.

± 13.5 years; height, 164.4 ± 11.5 cm; body weight, 59.74 ± 13.08 kg and BMI 21.86 ± 2.56 kg/m^2, and (2) for the 5-fold-dose group, age, 41.6 ± 14.0 years, height, 165.7 ± 6.4 cm, body weight, 60.20 ± 4.98 kg, and BMI 22.02 ± 2.50 kg/m^2.

During this study, the following adverse events were observed: headache (n=1), fullness feeling (n=1), loose stool (n=1), and stomatitis (n=1) in the 3-fold-dose group and fullness feeling (n=1), loose stool (n=1), diarrhea (n=1), and abdominal pain loose stool (n=1) in the 5-fold-dose group. Each of these adverse events was mild and judged by the principle investigator as being unrelated to test food consumption.

There were several slight but significant changes (from pretrial values) in parameters in tests for hematology (Table 4) and blood biochemistry (Table 5), whereas the principle investigator judged that none of these deviations was of any clinical significance.

Table 5. Effects of excessive intake of *L. paracasei* K71 on parameters in blood biochemistry examination

Item	Group	Pretrial	Week 2	Week 4	Posttrial
AST (U/l)	L	17.4 ± 5.0	17.2 ± 3.4	19.2 ± 10.0	16.4 ± 3.4
	H	17.2 ± 5.3	16.7 ± 6.1	17.0 ± 4.7	17.1 ± 5.0
ALT (U/l)	L	16.7 ± 8.7	14.9 ± 5.7	17.2 ± 8.6	13.8 ± 5.9
	H	15.4 ± 5.6	14.6 ± 5.8	14.9 ± 5.3	15.7 ± 9.3
LDH (U/l)	L	156.7 ± 19.6	157.5 ± 20.8	156.7 ± 17.1	154.8 ± 20.9
	H	164.7 ± 18.1	163.4 ± 28.5	168.6 ± 25.4	164.9 ± 25.9
ALP (U/l)	L	218.0 ± 72.2	212.7 ± 61.5	199.6 ± 54.7*	201.8 ± 58.5
	H	217.0 ± 72.1	215.6 ± 79.0	205.5 ± 76.5	220.3 ± 79.4
γ-GTP (U/l)	L	26.3 ± 10.8	27.5 ± 14.3	26.4 ± 11.9	25.6 ± 13.7
	H	31.3 ± 26.6	30.6 ± 24.6	30.8 ± 27.1	33.0 ± 29.3
Total bilirubin (mg/dl)	L	0.85 ± 0.25	0.79 ± 0.19	0.78 ± 0.09	0.76 ± 0.38
	H	0.86 ± 0.37	0.80 ± 0.35	0.84 ± 0.28	0.71 ± 0.23
Albumin (g/dl)	L	4.39 ± 0.21	4.39 ± 0.17	4.30 ± 0.19	4.30 ± 0.23
	H	4.40 ± 0.33	4.33 ± 0.19	4.43 ± 0.25	4.43 ± 0.26
Total protein (g/dl)	L	7.09 ± 0.32	7.11 ± 0.43	6.93 ± 0.33	6.97 ± 0.36
	H	7.31 ± 0.47	7.13 ± 0.40	7.21 ± 0.42	7.28 ± 0.44
Blood urea nitrogen (mg/dl)	L	12.69 ± 3.18	14.22 ± 4.07	13.13 ± 3.32	13.31 ± 4.25
	H	11.78 ± 1.35	11.37 ± 3.18	12.44 ± 2.89	12.27 ± 2.51
Creatinine (mg/dl)	L	0.736 ± 0.106	0.726 ± 0.157	0.737 ± 0.137	0.725 ± 0.121
	H	0.683 ± 0.129	0.679 ± 0.145	0.646 ± 0.163	0.680 ± 0.160
Uric acid (mg/dl)	L	5.52 ± 1.24	5.51 ± 1.25	5.24 ± 1.16	5.24 ± 1.28
	H	4.81 ± 0.91	4.95 ± 1.26	4.93 ± 1.30	4.81 ± 1.14
Total cholesterol (mg/dl)	L	188.7 ± 28.6	193.0 ± 34.5	179.6 ± 28.6	188.6 ± 38.4
	H	196.0 ± 41.3	195.3 ± 41.4	194.6 ± 45.9	201.9 ± 42.8
LDL-C (mg/dl)	L	113.9 ± 30.2	114.0 ± 31.0	104.5 ± 23.7	114.0 ± 38.2
	H	121.0 ± 29.7	122.1 ± 36.0	115.6 ± 36.1	125.2 ± 34.7
HDL-C (mg/dl)	L	60.6 ± 12.1	62.7 ± 15.7	57.2 ± 12.5**	59.7 ± 11.8
	H	58.5 ± 11.7	59.1 ± 8.9	59.8 ± 9.4	60.1 ± 10.6
Triglycerides (mg/dl)	L	99.1 ± 42.6	91.0 ± 69.3	103.5 ± 68.4	100.0 ± 66.6
	H	93.7 ± 56.2	86.1 ± 47.8	102.0 ± 50.4	102.4 ± 54.6
Glucose (mg/dl)	L	85.1 ± 6.0	87.4 ± 6.0	88.7 ± 7.9	86.0 ± 4.9
	H	85.3 ± 6.6	86.4 ± 6.8	85.7 ± 8.3	87.3 ± 4.6
Na (mEq/l)	L	140.7 ± 1.6	140.4 ± 1.6	141.5 ± 2.3	141.5 ± 1.6
	H	140.8 ± 1.9	141.1 ± 1.1	141.1 ± 1.7	141.7 ± 1.8*
K (mEq/l)	L	4.23 ± 0.27	4.21 ± 0.21	4.21 ± 0.28	4.11 ± 0.24
	H	4.23 ± 0.22	4.18 ± 0.21	4.32 ± 0.22	4.38 ± 0.40
Cl (mEq/l)	L	104.4 ± 0.7	104.5 ± 1.7	106.1 ± 2.4	105.0 ± 1.2
	H	104.8 ± 2.8	104.8 ± 1.7	105.2 ± 1.9	104.9 ± 2.1

Group L: 3-fold-dose group; group H: 5-fold-dose group, AST: aspartate transaminase; ALT: alanine transaminase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; γ-GTP: γ-glutamyltranspeptidase; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol. Each value represents the mean ± SD. n=10 for both groups. *p<0.05; **p<0.01 by paired Student's t-test compared with the respective pretrial values.

DISCUSSION

We conducted a double-blind, randomized, controlled trial and confirmed an immunomodulatory function of the *L. paracasei* K71-containing dietary supplement. The ΔsIgA release rate was significantly higher in the K71 tablet group at week 8 than in the placebo group. When compared with the pretrial level, an increase in the sIgA release rate was observed in both the K71 tablet group and placebo group. We speculate that these variations in both groups may be due to lifestyle regularization resulting from clinical trial participation or to a seasonal change

(the study was conducted from June to December). At week 12, a marked decrease in sIgA release rate was observed in both groups. Blood drawing, which was performed in week 12 but not in weeks 4 and 8, might evoke psychological stress, and this might have affected sIgA release in week 12. Indeed, it has been shown that a seasonal change and psychological factor affect salivary sIgA release. In subjects wearing knee-length skirts, the levels of salivary sIgA release increased with the advance of the autumn, from September to November [18]. Negative psychological factors caused a decrease in sIgA concentration in saliva [19].

The enrolled subjects (n=62) all had relatively low rates of salivary sIgA release in the pretrial test (n=187), and we therefore performed stratified analyses based on the subjects' baseline sIgA release rates. The ΔsIgA release rate in the K71 tablet group tended to be higher than in the placebo group among subjects with sIgA release rates above the average of the enrolled subjects, though the statistical significance was marginal. In contrast, there was no significant difference in ΔsIgA release rates between the groups in subjects with baseline sIgA release rates below the average. However, the time-course curves for ΔsIgA release rates in the two stratified analyses were similar to that for the whole-subject analysis. Moreover, the failure to detect significant changes in the stratified analyses may have been a result of the limited sample sizes, which reduced the statistical power. Further studies with larger sample sizes are therefore required to determine if the immunomodulatory effect of *L. paracasei* K71 is limited to certain populations, depending on their baseline sIgA secretion rates.

sIgA is the major component of mucosal immunity, which functions in the gastrointestinal tract as well as the upper respiratory tract, and constitutes the first line of defense against pathogens by interfering with pathogen adhesion to mucosal cells and promoting pathogen neutralization [20, 21]. Lactic acid bacteria can bind to pattern-recognition receptors that recognize conserved molecular structures known as microbe-associated molecular patterns and signals to induce the production of cytokines, chemokines, and other innate effectors [7, 22, 23]. Pattern recognition is greatly affected by cell surface exopolysaccharides, teichoic acids, and peptidoglycans [7, 22, 23], and the potential to elicit immunomodulation therefore differs among species and strains of bacteria. Activated antibody-producing cells migrate to destination tissues where they produce IgA to constitute a local defense system [24]. We have been studying the mechanism of enhancement of IgA when *L. paracasei* K71 is ingested orally and have observed the following results: incorporation of *L. paracasei* K71 into M cells, increase of IgA^+ B cells in mesenteric lymph nodes and the villus, and increase of IgA in feces in an animal study (unpublished data). The immunomodulatory effects of intake of *L. paracasei* K71 are also suggested by a trial in subjects with atopic dermatitis [13], as well as in animal experiments testing suppression of IgE production in mice [12], augmentation of fecal excretion of IgA in mice, and protection against viral infection in mice (unpublished observations). Further examination, however, is needed to investigate the immunomodulatory activities of *L. paracasei* K71.

Regarding the safety of intake of *L. paracasei* K71, we observed no clinically significant change during the intake period in Trial 1. This observation is consistent with the results of Trial 2, in which subjects consumed excessive amounts of *L. paracasei* K71-containing food. In Trial 2, the doses of 300 and 500 mg (approximately 6 to 10×10^{11} bacteria) daily for 4 weeks were shown to be safe. Thus, intake of *L. paracasei* K71 can be a dietary approach to enhance mucosal immune function.

ACKNOWLEDGEMENTS

TTC Co., Ltd. (Tokyo, Japan), supported this clinical trial as a contract research organization. A part of this work was done as part of the Regional Innovation Creation R&D Programs supported by the administration of Niigata Industrial Creation Organization and funded by the Ministry of Economy, Trade and Industry, Japan.

REFERENCES

1. Quinto E, Jiménez P, Caro I, Tejero J, Mateo J, Girbés T. 2014. Probiotic lactic acid bacteria: a review. Food Nutr Sci 5: 1765–1775. [CrossRef]
2. Dicks LM, Botes M. 2010. Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. Benef Microbes 1: 11–29. [Medline] [CrossRef]
3. Wedajo B. 2015. Lactic acid bacteria: benefits, selection criteria and probiotic potential in fermented food. J Prob Health 3: 129. [CrossRef]
4. Hayek SA, Ibrahim SA. 2013. Current limitations and challenges with lactic acid bacteria: a review. Food Nutr Sci 4: 73–87. [CrossRef]
5. Ebel B, Lemetais G, Beney L, Cachon R, Sokol H, Langella P, Gervais P. 2014. Impact of probiotics on risk factors for cardiovascular diseases. A review. Crit Rev Food Sci Nutr 54: 175–189. [Medline] [CrossRef]
6. Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, Fondén R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T. 1998. Demonstration of safety of probiotics—a

review. Int J Food Microbiol 44: 93–106. [Medline] [CrossRef]
7. Wells JM. 2011. Immunomodulatory mechanisms of lactobacilli. Microb Cell Fact 10 Suppl 1: S17. [Medline] [CrossRef]
8. Redman MG, Ward EJ, Phillips RS. 2014. The efficacy and safety of probiotics in people with cancer: a systematic review. Ann Oncol 25: 1919–1929. [Medline] [CrossRef]
9. Coombes JL, Maloy KJ. 2007. Control of intestinal homeostasis by regulatory T cells and dendritic cells. Semin Immunol 19: 116–126. [Medline] [CrossRef]
10. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, Nam JH, Rhee JH, Hwang KC, Im SH. 2010. Generation of regulatory dendritic cells and $CD4^{+}Foxp3^{+}$ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci USA 107: 2159–2164. [Medline] [CrossRef]
11. Torii A, Torii S, Fujiwara S, Tanaka H, Inagaki N, Nagai H. 2007. *Lactobacillus acidophilus* strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines. Allergol Int 56: 293–301. [Medline] [CrossRef]
12. Kumagai T, Iguchi M, Shigeyama N, Okada S, Joh T, Hara T. 2013. *Lactobacillus paracasei* K71 isolated from sake lees suppresses serum IgE levels in ovalbumin-immunized Balb/c mice. Food Sci Technol Res 19: 127–132. [CrossRef]
13. Moroi M, Uchi S, Nakamura K, Sato S, Shimizu N, Fujii M, Kumagai T, Saito M, Uchiyama K, Watanabe T, Yamaguchi H, Yamamoto T, Takeuchi S, Furue M. 2011. Beneficial effect of a diet containing heat-killed *Lactobacillus paracasei* K71 on adult type atopic dermatitis. J Dermatol 38: 131–139. [Medline] [CrossRef]
14. Woof JM, Mestecky J. 2005. Mucosal immunoglobulins. Immunol Rev 206: 64–82. [Medline] [CrossRef]
15. Dodds MW, Johnson DA, Yeh CK. 2005. Health benefits of saliva: a review. J Dent 33: 223–233. [Medline] [CrossRef]
16. Gleeson M, Cripps AW, Clancy RL. 1995. Modifiers of the human mucosal immune system. Immunol Cell Biol 73: 397–404. [Medline] [CrossRef]
17. Brandtzaeg P. 2007. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann N Y Acad Sci 1098: 288–311. [Medline] [CrossRef]
18. Li X, Tokura H. 1996. Seasonal changes of salivary immunoglobulin A under the influences of two types of clothing. Appl Human Sci 15: 123–125. [Medline] [CrossRef]
19. Izawa S, Hirata U, Kodama M, Nomura S. 2007. The effects of daily events and moods on secretory immunoglobulin A in saliva. Jpn J Physiol Psychol Psychophysiol 25: 237–244. [CrossRef]
20. Russell MW, Hajishengallis G, Childers NK, Michalek SM. 1999. Secretory immunity in defense against cariogenic mutans streptococci. Caries Res 33: 4–15. [Medline] [CrossRef]
21. Woof JM, Kerr MA. 2006. The function of immunoglobulin A in immunity. J Pathol 208: 270–282. [Medline] [CrossRef]
22. Abreu MT. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 10: 131–144. [Medline] [CrossRef]
23. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373–384. [Medline] [CrossRef]
24. Mora JR, von Andrian UH. 2008. Differentiation and homing of IgA-secreting cells. Mucosal Immunol 1: 96–109. [Medline] [CrossRef]