Effects of *Lactobacillus plantarum* No.14 (LP14) on Several Clinical Parameters and Influences of Gastrointestinal Transit on LP14

Lactobacillus plantarum No.14 (LP14) の各臨床項目に対する効果と LP14 が消化管において受ける影響

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Abbreviations

ADRB2, β2 adrenergic receptor

ADRB3, β3 adrenergic receptor

ANOVA, analysis of variance

BAT-SNA, sympathetic nerve activity innervating the brown adipose tissue

BMI, body mass index

CRP, C-reactive protein

EPS, exopolysaccharide

GALT, gut-associated lymphoid tissue

GDP, guanosine diphosphate

IFN, interferon

IgE, immunoglobulin E

IL, interleukin

LP14, *Lactobacillus plantarum* No.14

LPS, lipopolysaccharide

POMS, Profiles of Mood States

SACL, Stress Arousal Checklist

SCN, suprachiasmatic nucleus

SMS, symptom-medication score

SSS, Stanford Sleepiness Scale

STAI, State Trait Anxiety Inventory

Th1, T helper type 1

Th2, T helper type 2

UCP1, uncoupling protein 1

U-K, Uchida-Kraepelin

Introduction

Pollen of *Cryptomeria japonica* (Japanese cedar) is scattered in the atmosphere from February to May in Japan, and it causes pollinosis, which is characterized by sneezing, itchy eyes, watery eyes and runny nose. According to the 2008 survey conducted by Baba *et al.*,[1] the annual average prevalence of Japanese cedar-pollen allergy is 26.5% in Japan. Daily activity and quality of life are reduced during the pollen season due to rhinoconjunctival symptoms or pharmacological side-effects.[2] Pollinosis negatively affects the economy due to increased medical expenses, decreased labor productivity, and decreased domestic consumption as the affected individuals often refrain from going out. Thus, seasonal allergic disease induced by Japanese cedar pollen is problematic both to affected individuals and society as a whole.

Obesity is also a problem in many countries. Obesity is a risk factor for hypertension, hypercholesterolemia, diabetes, cardiovascular diseases, respiratory problems (asthma), musculoskeletal disease (arthritis) and some forms of cancer. Based on the current WHO guidelines,[3] adults with a body mass index (BMI) between 25 and 30 kg/m^2 are overweight, and adults with a BMI greater than 30 kg/m^2 are obese. Based on OECD health data,[4] at least half of the adult population in the following 13 countries are overweight or obese: Mexico, the United States, the United Kingdom, Australia, Greece, New Zealand, Luxembourg, Hungary, the Czech Republic, Portugal, Ireland, Spain and Iceland. Although the overweight and obesity rates are much lower in Japan, Korea and some European countries (France and Switzerland), the overweight and obesity rates are also increasing in these countries. In Japan, 3.4% of adults are obese, and 21.8% of adults are overweight. One report has indicated that the impact of overweight and obesity upon medical care costs in Japan is as large as in Western countries, despite the much lower mean BMI in Japanese populations.[5]

Allergy and obesity appear to be related. The prevalence of asthma and atopy increased significantly with increasing quartiles of BMI.[6] The relationship between obesity and immunity is an area of active research. Because it takes a long time to treat both disorders, anti-allergy foods and anti-obesity foods with easy continuous intake

and without side effects are needed.

Lactobacillus plantarum No.14 (LP14) was isolated from pickled shallots, a traditional Japanese food, and has long been used in food processing. It is used in starter cultures in the food industry. The LD50 values of LP14 was not less than 2 g/kg in acute toxicity test. The mutagenicity of LP14 was negative. It is thought that the safety of LP14 is high. Then, the validity of LP14 has been investigated in human-being. LP14 was deposited as FERM P-11550 in the International Patent Organism Depositary (Ibaraki, Japan) by Momoya (Tokyo, Japan).

A placebo-controlled, double-blind study was conducted in order to evaluate the effects of LP14 on Japanese cedar pollen allergy in 2005.[7] A daily oral intake of LP14 was found to suppress Japanese cedar pollen-specific immunoglobulin E (IgE) levels, eosinophil counts, and subjective symptoms, and to reduce body fat percentage. No lactic acid bacterium able to reduce body fat percentage in humans had been reported previously, and thus LP14 is the first reported lactic acid bacterium that decreases body fat percentage in humans. Afterwards, it is found that *Lactobacillus gasseri* SBT2055 has the effect of lowering abdominal adiposity.[8]

It was suggested that LP14 was useful which improved both allergy and overweight. These effects of LP14 were investigated further in this work. The administration studies were conducted on allergy in spring and autumn. The gastrointestinal tolerance of LP14 was evaluated *in vitro*. Body fat percentage was assumed to decrease because LP14 induced thermogenesis, and body temperature was measured in two administration studies. A novel processed food was developed through fermentation with LP14 based on above research, and an administration study of this food was conducted.

Chapter 1: Improvements in Seasonal Allergic Disease with LP14

Introduction

The most common cause of seasonal allergic disease in Japan is Japanese cedar pollen (February to April).[9] According to the 2008 survey conducted by Baba *et al.*,[1] the annual average prevalence of Japanese cedar-pollen allergy is 26.5% in Japan. The prevalence increased 10% by compared with ten-year before.

One of the explanations for the increased prevalence of allergies is the well-known hygiene hypothesis. The hygiene hypothesis postulates that a decrease in exposure to immunostimulating pathogens in early childhood causes an increased prevalence of allergic diseases.[10] Consequently, T helper type 1 (Th1) cells are insufficiently developed. A continuous marked skewing of the immune response toward cells of the T helper type 2 (Th2) lineage promotes humoral immunity, for example, IgE production, and eosinophilia.[11, 12]

Some studies have indicated that the clinical symptoms and inflammation markers for allergies are alleviated after administration of lactic acid bacteria.[13-16] Intestinal microflora, pathogenic and commensal, show similar capacities to modulate the local immunological environment, and this local modulation can influence systemic immunological function. Although the clinical implications are accepted, there have been few clinical trials and there is little information on the significance of lactic acid bacteria in allergic disorders. Furthermore, there have been few screening studies on serum IgE-modulating strains of lactic acid bacteria, hence the differences in the ability to suppress serum IgE among strains, species, and genera of lactic acid bacteria remain uncertain. In some tests, it has been reported that serum IgE suppression is a species-specific characteristic.[17] *Lactobacillus rhamnosus* GG is effective in preventing early atopic disease in children,[16] but oral treatment with *L. rhamnosus* GG has no effect on birch-pollen allergy.[18] Therefore, the combination of an allergic disorder and a lactic acid bacterium should also be considered in deciding on a treatment strategy.

The present intervention studies were carried out in order to evaluate the effects of LP14 on seasonal allergic diseases in the autumn of 2007 and the spring of 2008. It was

examined to determine whether there is a difference in the reactiveness due to the difference in the allergen when a sample of LP14 is taken.

Experimental methods

Study design. Both the spring and the autumn study were conducted using a randomized, placebo-controlled, double-blind system.

The spring study was carried out from January 11 to March 13. During a pre-intake period (January 11-24), the subjects were randomized into intervention and placebo groups. Those in the intervention group were administered LP14 (8.7×10^8 CFU/0.5 g), and those in the placebo group were administered 0.5 g of branched dextrin for 6 weeks (between January 25 and March 6). After 6 weeks of intake, there was a 1-week period with no intake. The subjects were instructed to keep a diary of allergic symptoms, medication, and stool and abdominal condition throughout the experimental period. During the study period, blood samples were obtained 3 times (pre-intake, January 10; cessation of intake, March 6; and post-intake, March 13), and fecal samples 2 times (pre- and post-intake).

The autumn study was carried out between October 12 and December 13. The protocol of the autumn study was the same as for the spring study. The pre-intake period was October 12-25. The intake period was from October 26 to December 6. And the post-intake period was December 7-13.

The studies were conducted in accordance with the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Kagawa Nutrition University. Written informed consent was obtained from all subjects.

Subjects. The first study was held during the spring of cedar pollen season, and enrolled a total of 35 female college students allergic to Japanese cedar pollen (age range, 18-27 years). The subjects were randomized into two groups: LP14 intervention and placebo. All subjects in both groups were students from the same campus, and exposure to pollen was similar between the two groups. Two subjects withdrew for personal reasons, one from the intervention group and one from placebo group. Thus, 16 subjects comprised the intervention group and 17 the placebo group. Neither the placebo group nor the LP14 group showed significant differences in any of the

measured parameters when the subjects began the experiment (Table 1).

The second study was held in the autumn enrolled 20 female students, 10 in the intervention and in the placebo group. There were no significant differences between the placebo and the intervention group in any of the measured parameters when the subjects began the experiment (Table 1).

Preparation of active and placebo sample. LP14 powder and placebo were prepared in the same manner in the spring and autumn studies. One percent LP14 starter was inoculated into Rogosa medium, modified by replacement of glucose with fructose and then pre-fermentation overnight at 30°C before fermentation. The culture medium was centrifuged at 13,000×*g* 3 times and washed with saline solution. The precipitate was resuspended in 4% branched dextrin solution and lyophilized. The active sample consisted of 20% w/w bacterial lyophilisate (1.7×10^9 CFU/g) and 80% w/w branched dextrin. The placebo contained only branched dextrin.

Pollen count. Pollen counts in the atmosphere were obtained from the data acquisition system of the Ministry of the Environment of Japan (http://kafun.taiki.go.jp/). Levels were measured from February 1 to March 6 in 2008 in the experimental area at the Hanno City Office, Saitama, Japan (13 km from the experiment site). Values are presented as particle number per cubic meter per day.

Subjective allergy symptoms. Nasal and ocular symptoms described by the subjects were classified as follows: sneezing, runny nose, stuffy nose, itchy eyes, and watery eyes. Each of these was scored on a scale from 0 to 4 (Table 2). The highest score for sneezing, runny nose and stuffy nose was used as the nasal symptom score, and the highest score for itchy eyes and watery eyes was used as the ocular symptom score. Although no medication to prevent the allergy itself was administered before the pollen season, the subjects in both studies were concomitantly treated with commercial and/or prescribed medicine to relieve their symptoms. The use of medication was scored from 0 to 3 (Table 3). The sum of the symptom and medication score was used as the

symptom-medication score (SMS), as defined by the Japanese Society for Allergology,[19] to assess the severity of the symptoms of Japanese cedar pollinosis.

Blood examination. Blood samples were taken in order to determine total IgE, anti-Japanese cedar pollen IgE, Th1 percentage, Th2 percentage, Th1/Th2 ratio, eosinophil counts, and C-reactive protein (CRP). The Th1 and Th2 cells were analyzed by flow cytometer. The $CD4^+$, interferon (IFN)-γ^+, and interleukin (IL)4^- populations and the $CD4^+$, IFN-γ^-, and $IL4^+$ populations were defined as Th1 and Th2 cells respectively. The Th1/Th2 ratio was calculated by dividing the percentage of the Th1 population by the percentage of the Th2 population. In the autumn study, anti-ragweed pollen IgE and anti-house dust IgE was also measured. All blood tests were performed by SRL (Tokyo, Japan).

Fecal microflora. Feces were collected pre- and post-intake from five subjects of each group. Fresh fecal samples were placed in tubes with an oxygen absorber, transferred on ice, and analyzed for the presence of *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and *Bacteroidaceae*. Fecal analysis was performed by Mitsubishi Chemical Medience (Tokyo, Japan).

Condition of stool and abdomen. Each subject recorded stool and abdominal conditions throughout the experimental period. The diary included frequency of defecation, the fecal amount, shape, color, and odor, and sensation after defecation (Table 4). Each subject was also asked questions on abdominal distention, gassiness, cramps, and diarrhea.

Other measurements. The other measured parameters were body weight, body fat weight, lean body weight, blood pressure, and general blood components.

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Statistical analysis. In the clinical test, for the null hypothesis the Shapiro-Wilk test was used. Total IgE, IgE specific for Japanese cedar pollen, Th1/Th2 ratio, eosinophil

counts, and CRP were non-normality. Thus nonparametric analysis was applied to these data and the categorical data (subjective symptoms of pollinosis and stool condition). Comparisons between the intervention and placebo groups were made by the Mann-Whitney U-test for non-normal data. In the case of the normal data, the unpaired t-test was used. Comparisons between pre-intake and intake cessation were made by the Wilcoxon t-test for the non-normal data. Comparisons between intake cessation and post-intake were also made by the Wilcoxon t-test for the non-normal data. In the case of the normal data, the paired t-test was used. All statistical tests were two-sided. Statistical significance was set at $P<0.05$. Statistical analysis employed SPSS 6.1 software (SPSS, Chicago, IL, USA) and ystat2006 (Igakutosho-shuppan, Tokyo, Japan).

Results

1. Spring study

Pollen count

Figure 1 shows the weekly averages of Japanese cedar pollen count during the spring season of 2008.

Subjective allergy symptoms

The effects of orally administered LP14 on symptoms were examined by comparing time-course changes in the mean SMS value for the LP14 group with that for the placebo group during the experimental period (Fig. 2). The mean ocular SMS for the LP14 group was lower than that for the placebo group during the intake period, and there was a significant difference between the two groups in ocular SMS during the first week of intake (P=0.0033). In particular, the symptom score for itchy eyes was significantly lower in the LP14 group than in the placebo group (P=0.014). No significant differences were observed in the medication scores.

Blood examination

In order to exclude the influence of medicine on allergy symptoms, data from the subjects who had not taken medicine within one week prior to blood examination was analyzed (Table 5). The remaining subjects numbered nine in the LP14 group, and 10 in the placebo group. No significant differences between the placebo and the LP14 group were detected in the pre-intake data. The distribution of data after excluding the subjects who had taken medicine was the same as before exclusion. There were no significant differences between the two groups in the data at intake cessation, either. However, in the placebo group, the Th1/Th2 ratio tended to decrease after intake cessation (P=0.075). In the LP14 group, the percentage of Th1 cells increased significantly (P=0.013). Post-intake eosinophil counts increased significantly in comparison with those during intake in the placebo group (P=0.018), but it appeared to be suppressed in the LP14 group (P=0.067). The CRP in the LP14 group was unchanged.

Fecal microflora

No significant differences in fecal microflora were observed between the LP14 and the placebo group, or between pre-intake and at intake cessation in either group (Table 6).

Condition of stool and abdomen

The score for sensation after defecation in the LP14 group after 3 weeks of intake was significantly higher than in the placebo group (P=0.047, Fig. 3). Frequency of defecation, fecal amount, and other scores did not vary between the groups during the experiment period (data not shown).

Other measurements

There were no clinically significant differences in body weight, body fat weight, lean body weight, blood pressure, or other general blood parameters (data not shown).

2. Autumn study

Subjective allergy symptoms

In the autumn study, data in subjects that were positive for IgE against ragweed pollen and/or house dust was analyzed (Table 7). There were seven subjects in both the LP14 and the placebo group, and none had taken any medication for allergy symptoms. The level of exposure to ragweed pollen and house dust was unclear. However, in the placebo group, peak allergic symptoms were observed at weeks 3-5 of intake (Fig. 4). There were no significant differences among symptom scores, but the nasal and ocular symptom scores in the LP14 group appeared to be lower than those in the placebo group during the intake period. In particular, the score for stuffy nose tended to decrease at week 3 of intake (P=0.086).

Blood examination

Subjects who were positive for IgE against ragweed pollen and/or house dust were examined. No differences in changes in blood parameters were observed between the

LP14 and the placebo group (data not shown).

Three subjects in each group were positive for IgE against Japanese cedar pollen only. No adverse events were reported for any parameters (data not shown).

Fecal microflora

No significant differences in fecal microflora were observed between the LP14 and the placebo group, or between pre-intake and intake cessation in either group (data not shown).

Condition of stool and abdomen

The sensation score after defecation declined in the LP14 group at post-intake (P=0.040, Fig. 5). The other data on stool condition were similar in the two groups during the intake period (data not shown).

Other measurements

BMI increased significantly in the LP14 group (+0.36, P=0.041), and tended to increase in the placebo group (+0.30, P=0.060) after 6-week intake period (data not shown). The increase in body fat weight in the LP14 group appeared to be slightly lower than in the placebo group (Fig. 6), while lean body weight was significantly higher in the LP14 group (P=0.014).

There were no clinically significant differences in blood pressure or other general blood parameters.

Discussion

This spring study was planned to end before the spring vacation (the middle of March). The purpose was to expose subjects, female students, to pollen in a similar environment, and to examine under constant life conditions, but pollen dispersal was late, and the amount was also small that year. Thus, the subjects were not heavily exposed to pollen for the administration period.

Mean ocular SMS for the LP14 group decreased significantly as compared with the placebo group during the first week of intake in spring, although no significant differences were observed in the medication score. This suggests that ingestion of LP14 instead of medication might have an effect on allergic symptoms.

Furthermore, excluding the influence of allergy medicines, the Th1/Th2 ratio in the placebo group tended to decrease after 6-week intake period based on the increase in pollen dispersal, but in the LP14 group, the percentage of Th1 cells increased significantly. Pollen dispersal in the post-intake period increased greatly as compared with that during the intake period, and the eosinophil counts increased significantly in the placebo group, but appeared to be suppressed in the LP14 group. LP14 has immunostimulatory activity, including mitogenicity and strong induction of Th1-type cytokines.[20] Allergen exposure activates mast cells, and consequently various chemical mediators and cytokines capable of inducing the recruitment of allergy-related inflammatory cells, such as eosinophils and Th2 cells, are released.[21] Abnormal generation of IgE antibodies in the serum is thought to be initiated by the activation of Th2 cells.[22-24] LP14 might function as an effective counter-regulator of Th2-skewed immunity in patients with pollinosis when administered orally.

LP14 induced gene expression of inflammatory cytokines such as IL-1β, but it also induced anti-inflammatory cytokines such as IL-10.[20] In the administration study on humans, CRP in the LP14 group was unchanged, and no excess inflammatory reactions due to LP14 occurred.

In a previous study, Japanese cedar pollen-specific IgE levels, eosinophil counts, and subjective symptoms were reported to be suppressed in subjects taking LP14 during the spring season, when cedar pollen dispersal was heavy.[7] The production of IgE

antibodies specific for Japanese cedar pollen is considered to play an important role in cedar pollen allergy. IgE is directly involved in allergic reactions. The total IgE and cedar pollen-specific IgE levels were insignificantly decreased by intake of LP14 in the present study. Allergy symptoms deteriorated during the late intake term as the amount of pollen increased, and the difference between the LP14 group and the placebo group disappeared. One of the reasons for the difference between the results of these studies might be the amount of LP14. Subjects took 8.7×10^8 CFU/day of LP14 in this study, and 2.0×10^{10} CFU/day of it in the previous study.[7] It is thought that more than 10^{10} CFU/day intake is necessary to obtain a clear improvement effect in Japanese cedar pollinosis.

The most common cause of pollinosis in autumn in Japan is ragweed. House dust allergy is a perennial allergic disease, but the peak of allergic symptoms is generally observed in autumn. In the autumn study, no statistically significant anti-allergic effects were seen for LP14 with regard to the blood parameters or symptom scores. However, the nasal and ocular symptom scores in the LP14 group appeared to be lower than those in the placebo group during the intake period. One of the reasons might be the limited number of subjects. Thus, because there is a possibility that LP14 improves seasonal allergic diseases in autumn, it is necessary to do the further investigation with a larger number of subjects that were positive for IgE against ragweed pollen and against house dust.

It has been suggested that changes in intestinal microflora resulting from changes in diet and hygiene are the cause of the progressive increase in the frequency of allergic diseases in developed countries,[25] as microflora are considered to affect host Th1/Th2 balance.[26, 27] Analysis of intestinal microflora in fecal samples from 2-year-old children in Sweden and Estonia indicated a lower rate of colonization by lactobacilli in allergic children as compared with non-allergic children.[28] The proportion of aerobic bacteria to total bacterial count, particularly for coliforms and *Staphylococcus aureus*, was elevated in the intestinal microflora of the allergic children. In a recent case-control study, Matricardi *et al.*[29] found that inappropriate stimulation by commensal intestinal bacteria or pathogens affecting gut-associated lymphoid tissue (GALT) enhanced the risk of

atopy. Some lactic acid bacteria are thought to be a tool for establishing Th1 predominance through changes in the composition of host intestinal microflora by their effects or by their direct action on GALT. No significant changes in fecal microflora due to LP14 intake were observed in either the spring or the autumn study. It indicates that LP14 does not affect host intestinal microflora. Therefore, the underlying mechanism of the anti-allergic effects of orally administered LP14 can be explained by direct action on the host systemic immune system.

In the autumn study, BMI significantly increased in the LP14 group and tended to increase in the placebo group after the intake period. However, the increase in body fat weight in the LP14 group appeared to be less than that in the placebo group, while lean body weight was significantly higher in the LP14 group. Although body fat percentage did not decrease in the present study, body composition appears to be affected by oral intake of LP14.

As for the condition of the stools, the sensation score after defecation of the LP14 group improved in the spring study, and declined in the autumn study. The change in the condition of stools was a different result at each examination in this study. Hence, it was considered to be a chance result. Neither increases in fecal amount nor softening of stool, which happened generally because of the lactic acid bacterium intake was seen in this study.

There were no clinically important abnormal values in any of the data in either study. No adverse experience was noted when the subjects who were positive for anti-Japanese cedar pollen IgE and negative for IgE against ragweed pollen and house dust took LP14 in the autumn, either. LP14 was isolated from a traditional food source, and has long been used in food processing. Therefore, the safety profile of LP14 in the treatment of seasonal allergic diseases appears favorable.

In conclusion, this study indicates a beneficial clinical effect of LP14 ingestion on the symptoms of seasonal allergic diseases. The underlying mechanism might be explained by direct action on the host systemic immune system.

List of Tables and Figures

Table 1. Clinical Characteristics of the Subjects.

	Spring study				Autumn study			
	Placebo		LP14		Placebo		LP14	
Number of subjects	17		16		10		10	
Age (years)	21.9 ± 5.6		22.0 ± 3.9		20.7 ± 1.3		22.6 ± 4.8	
Total IgE (IU/ml)	450.4 ± 627.4		390.4 ± 570.9		291.1 ± 333.0		402.3 ± 550.1	
IgE specific for Japanese cedar pollen (UA/ml)	30.1 ± 32.9		19.3 ± 25.7		36.7 ± 33.2		27.9 ± 30.9	
IgE specific for house dust (UA/ml)	–		–		21.9 ± 38.6	(6)[a]	24.5 ± 36.7	(7)
IgE specific for ragweed pollen (UA/ml)	–		–		2.0	(1)	3.7 ± 4.3	(4)
Eosinophil counts (/μl)	189.4 ± 113.5		196.0 ± 131.4		227.0 ± 108.8		218.0 ± 119.2	
Th1/Th2 ratio (−)	12.8 ± 5.0		15.7 ± 7.0		11.6 ± 4.4		12.0 ± 6.1	
BMI (kg/m^2)	21.5 ± 2.2		22.5 ± 3.0		21.2 ± 2.1		21.1 ± 2.3	
Body fat parcentage (%)	29.4 ± 4.3		29.3 ± 5.6		28.0 ± 4.9		27.1 ± 5.2	
Severity of nasal symptoms at baseline (−)	0.88 ± 0.67		1.26 ± 0.95		1.41 ± 1.03		1.24 ± 0.86	
Severity of ocular symptoms at baseline (−)	0.49 ± 0.73		0.22 ± 0.34		0.69 ± 0.75		0.80 ± 0.75	

Data are expressed as means ± SD. [a]Number of subjects who were positive for allergen-specific IgE.

Table 2. Scores for Allergic Symptoms.

	Severity score				
	0	1	2	3	4
Sneezing[a]	0	1–5	6–10	11–20	21≦
Ruuny nose[b]	0	1–5	6–10	11–20	21≦
Stuffy nose	None	Mild	Moderate	Severe	Violent
Itchy eyes	None	Mild	Moderate	Severe	Violent
Watery eyes	None	Mild	Moderate	Severe	Violent
Obstacles in daily life	None	Mild	Moderate	Severe	Violent

[a] Mean number of sneezing attacks per day. [b] Mean number of nose blows per day.

Table 3. Medicine Scores.

	Score
Oral antihistamines	1
Oral histamine release inhibitors	1
Nose drops (excluding focal steroids)	1
Eys drops (excluding focal steroids)	1
Focal administration of steroids	2
Oral antihistamines and focal steroids	3

Prescribed medications and commercial drugs recorded daily were scored according to the description in this table.

Table 4. Scores for Fecal Characteristics.

	1	2	3	4	5	6
Fecal shape	very hard	hard	banana-shaped[a]	soft[a]	muddy	watery
Fecal color	brownish yellow[a]	brown-dark brown	black	–	–	–
Fecal odor	very strong	strong	unchanged[a]	weak[a]	very weak[a]	–
Sensation after defecation	refreshing[a]	usual[a]	unrefreshing	–	–	–

[a] Considered the normal condition.

Table 5. Blood Components of Allergy Medicine-Free Subjects of within One Week Prior to Blood Examination in the Spring Study.

		Pre-intake	p [a]	At intake cessation	p [b]	Post-intake
Total IgE (IU/ml)	Placebo	306.3 ± 523.0	0.005 **	238.4 ± 384.8	0.513	248.8 ± 415.6
	LP14	480.2 ± 710.6	0.139	515.9 ± 850.5	0.173	487.4 ± 801.3
IgE specific for Japanese cedar pollen (UA/ml)	Placebo	14.7 ± 17.5	0.161	13.9 ± 16.1	0.677	14.6 ± 18.3
	LP14	15.7 ± 28.4	0.354	16.3 ± 32.0	0.504	15.7 ± 29.9
CRP (mg/dl)	Placebo	0.03 ± 0.02	0.075	0.07 ± 0.08	0.398	0.05 ± 0.04
	LP14	0.03 ± 0.03	0.360	0.04 ± 0.03	0.625	0.04 ± 0.02
Eosinophil counts (/ μ l)	Placebo	174.0 ± 121.2	0.217	215.0 ± 108.4	0.018 *	341.0 ± 262.0
	LP14	231.1 ± 156.4	0.879	225.6 ± 144.5	0.067	323.3 ± 174.6
Th1/Th2 ratio (-)	Placebo	13.6 ± 5.9	0.075	10.6 ± 4.8	0.513	9.9 ± 5.5
	LP14	17.5 ± 7.5	0.139	15.4 ± 6.3	0.595	14.8 ± 10.0
Th1 (%)	Placebo	18.2 ± 6.1	0.110	20.2 ± 6.4	0.319	22.1 ± 10.0
	LP14	19.5 ± 4.1	0.013 *	23.2 ± 4.7	0.681	23.9 ± 7.2
Th2 (%)	Placebo	1.4 ± 0.4	0.005 **	2.0 ± 0.4	0.227	2.7 ± 1.4
	LP14	1.3 ± 0.5	0.008 **	1.8 ± 0.8	0.210	2.1 ± 1.1

Data are expressed as means ± SD. There were 9 subjects in LP14 group, and 10 subjects in the placebo group. [a]Comparisons between pre-intake and intake cessation were made by the Wilcoxon t-test. [b]Comparisons between intake cessation and post-intake were made by the Wilcoxon t-test. * P<0.05; ** P<0.01.

Table 6. Compositions of Fecal Flora in the Spring Study.

		Pre-intake	At intake cessation
Bacteroidaceae	Placebo	9.9 ± 0.4	8.7 ± 2.5
	LP14	10.0 ± 0.4	10.0 ± 0.6
Bifidobacterium	Placebo	9.6 ± 0.3	9.1 ± 1.7
	LP14	9.6 ± 0.3	9.6 ± 0.4
Lactobacillus	Placebo	5.4 ± 1.9	5.3 ± 1.8
	LP14	4.5 ± 1.2	3.8 ± 1.4
Clostridium	Placebo	7.1 ± 0.7	5.9 ± 2.2
	LP14	7.0 ± 0.3	6.8 ± 0.7

Each value represents the means ± SD in terms of log10/g. There were 5 subjects in each group. No significant differences were observed between the two groups in these measurements (Mann-Whitney U-test). No significant differences were observed between pre-intake and intake cessation in these measurements (Wilcoxon t-test).

Table 7. Number of Subjects Positive for IgE against Specific Allergens in the Autumn Study.

	Japanese cedar pollen	Ragweed pollen	House dust	All three	Ragweed pollen and/or housedust
Placebo	10	1	6	0	7
LP14	10	4	7	4	7

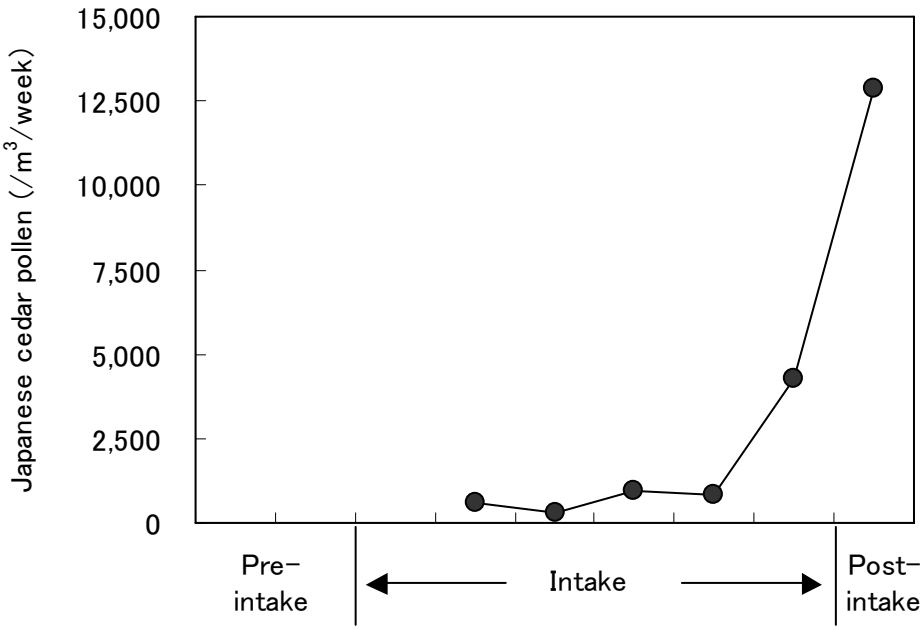

Fig. 1. Japanese Cedar Pollen at the Experimental Site during the Spring Study.

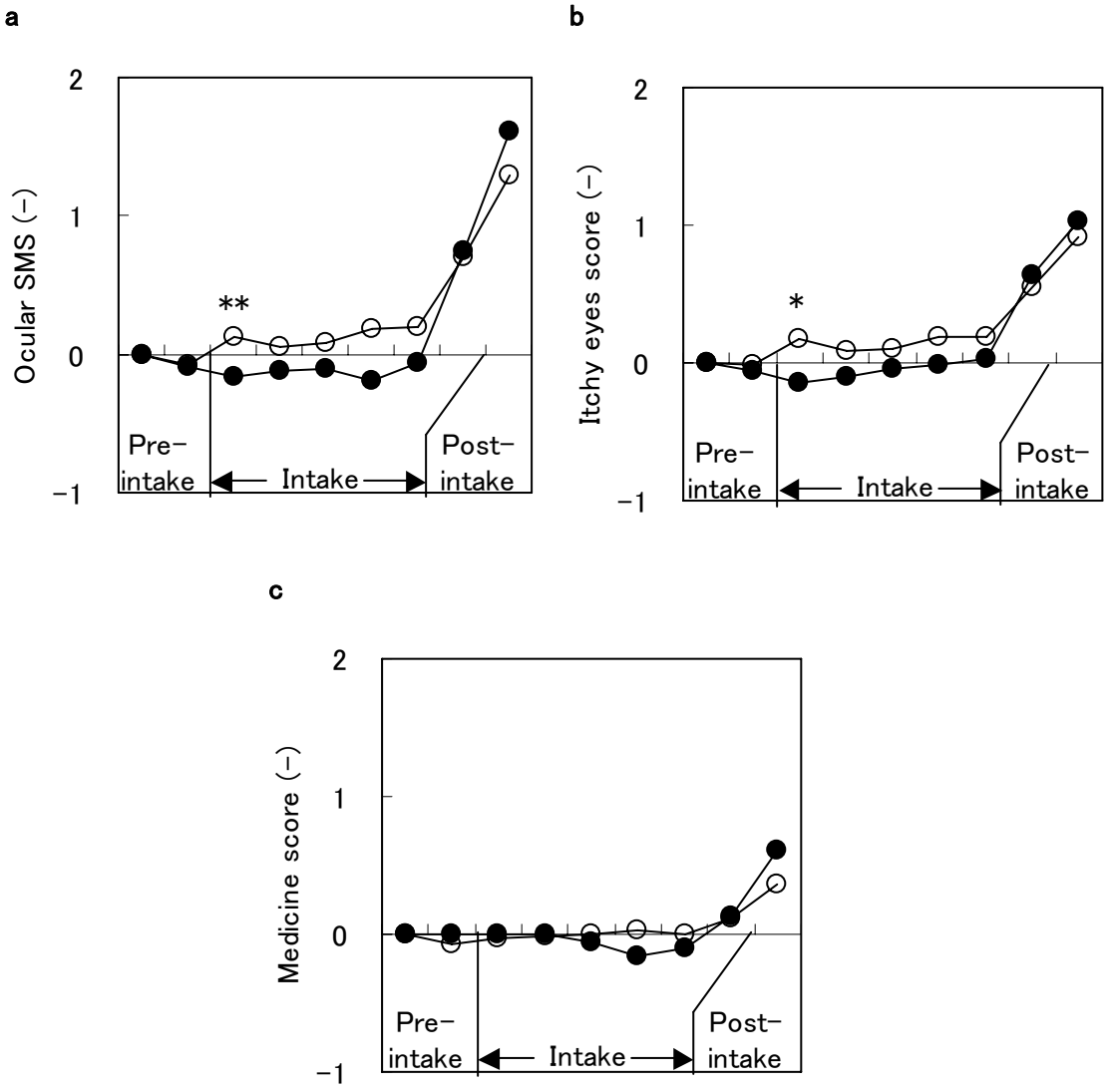

Fig. 2. Changes in Weekly Average Scores for Ocular SMS (a), Itchy Eyes (b) and Medicine Intake (c) during the Spring Study.

Data are expressed as ((Experimental period) - (1st week of pre-intake)) of 17 subjects in the placebo group (○) and 16 subjects in the LP14 group (●). For comparisons between the placebo and LP14 group, the Mann-Whitney U-test was performed. * $P<0.05$; ** $P<0.01$.

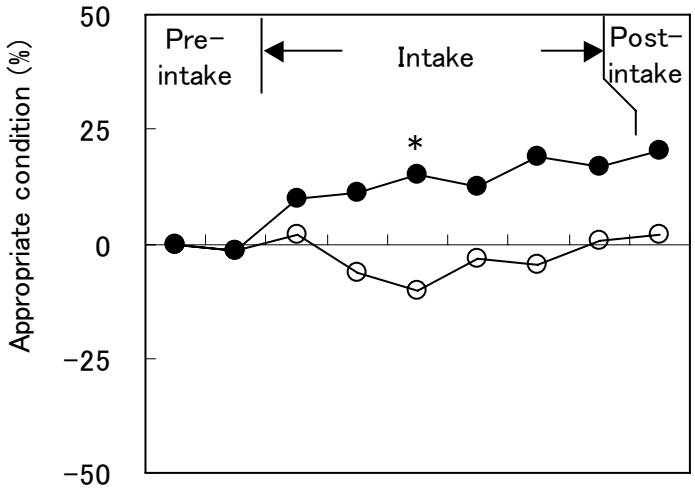

Fig. 3. Changes in Weekly Average Scores for Sensation after Defecation during the Spring Study.

Data are expressed as ((Experimental period) - (1st week of pre-intake)) of 17 subjects in the placebo group (○) and 16 subjects in the LP14 group (●). For comparisons between the placebo and the LP14 group, the Mann-Whitney U-test was performed. * $P<0.05$.

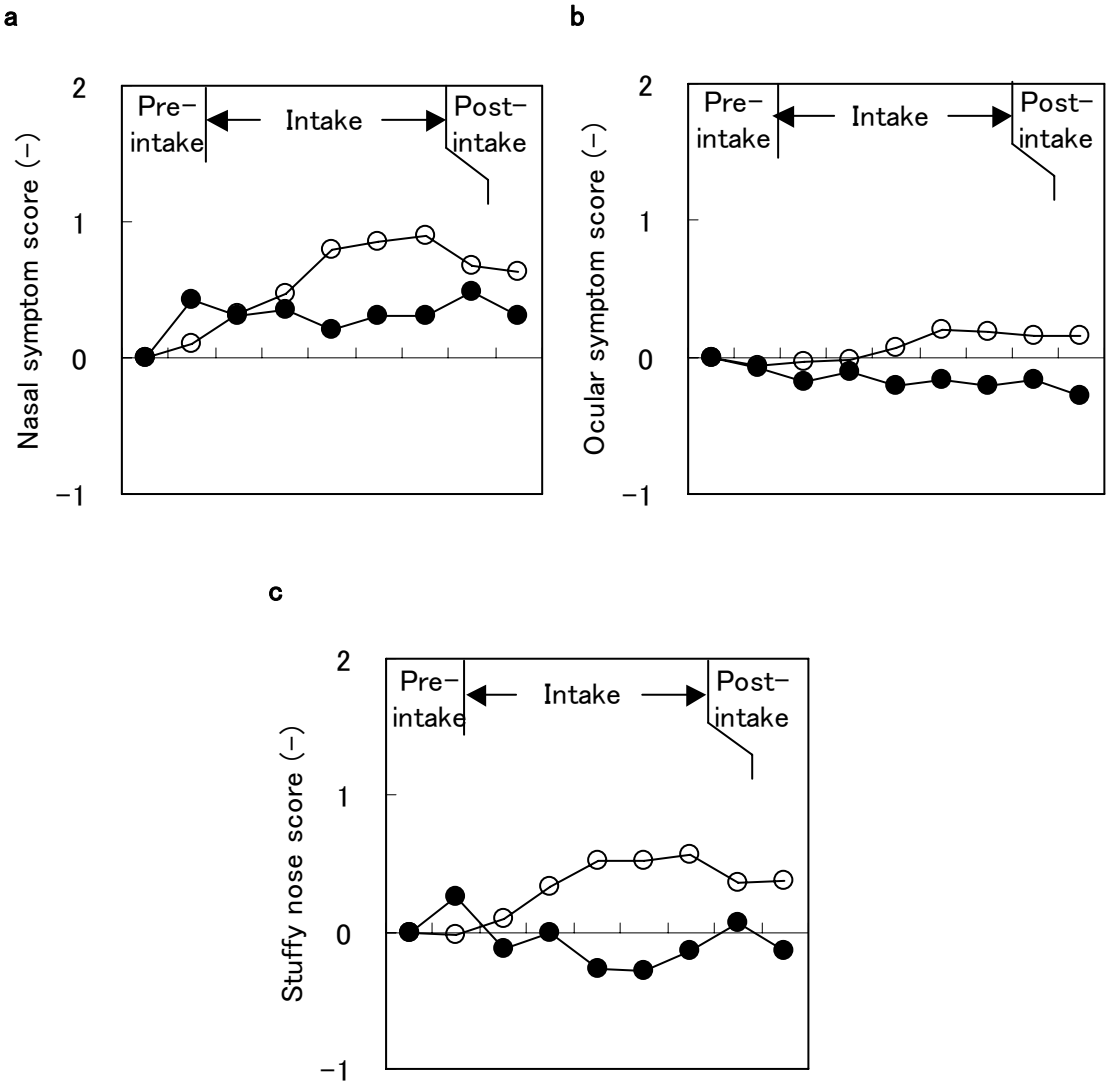

Fig. 4. Changes in Weekly Average Scores for Nasal Symptoms (a), Ocular Symptoms (b), and Stuffy Nose (c) during the Autumn Study.

Data are expressed as ((Experimental period) - (1st week of pre-intake)) of 10 subjects in the placebo group (○) and 10 subjects in the LP14 group (●). For comparisons between the placebo and the LP14 groups, the Mann-Whitney U-test was performed. There were no significant differences.

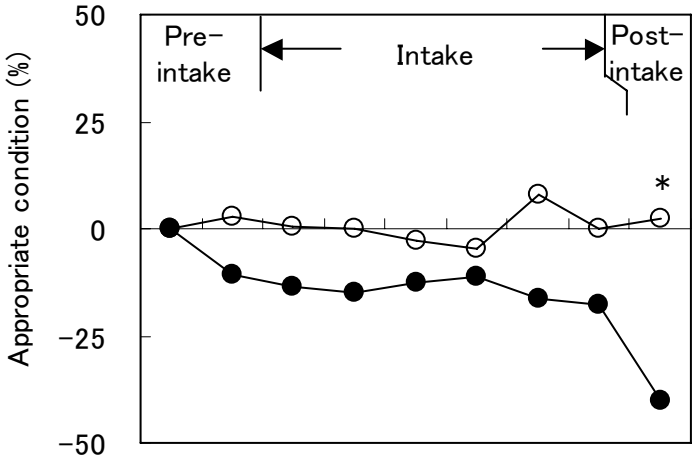

Fig. 5. Changes in Weekly Average Scores for Sensation after Defecation during the Autumn Study.

Data are expressed as ((Experimental period) - (1st week of pre-intake)) of 10 subjects in the placebo group (○) and 10 subjects in the LP14 group (●). For comparisons between the placebo and the LP14 group, the Mann-Whitney U-test was performed. * $P<0.05$.

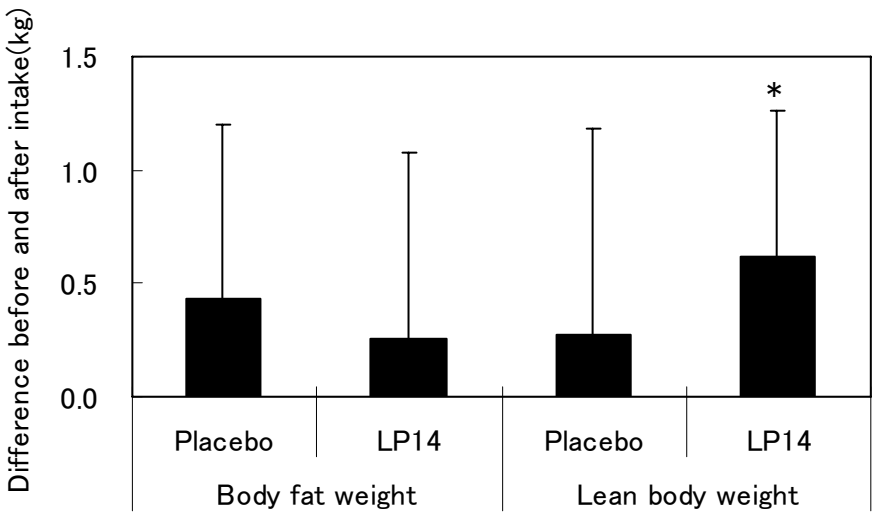

Fig. 6. Increases in Body Fat Weight and Lean Body Weight in the Autumn Study.

Data are expressed as means ± SD of ((intake cessation) - (pre-intake)) of 10 subjects in the placebo group and 10 subjects in the LP14 group. Comparisons between the pre-intake and intake cessation were made by the paired t-test. * $P<0.05$.

Chapter 2: Gastrointestinal Transit Tolerance of LP14

Introduction

LP14 is a plant-origin lactic acid bacterium that is generally considered to be capable of surviving in severe environments.[30] It has been reported that the gastrointestinal transit tolerance of some strains of *L. plantarum* isolated from rice is high.[31]

Roy Fuller's definition of probiotic (1989),[32] "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance," is widely used. However, the definition of probiotic has since become broader, to the extent that almost anything, living or dead, can be included. According to Salminen *et al.* (1999),[33] "probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host." It has been found that heat-killed lactic acid bacteria cells affect the host systemic immune system. For example, the symptoms of Japanese cedar-pollen allergy improved significantly in a group of volunteers who consumed fermented milk containing *Lactobacillus acidophilus* strain L-92 cells (heat-treated).[34] When microorganisms are consumed orally, it is important to consider whether the microorganisms are dead or alive in the intestine, as this should provide insight into the mechanisms of the effects on the host.

Probiotic bacteria delivered through food systems must first survive transit through the upper gastrointestinal tract and then persist in the gut in order to provide the beneficial effects for the host.[35] The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against the entry of many bacteria into the intestinal tract.[36] The pH of the stomach can be as low as pH 1.5,[37] or as high as pH 6.0 after food intake,[38] but generally ranges from pH 2.5 to 3.5.[36] The nature of the food affects the transit time through the stomach. Food typically stays in the stomach for between 2 and 4 h.[39] Another barrier against probiotic bacterial transit is the intestine. Adverse conditions in the intestine include the presence of bile salts.[40] The transit time of food through the intestine is generally between 18 and 36 h.[39] A bile salt concentration of 0.15-0.3% has been recommended as a suitable concentration for

selecting probiotic bacteria for human use.[41)]

In this study, the gastrointestinal tolerance of LP14 was evaluated *in vitro*. In general, in lactic acid bacterium cultures, glucose is used as carbon and energy source, but fructose was used in the preparation of active samples for the administration of LP14, since LP14 produces viscous material when cultured with glucose, which makes it difficult to produce samples. When fructose is used as carbon source, LP14 produces smaller amounts of viscous material. There are several reports on lactic acid bacteria that make different amounts of viscous material, or exopolysaccharide (EPS), depending on the carbon source. For instance, there are *Lactobacillus amylovorus* DU-21[42)] and *Lactobacillus casei* CRL 87.[43)] The transit tolerance and yield of EPS was evaluated when LP14 was cultured with glucose or fructose as carbon source.

Experimental methods

Preparation of bacteria. In simulated gastrointestinal transit assays, LP14 was serially transferred twice into MRS medium (2% glucose or fructose, 1% peptone, 0.8% LAB-Lemco, 0.5% $CH_3COONa \cdot 3H_2O$, 0.4% yeast extract, 0.2% K_2HPO_4, 0.2% triammonium citrate, 0.1% Tween80, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnSO_4 \cdot 4H_2O$, pH 6.8), followed by aerobic incubation at 30°C for 20 h. For comparison, *Leuconostoc* sp. GLT 36 cultured with glucose was also treated. *Leuconostoc* sp. GLT 36 has been deposited as FERM P-13131. It was isolated from soil. To determine the simulated gastric tolerance of LP14 in the absence of EPS, cultures incubated with glucose or fructose were centrifuged for 10 min at 9,000×*g*. The precipitates were suspended by vortexing in fresh MRS broth (volume equal to original culture volume), and then centrifuged again under the same conditions. Centrifugation and suspension were repeated 3 times.

Preparation of simulated gastric and intestinal juices. Simulated gastric and intestinal juices were prepared fresh daily. Simulated gastric juices were prepared by suspending pepsin (1:10,000, Sigma, St Louis, MS, USA) in sterilized MRS broth (glucose was used as carbon source, Oxoid, Basingstoke, UK) at a final concentration of 0.04%, and by adjusting the pH to 2.5, 3.0, or 3.5 with concentrated HCl or sterilized 0.1 mol/l NaOH. Simulated intestinal juices were prepared by suspending 0%, 0.2%, or 0.4% oxgall (Difco, Detroit, MI, USA) in sterilized MRS broth (glucose was used as carbon source, Oxoid).

Gastrointestinal transit tolerance assay. Simulated gastric and intestinal transit tolerance was determined by the methods of Azuma *et al.* (2001)[44] and Kumagai *et al.* (2001).[31] LP14 was cultured with glucose and with fructose, as described above, and then mixed with simulated gastric (pH 2.5, 3.0, or 3.5) or intestinal juices (0%, 0.2%, or 0.4% oxgall) at a final concentration of 1%. As a control, *Leuconostoc* sp. GLT 36 was culture with glucose. The mixture was then vortexed at maximum speed for 10 s and incubated at 37°C. To determine the tolerance of combined gastric and intestinal transit,

3% of the simulated gastric juice mixture (pH 3.0, 3 h) was added to the intestinal juices (0%, 0.2%, or 0.4% oxgall). The culture medium of LP14 cultured with glucose or fructose and removed EPS was used only for the examination of simulated gastric transit tolerance at pH2.5.

Gastric transit tolerance was evaluated based on the total viable count after incubation for 0, 1, 2, or 3 h. Total viable counts of bacteria were determined by the pour-plate method using MRS agar supplemented 0.5% $CaCO_3$ after serial 10-fold dilution in maximum recovery diluents. MRS plates were incubated anaerobically at 30°C for 2 days, and the colonies on the MRS plates were counted using a colony counter.

Intestinal transit tolerance was evaluated based on optical densities (OD_{660}), which were determined after incubation for 0 or 18 h. Growth rates were expressed as the percentage of turbidity in culture liquid containing 0.2% or 0.4% oxgall after incubation for 18 h, as compared with those of broth containing no oxgall. Gastrointestinal transit tolerance was evaluated based on turbidity. The mixtures were observed under a microscope.

Determination of EPS from LP14. EPS was isolated and purified by the method of Kitazawa *et al.* (1998).[45] LP14 was serially transferred twice in MRS medium (glucose or fructose), and incubated aerobically at 30°C for 20 h. The cultures were heated at 100°C for 20 min, and bacterial cells were removed by centrifugation (13,000×*g*, 20 min, 4°C). The precipitates were then dissolved in PBS and centrifuged twice at 13,000×*g* for 20 min at 4°C. The supernatant was neutralized and concentrated 10-fold, and an equal volume of cold ethanol was added, followed by centrifugation at 13,000×*g* for 20 min at 4°C. Precipitated polysaccharide materials were collected and dissolved in distilled water, followed by the removal of insoluble material by centrifugation (13,000×*g*, 20 min, 4°C). Ethanol was then added to the resulting solution in order to precipitate the dissolved materials, as described above. The precipitates were treated for 6 h at 37°C with DNase and RNase (each at 7 μg/ml, Sigma) in 5 mmol/l Tris-HCl buffer (pH 8.0) containing 1 mmol/l $MgCl_2$, and were subjected to digestion by

proteinase K (200 μg/ml, Sigma) overnight at 37°C. After inactivation of the enzyme by heating for 10 min at 100°C, polysaccharide samples were precipitated with cold ethanol. The supernatants were centrifuged twice for 20 min at 13,000×*g* at 4°C. The precipitated polysaccharide materials were collected and dissolved in distilled water, and after removal of the insoluble material by centrifugation (13,000×*g*, 20 min, 4°C), the solution was dialyzed against distilled water for 48 h at 4°C. Polysaccharides were quantified by the phenol-sulfuric method.

Statistical analysis. The results were expressed as means ± SD. For gastric tolerance, two-way analysis of variance (ANOVA), one-way ANOVA, and Dunnett's test were performed. For intestinal tolerance and gastrointestinal tolerance, two-way ANOVA and unpaired t-test were performed. For simulated gastric tolerance of LP14 without EPS, two-way ANOVA, one-way ANOVA, and Student-Newman-Keuls test were performed. To compare the amounts of EPS, an unpaired t-test was performed. *P* values of less than 0.05 were regarded as indicating a significant difference.

Results

1. Gastrointestinal Transit Tolerance of LP14

Tolerance to simulated gastric juices at different pH levels

The effects of simulated gastric juices at different pH levels on viability are presented in Fig. 1. The average final pH of the simulated transit mixture did not change. All samples showed lower viability in simulated gastric juice at pH 2.5 as compared with pH 3.0 or pH 3.5.

In the simulated gastric juice at pH 2.5, the viability of LP14 was reduced by 1-log unit after at was incubated in MRS medium (LP14-Glc) for 3 h. LP14 incubated in MRS medium with fructose (LP14-Fru) showed a 4-log reduction in viability after 3 h. One-way ANOVA showed no differences among the time courses under glucose conditions, but in the case of LP14-Fru, there was a significant difference (P<0.01). Dunnett's test indicated that the viability of LP14-Fru decreased after 1 h. *Leuconostoc* sp. GLT 36 incubated in MRS medium (GLT36-Glc) lost viability after 1 h of simulated gastric tract transit.

When the pH of the simulated gastric juice was raised to pH 3.0, LP14-Glc retained a similar level of viability during simulated gastric tract transit for up to 3 h. In contrast, LP14-Fru showed a 1-log reduction in viability after 3 h. One-way ANOVA indicated that there were no differences among the time courses under glucose conditions, but in the case of LP14-Fru, there was a significant difference (P<0.01). Dunnett's test indicated that the viability of LP14-Fru decreased after 2 h. GLT36-Glc showed a 2.5-log reduction in viability after 1 h.

When the pH of the simulated gastric juice was further raised to 3.5, all the samples retained the same level of viability during the 3 h of simulated gastric tract transit, and there were no significant differences among the time courses.

Tolerance of simulated intestinal juices

The effects of different oxgall concentrations in the simulated intestinal juices on growth rate are presented in Table 1. The pH of all samples was approximately 5.8. The growth rates of LP14-Glc and LP14-Fru in the presence of 0.2% oxgall were 37.7% and

37.5% respectively. The growth rates of LP14-Glc and LP14-Fru in the presence of 0.4% oxgall were 24.0% and 21.8% respectively. With 0.2% oxgall, there were no significant differences between LP14-Glc and LP14-Fru, but with 0.4% oxgall there was a significant difference between them. The growth rate of GLT36-Glc was 30.3% in 0.2% oxgall and 12.4% in 0.4% oxgall.

Tolerance of simulated gastric and intestinal juices

The pH values in all samples were approximately 5.3. The growth rates of LP14-Glc and LP14-Fru in gastric and intestinal juices (0.2% oxgall) were 31.8% and 29.5% respectively (Table 2). In gastrointestinal juices with 0.4% oxgall, the growth rates of LP14-Glc and LP14-Fru were 12.1% and 6.6% respectively. The growth rate in intestinal juices alone and that in gastrointestinal juices containing oxgall at any concentration were significantly different (unpaired t-test, $P<0.01$). Under gastrointestinal conditions with 0.2% oxgall, there was no significant difference between LP14-Glc and LP14-Fru, but with 0.4% oxgall, a significant difference was noted. The growth rate of GLT36-Glc was 0% in both 0.2% and 0.4% oxgall.

It has been reported that *L. casei* in bile-containing medium forms chains of 10-15 cells, and that this results in difficulties in counting colonies and viable cells.[46)] When the simulated gastrointestinal juices treated with LP14-Glc were observed under a microscope, the chains of LP14 were about 1-10 cells in 0% oxgall (Fig. 2). The ratio of 5-10 cell chains increased in 0.2% oxgall, while the chains of LP14 were about 10-20 cells in 0.4% oxgall. The lengths of the chains of LP14-Fru were similar to those of LP14-Glc in 0.2% oxgall, but in 0.4% oxgall the chains of LP14-Fru were longer than those of LP14-Glc (data not shown).

Tolerance of simulated gastric juices of LP14 when EPS was removed

In order to remove EPS from LP14, cultures were incubated with glucose or fructose, centrifuged 3 times at 9,000×*g* for 10 min, and then washed with fresh MRS broth. Viable counts of LP14 before the gastric tolerance test did not change after centrifugation (data not shown). The tolerance of washed LP14-Glc of simulated gastric

juices at pH 2.5 decreased significantly, but that of washed LP14-Fru did not (Fig. 3). There was no significant difference between washed LP14-Glc and untreated LP14-Fru.

2. Determination of EPS from LP14

The amounts of EPS from LP14 after growth in glucose and in fructose were compared. The concentrations of EPS obtained after culture in glucose and in fructose were 146.5±8.1 mg/l and 20.1±17.0 mg/l respectively (Table 3), and the difference was found to be significant. The concentration of EPS per viable count was 4.6×10^{-11} mg/CFU in glucose and 2.4×10^{-11} mg/CFU in fructose.

Discussion

Initially, this study evaluated the effects of the pH of simulated gastric juices on the viability of LP14 during 3 h of simulated gastric transit. There was no loss of viability for LP14 at pH 3.5 regardless of the carbon source. On the other hand, at pH 3.0, LP14-Glc retained the same level of viability, while LP14-Fru showed reduced viability after 2 h. At pH 2.5, all the samples showed reduced viability, and a difference between LP14-Glc and LP14-Fru was seen after 1 h. Kumagai *et al.*[31] found that at pH 3.5, several strains of *L. casei* subsp. *casei*, *L. plantarum, L. acidophilus,* and *L. gasseri* retained the same level of viability. However, at pH 2.5, six strains of *L. casei* subsp. *casei* showed a 5-log reduction in viability, while the other species showed a 0- to 7-log reduction, depending on the strain. These results indicate that the gastric transit tolerance of LP14-Glc is sufficiently high for it to transit successfully through the human stomach. However, the gastric transit tolerance of LP14 varied with the carbon source, and when incubated with fructose, the viability of LP14 decreased.

The growth rate of LP14 was evaluated in simulated intestinal juices containing various concentrations of oxgall during an 18-h simulated intestinal transit. The growth rates of LP14-Glc and LP14-Fru in the presence of 0.2% oxgall decreased to 37.7% and 37.5% respectively. Other reports have indicated that the growth rates of some strains of *L. gasseri*, *L. casei*, *L. sakei*, and *L. acidophilus* decreased to 11-91% in simulated intestinal juices with 0.2% oxgall.[44, 47] Although the growth rate of LP14 in simulated intestinal juice decreased, the present results indicate that LP14 has the potential to grow in human intestinal juices. In 0.2% oxgall, there was no significant difference between LP14-Glc and LP14-Fru, but in 0.4% oxgall, a significant difference in growth rates was seen. The viable cell count of LP14 after it was mixed with intestinal juice could not be measured because the colony count is difficult in a medium adding bile, and the time course of tolerance is uncertain.

Potential probiotics are evaluated according to tolerance of gastric and intestinal transit respectively, but the intestinal tolerance of microorganisms after passage through the stomach is thought to decrease. Hence, intestinal tolerance was evaluated after the microorganisms were treated under stomach conditions, by the method of Kumagai *et*

al.[31] The intestinal tolerance of LP14 decreased after it was treated under stomach conditions, and the gastrointestinal transit tolerance of LP14 was also found to depend on the carbon source.

LP14 produced much viscous material when cultured with glucose, while it produced little when cultured with fructose. The physiological function of the EPS produced by *Lactococcus lactis* was studied by comparing the tolerance of the non-EPS-producing strain *Lactococcus lactis* ssp. *cremoris* MG1614 with an EPS-producing isogenic variant of this strain against several anti-microbial factors.[48] There was no difference in the sensitivity of the strains to increased temperatures, freezing, or freeze-drying, or to antibiotics such as penicillin and vancomycin. A model system also showed that EPS production did not affect the survival of *Lactococcus lactis* during passage through the gastrointestinal tract. In contrast, *Escherichia coli* O157:H7 W6-13 and its colanic acid EPS-deficient mutant M4020 were examined for gastrointestinal tolerance.[49] The results showed that colanic acid EPS might serve as a protective barrier for *E. coli* O157:H7, improving its survival in the human gastrointestinal tract. Another study was carried out on whether the EPS released by microorganisms have a protective effect on other members of similar and neighboring microbial communities.[50] The results clearly demonstrated the importance of exogenous EPS in desiccation tolerance, while mixed results were obtained in freezing trials.

It was hypothesized that the gastrointestinal tolerance of LP14 is related to EPS production and is affected by the EPS yield. The EPS yield of LP14 cultured with glucose was greater than that during fructose culture. The simulated gastric tolerance when EPS was removed by centrifugation was evaluated. Viable counts of LP14-Glc decreased significantly during treatment in gastric juice at pH 2.5 for 3 h after centrifugation. Viable counts of LP14-Fru, which produced little EPS, did not significantly decrease after centrifugation. There was no significant difference between washed LP14-Glc and untreated LP14-Fru. It was suggested that the gastrointestinal transit tolerance of LP14 is related to EPS.

In this study, when LP14 cultured with glucose or fructose was mixed with simulated gastric or intestinal juices based on MRS broth in which glucose was the

carbon source, at a final concentration of 1%, there was a difference in tolerance with carbon source. Perhaps human gastrointestinal transit tolerance differs according to how the microorganism content food is made. Moreover it appears that the protective capacity is different according to whether the bacterial cell is wrapped in EPS when it faces gastrointestinal juice, because the gastric transit tolerance of LP14 decreased with removal the EPS. But the physical and chemical relations between the bacterial cells and EPS of LP14 is not yet clear.

Most studies of EPS produced by lactic acid bacteria have focused on the influence of physiological growth conditions on EPS biosynthesis, the genetics of EPS biosynthesis, and elucidation of the composition and primary structures of these EPS. Information on the physiological role of EPS themselves is almost completely lacking. Some bacteria invest more than 70% of their energy in EPS production, presumably to obtain a selective advantage in the environment.[51] Most proposed functions of EPS in general are of a protective nature, such as protection against dehydration, macrophages, bacteriophages, antibiotics, and toxic compounds. However, the protective mechanism of EPS against these anti-microbial factors has not been clarified.

In conclusion, this study indicates that the tolerance of gastric and intestinal transit of LP14 is dependent on the carbon source, and that the gastrointestinal transit tolerance of LP14 is related to EPS production.

List of Tables and Figures

Table 1. Effects of Oxgall on the Growth of LP14 and *Leuconostoc* sp. GLT 36 in MRS Broth.

	Growth rate (%)		
% oxgall	0.2	0.4	
LP14-Glc	37.7 ± 0.1	24.0 ± 0.6	**
LP14-Fru	37.5 ± 0.6	21.8 ± 0.3	
GLT36-Glc	30.3 ± 0.3	12.4 ± 1.3	

Growth rates are expressed as percentage of turbidity in culture medium containing 0.2% or 0.4% oxgall after incubation for 18 h, as compared to those in broth containing no oxgall. Each value represents the means ± SD of triplicate determinations. Two-way ANOVA was performed for LP14-Glc and LP14-Fru in 0.2% and 0.4% oxgall. There were significant differences in oxgall concentrations and between carbon sources (P<0.01). A *post-hoc* unpaired t-test was performed for LP14-Glc and for LP14-Fru at 0.2% and 0.4% oxgall. * P<0.05; ** P<0.01.

Table 2. Effects of Oxgall on the Growth of LP14 and *Leuconostoc* sp. GLT 36 in MRS Broth after Treatment at pH 3.0 in MRS Broth Containing 0.04% Pepsin.

	Growth rate (%)		
% oxgall	0.2	0.4	
LP14-Glc	31.8 ± 4.0	12.1 ± 1.3	**
LP14-Fru	29.5 ± 1.3	6.6 ± 1.3	
GLT36-Glc	0.0 ± 0.0	0.0 ± 0.0	

Growth rates are expressed as percentage of turbidity in culture liquid containing 0.2% or 0.4% oxgall after incubation for 18 h, as compared to those in broth containing no oxgall. Each value represents the means ± SD of triplicate determinations. Two-way ANOVA was performed for LP14-Glc and LP14-Fru at 0.2% and 0.4% oxgall. There were significant differences in oxgall concentrations and between carbon sources (P<0.01). A *post-hoc* unpaired t-test was performed for LP14-Glc and for LP14-Fru at 0.2% and 0.4% oxgall. * P<0.05; ** P<0.01.

Table 3. Effects of Carbon Source on EPS Production by LP14.

	mg/l		mg/CFU
LP14-Glc	146.5 ± 8.1	**	$4.6 \times 10^{-11} \pm 2.5 \times 10^{-12}$
LP14-Fru	20.1 ± 17.0		$2.4 \times 10^{-11} \pm 2.1 \times 10^{-11}$

Each value represents the means ± SD of triplicate determinations. An unpaired t-test was performed for LP14-Glc and for LP14-Fru. * $P<0.05$; ** $P<0.01$.

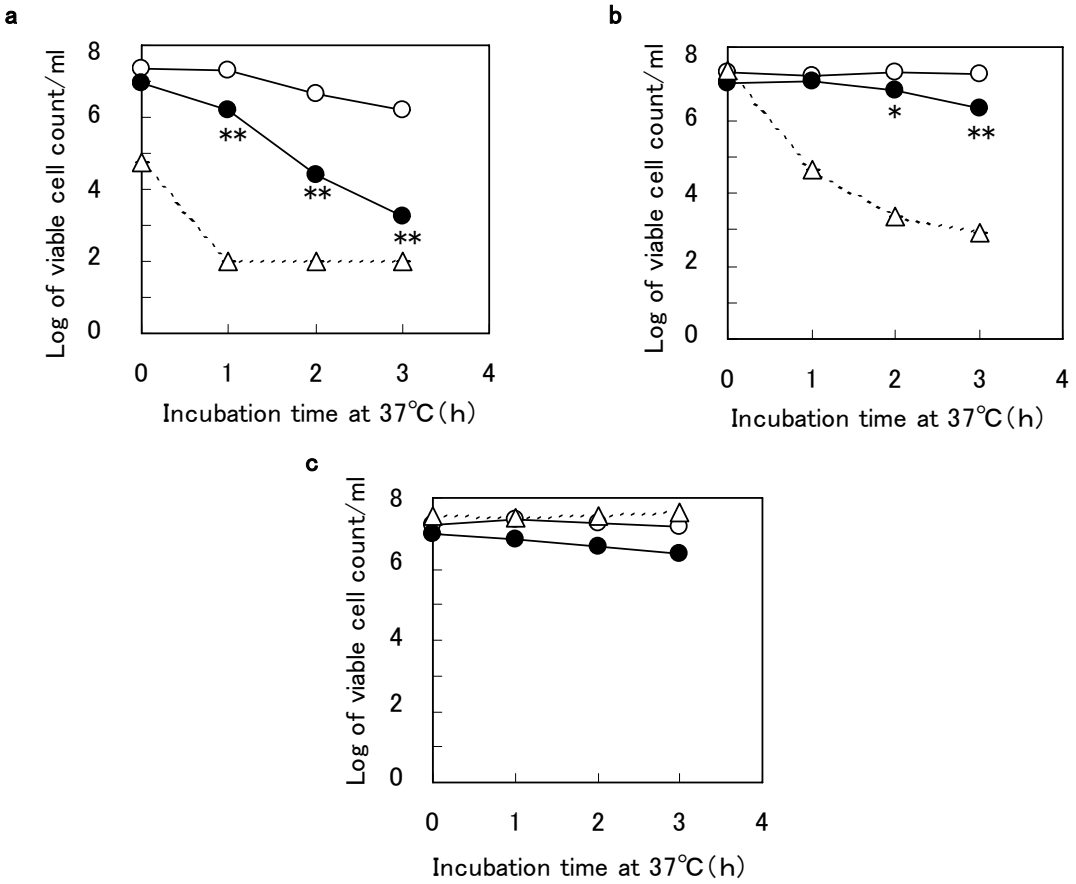

Fig. 1. Survival of LP14 and *Leuconostoc* sp. GLT 36 at pH 2.5, 3.0, and 3.5 in MRS Broth Containing 0.04% Pepsin.

Viable cell counts in simulated gastric juices are shown in a (pH 2.5), b (pH 3.0) and c (pH 3.5). Each value represents the means ± SD of triplicate determinations in terms of log10/ml. Two-way ANOVA was performed for LP14-Glc and LP14-Fru at pH 2.5, 3.0, and 3.5. There were significant differences in time course and between the carbon sources at pH 2.5 and 3.0 (P<0.01). There were no differences in time course at pH 3.5. One-way ANOVA and *post-hoc* Dunnett's test (*vs.* 0 h) were performed for LP14-Glc and LP14-Fru at pH 2.5 and 3.0 (* P<0.05; ** P<0.01). Symbols: ○, LP14-Glc; ●, LP14-Fru; Δ, GLT36-Glc.

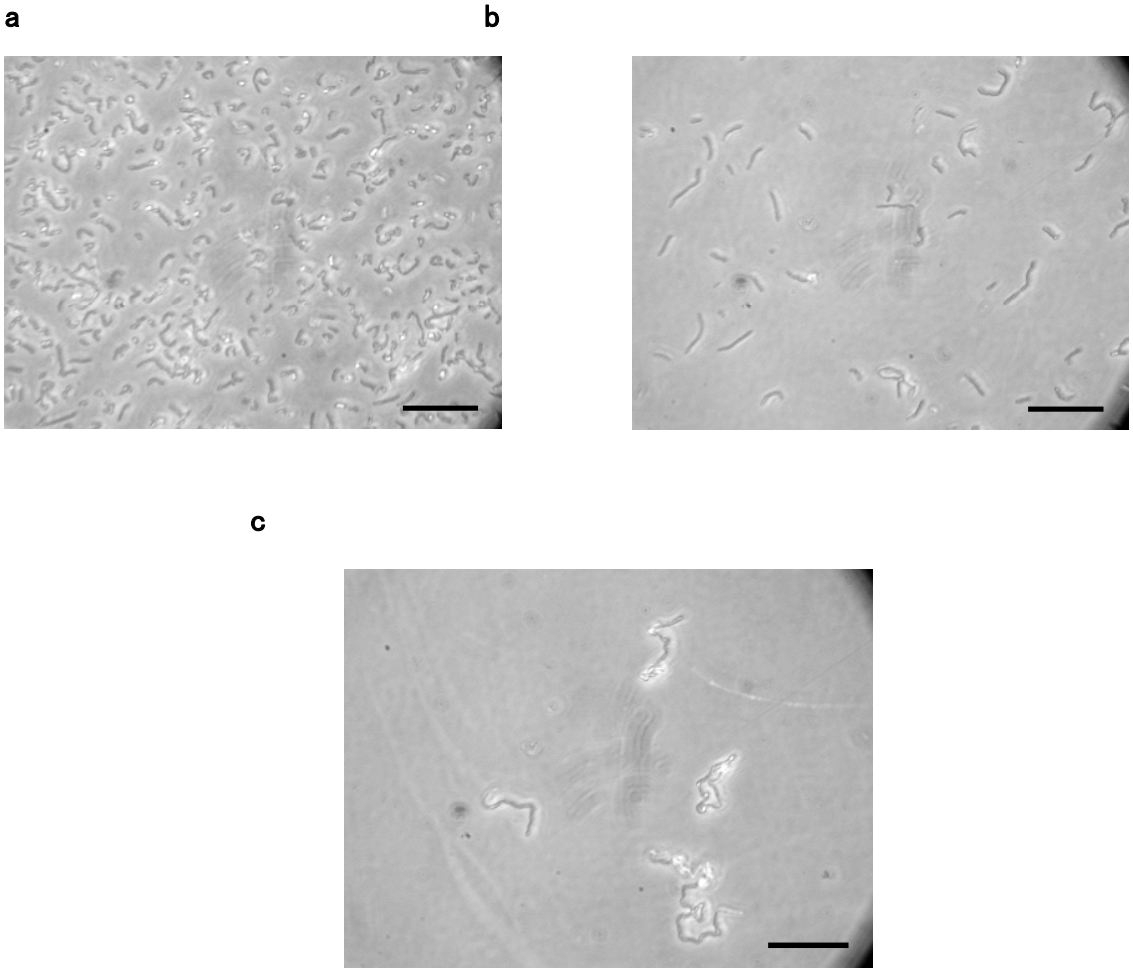

Fig. 2. Cell Morphology of LP14 Cultured in MRS with Glucose and Grown in Simulated Intestinal Juice Containing 0, 0.2, or 0.4% Oxgall (×1,000).

Chains of LP14 comprised about 1-10 cells in 0% oxgall (a). The ratio of 5-10-cell chains increased in 0.2% oxgall (b), while the chains of LP14 comprised about 10-20 cells in 0.4% oxgall (c). The chain length of LP14-Fru was similar to that of LP14-Glc in 0.2% oxgall, but in 0.4% oxgall, the chains of LP14-Fru were longer than those of LP14-Glc (data not shown). The bar in each image represents 10 μm.

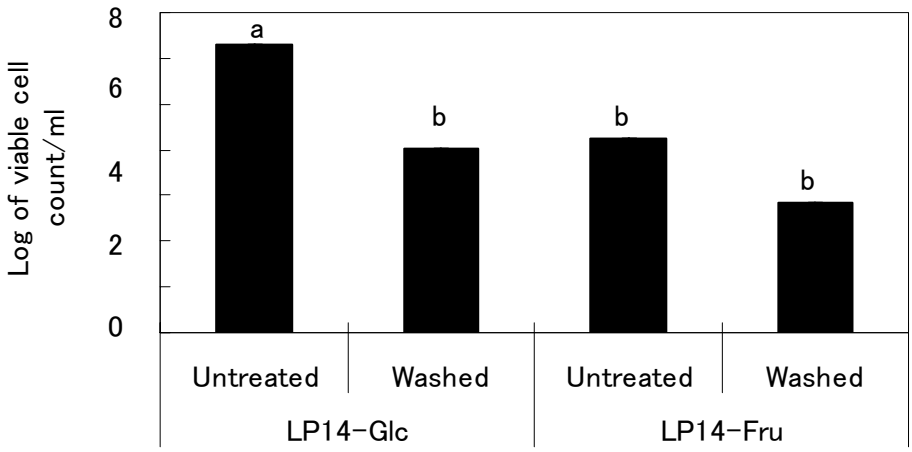

Fig. 3. Survival of LP14 Cultured with Glucose or Fructose at pH 2.5 in MRS Broth Containing 0.04% Pepsin for 3 h after Centrifugation.

Each value represents the means ± SD of triplicate determinations in terms of log10/ml. Two-way ANOVA was performed for LP14-Glc and LP14-Fru at 0 and 3 h. There were significant differences in carbon source and between treatment methods at 3 h (P<0.05). One-way ANOVA was performed at 3 h, and a significant difference was confirmed (P<0.01). A *post-hoc* Student-Newman-Keuls test was performed at 3 h. a, b: same letter indicates significant difference.

Chapter 3: Thermogenesis by LP14

Introduction

LP14 is the first reported lactic acid bacterium that decreases the body fat percentage in humans. In mice fed a high-fat diet, LP14 administration reduced the mean adipocyte size and the white adipose tissue weight.[52] It seems unlikely that previously reported mechanisms for the loss of body fat by other bacterial strains (*e.g.*, conjugated linoleic acids and inhibition of dietary fat absorption) are involved in the action of LP14.

Nagata *et al.* reported that LP14 induced gene expression of an endogenous pyrogen, *IL-1β*.[20] In regard to the mechanism that sends intra-intestinal information to the brain, two routes including hormonal information in blood and afferent neural signals, are known. Proinflammatory cytokines, such as IL-1β, have potent effects on the brain. When administrated systemically or directly into the brain, IL-1β induce fever.[53-55] Immune signals, such as IL-1β, induced prostaglandin (PG) E_2 in the brain endothelial cells.[56] PGE_2 is released into the brain parenchyma, activates PGE receptors located on neurons, and then triggers the neural circuitry for fever induction. When sympathetic nerve innervating the brown adipose tissue is activated, metabolic heat production increases.[57] When sympathetic nerve innervating the vascular smooth muscle is activated, the blood vessels shrink in order to minimise heat loss. When motor nerve is activated, the skeletal muscles create the shivering thermogenesis. Similar effects are observed when endogenous cytokines are released in response to administration of lipopolysaccharide (LPS) from gram-negative bacteria. Therefore, the body fat percentage was assumed to decrease because LP14 induces *IL-1β*, activation of the sympathetic nerve and thermogenesis.

The effect of LP14 on human body temperature in 4-week administration study and a single-time administration study were measured. In addition, the effect of intragastric injection of LP14 on sympathetic nerve activity innervating the brown adipose tissue (BAT-SNA) of rats was examined. In Chapter 2, it was reported that the gastrointestinal transit tolerance of LP14 depended on the carbon source.[58] Although all previous

studies[7, 20] used living LP14, it was considered that the sample didn't have sufficient tolerance for gastrointestinal transit under the conditions of the sample preparation in these studies. Therefore, the effect of heat-sterilized LP14 on allergy and body fat percentage in 4-week administration study was examined.

Experimental methods

1. 4-week administration study

Study design. This study had a randomized, placebo-controlled, double-blind design. The study was conducted during cedar pollen season in spring. After a pre-intake period of 4 weeks (from January 9 to February 5, 2009), the intervention group received one LP14 capsule (1.8×10^{10} CFU/0.3 g), and the placebo group received one placebo capsule (that did not contain LP14) every day for 4 weeks (from February 6 to March 5). Subjects were instructed to keep a diary of allergic symptoms, medication, basal body temperature and stool and abdominal conditions throughout the experimental period. Blood samples, body composition and blood pressure data were measured two times (pre-intake, January 9; cessation of intake, March 6).

Studies were conducted in accordance with the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Kagawa Nutrition University. Written informed consent was obtained from all subjects.

Subjects. A total of 33 female college students who were invariably allergic to Japanese cedar pollen (age range, 20-27 years) were randomized into an intervention group (n=17) and a placebo group (n=16). As all subjects in both groups were students from the same campus, the extent of pollen exposure was estimated to be the same between the two groups of subjects. No significant differences in baseline clinical characteristics were found between the placebo group and intervention group (Table1).

Preparation of active and placebo samples. LP14 starter was pre-cultured and cultured in MRS medium (1% concentration) at 30°C for 24 h. Then, the culture medium was pasteurized at 100°C for 20 min. After cooling, ethanol was added at a final concentration of 70% and the mixture was kept at 4°C for 48 h. The precipitate was collected and suspended in distilled water. It was reprecipitated with a 70% volume of ethanol and centrifuged at 13,000×*g* for 20 min at 4°C. The precipitate was resuspended, mixed with branched dextrin solution, and lyophilized. Cellulose capsules were filled with 0.3 g of lyophilized LP14. This active sample consisted of 22.5% (w/w)

bacterial origin material, 4.8% (w/w) medium origin material, and 72.7% (w/w) branched dextrin (1.8×10^{10} CFU/0.3 g). Placebo capsules were prepared by the same method without inoculation. The placebo sample consisted of 3.8% (w/w) medium origin material, and 96.2% (w/w) branched dextrin.

Pollen count and atmospheric temperature. Pollen counts and temperature in the atmosphere were obtained from the data acquisition system of the Ministry of the Environment of Japan (http://kafun.taiki.go.jp/). Levels were measured in the experimental area at Saitama Medical University (Saitama, Japan), which was 10 km from the experiment site. Values were presented as particle number per cubic meter per day.

Personal recordings. Each subject self-recorded allergic symptoms, medication use and conditions of stool and abdomen throughout the experimental period. These were scored according to Chapter 1 (Chapter 1-Table 2, 3, 4).

Each subject measured her body temperature when getting up in the morning with an electronic oral thermometer (C502, Terumo, Tokyo, Japan).

Measurements. Subjects were not allowed to eat or drink (expect water) after 21:00 in the evening prior to blood collection. The levels of total IgE, anti-Japanese cedar pollen IgE, IFN-γ, IL-4 and eosinophil counts were measured. All blood tests were performed by SRL. (Tokyo, Japan).

The body weight, body fat weight, lean body weight and BMI were measured by using a body composition analyzer (InBody 3.0, BioSpace, Seoul, Korea). Blood pressure and pulse rate were measured with an upper arm automatic blood pressure monitor (HEM-7115, Omron Healthcare, Kyoto, Japan).

Questionnaire after study. After the administration period, each subject completed a questionnaire concerning alteration in body temperature and appetite. Self-reported alterations in body temperature unrelated to the menstrual cycle or illness were assessed

by asking each subject to select one of the following choices: “I continually felt feverish”; “I felt warmer than usual”; “I did not feel a change in body temperature”; “I felt a little colder than usual”; and “I felt considerably colder than usual”. The subjects were also asked to describe their appetite as “Increased”, “Increased a little”, “Not changed”, “Decreased a little”, or “Decreased”.

Statistical analysis. The data was expressed as means ± SD. In the clinical tests, the Shapiro-Wilk test was applied to the null hypothesis. Total IgE, anti-Japanese cedar pollen IgE, IFN-γ, IL-4 and eosinophil counts were distributed in non-normality. Thus, a nonparametric analysis was applied to these data and categorical data (subjective symptoms of pollinosis and stool condition). The non-normal data in the intervention group and the placebo one were compared with the Mann-Whitney U-test. In the case of normal data, the unpaired t-test was used. The Wilcoxon t-test was used for paired data analysis to compare the intake cessation with the pre-intake for non-normal data. The paired t-test was used for the normal data. Logistic regression was used to calculate odds ratios for each allergy symptom. Each symptom score of each subject was categorized into two groups based on the severity reported during the fourth week of intake. It was based on an average score 1.0. For the questionnaire survey, statistically significant differences were assessed by using the chi-square test with Yates correction for continuity for an m×n contingency table. All statistical tests were two-sided. Statistical significance was set at $P<0.05$. The statistical analysis was performed with SPSS 6.1 software (SPSS, Chicago, IL, USA) and ystat2006 (Igakutosho-shuppan, Tokyo, Japan).

2. Single-time administration study

Subjects and study design. The subject group consisted of 2 men and 19 women (mean age, 21.5±3.9 years; range, 18-31 years) in good health. The subjects were nonsmokers and did not take any medications at the time of the experiment.

The study had a randomized, double-blind, cross-over design. The study was held between the end of July and the beginning of October, 2010. At least 1 day elapsed

between the individual experimental runs. Subjects finished the examination of the effects of LP14 and placebo within 7 days. The menstruation period was excluded as an examination day.

The subjects were directed to eat the same supper at the same time and to sleep at the same time the day before the study. The subjects were also directed to get up at the same time in the morning and put on the same clothes on the day of the examination. Experiments were started at 8:30, after the subjects had refrained from consuming food and drink (other than water) for at least 10 h and refrained from vigorous exercise for at least 24 h. After thermo sensors were set on the chest and the dorsum of right foot, the subjects rested for 30 min in the sitting position. Baseline data were collected, and then the subjects ingested one capsule with 100 ml water (25°C). The examination was stopped 3 h later. The experiment room was air-conditioned (25±0.7°C, 66.2±6.4% relative humidity).

The studies were conducted in accordance with the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Kagawa Nutrition University. Written informed consent was obtained from all subjects.

Preparation of active and placebo samples. LP14 starter was pre-cultured and cultured in MRS medium (1% concentration) at 30°C for 24 h. Then, the culture medium was pasteurized at 100°C for 20 min. After cooling, ethanol was added at a final concentration of 70%, and the mixture was kept at 4°C for 48 h. The precipitate was collected and suspended in distilled water. It was reprecipitated with 70% volume of ethanol and centrifuged at 13,000×*g* for 20 min at 4°C. The precipitate was resuspended and lyophilized. Cellulose capsules were filled with 0.2 g (1.6×10^{11} CFU) of lyophilized LP14. This active sample consisted of 91.8% (w/w) bacterial-origin material and 8.2% (w/w) medium-origin material. Placebo capsules were prepared by the same method without inoculation. To adjust the weight of placebo samples, branched dextrin was added. The placebo sample consisted of 8.2% (w/w) medium-origin material and 91.8% (w/w) branched dextrin.

Clinical characteristics of subjects. The BMI, body fat percentage and muscular percentage were measured by using a digital weight scale incorporating a bioelectrical impedance analyzer (HBF-354, Omron Healthcare, Kyoto, Japan). The circumference of the chest, waist and hips of the subjects were also measured.

The polymorphisms of Trp64Arg in the beta3-adrerergic receptor (*ADRB3*) gene, A3826G in the uncoupling protein 1 (*UCP1*) gene and Arg16Gly in the beta2-adrerergic receptor (*ADRB2*) gene were examined. Buccal swabs were performed to obtain epithelial cells for the extraction of genomic DNA. Samples were analyzed by reverse polymerase chain reaction sequence-specific oligonucleotide hybridization on microbeads by using Luminex technology.[59] The measurement of genetic polymorphisms was performed by DHC (Tokyo, Japan).

Each subject completed a questionnaire about the intake frequency of lactic acid bacterium and body constitution (allergies, normal temperature, excessive sensitivity to cold and overall metabolism). Subjects selected the most suitable statement about their state.

Measurements. The skin temperature every 5 sec at two points (chest and dorsum of right foot) was measured with thermistors ITP010-22 and a data logger N542R (Nikkoso-Therm, Tokyo, Japan) with a precision of 0.01°C during the experiment time. Subjects measured blood pressure and pulse rate every 20 min by themselves with an upper arm automatic blood pressure monitor (HEM-7115, Omron Healthcare, Kyoto, Japan).

The Uchida-Kraepelin (U-K)[60] test was conducted every hour. The U-K test is a serial addition test that requires subjects to perform calculations as fast and accurately as possible. The subjects were given a pre-printed paper containing 15 lines of random, single-digit, horizontally aligned numbers. For each minute of the test, the subject was instructed to begin a new line regardless of their position on the current line. Each line contained an excess of calculations such that the subjects were not able to finish any line for a particular minute before being prompted to move on to the start of the next minute by the examiner's prompting. Although this test is usually performed in repeated

cycles of 15 min of work time and 5 min of rest time, the subjects in this study received only one 5-min work period and no rest periods.

After the U-K test, the Stanford Sleepiness Scale (SSS)[61] was administered every hour. Subjects selected one of seven statements to best describe their current state of alertness. The statements ranged from "1, Feeling active, vital, alert, or wide awake" to "7, No longer fighting sleep, sleep onset soon; having dream-like thoughts". The dependent variable was the self-rated sleepiness score on a scale of 1 to 7.

After SSS, the Stress Arousal Checklist (SACL)[62] was administered every hour. The SACL is a mood adjective checklist consisting of 17 items on stress and 13 items on arousal. Self-reported stressful feelings were examined by asking questions such as, "Describe your feelings at this moment: comfortable, calm, distressed, relaxed, contented, tense, easy, up-tight, cheerful, apprehensive, peaceful, dejected, nervous, bothered, pleasant, worried and jittery". The response scales were "Definitely feel", "Feel slightly", "Do not understand or cannot decide" and "Definitely do not feel". Self-reported arousal feelings were examined by asking questions such as, "Describe your feelings at this moment: restful, active, energetic, drowsy, vigorous, tired, passive, alert, lively, sluggish, sleepy, stimulated and activated". Responses to each adjective were coded on a scale of 0 to 3, the direction of the scale depending on whether the adjective was, positively or negatively associated with the mood scale. Total stress and arousal scores were calculated by the sum of scores for each contributing adjective. As 17 items contributed to the stress score, the maximum score was 51, and the minimum score was 0. The maximum arousal score was 39, and the minimum score was 0.

Questionnaire after study. Subjects were also asked if there was a difference in hunger and the alteration in body temperature between the first day and the second day. Subject blinding was confirmed by asking the subjects to indicate which sample they believed they had received (test sample, placebo, or do not know) after measurements on the second day.

Statistical analysis. According to a cross-over design, the order effects of all

parameters were examined by using ANOVA. A significant difference was found only in the total number of answers on the U-K test (P=0.037). The subjects seemed to be insufficiently accustomed to the U-K test, despite 3 min of practice immediately before the examination; therefore the total number of answers on the second day alone was analyzed (unpaired t-test). Other parameters, including the number of mistakes on the U-K test, were analyzed by combining the data obtained from the first day test and the second day test.

The deviation from the baseline was calculated for all data expressed as means ± SD. Nonparametric analysis was applied to categorical data (questionnaire survey and genetic polymorphisms). The Wilcoxon t-test was used for paired data analysis to compare LP14 and placebo intake for non-normal data. The paired t-test was used for the normal data. Correlation was evaluated with Spearman's correlation coefficient in case of non-normal data and Pearson's correlation coefficient in the case of normal data. Data from the questionnaire regarding hunger and altered body temperature were assessed by using the chi-square test with Yates correction for continuity for an m×n contingency table. All statistical tests were two-sided. Statistical significance was set at P<0.05. The statistical analysis performed with SPSS 6.1 software (SPSS, Chicago, IL, USA) and ystat2006 (Igakutosho-shuppan, Tokyo, Japan).

3. Measurement of sympathetic nerve activity innervating the brown adipose tissue (BAT-SNA) of rats

Animals. BAT-SNA was measured by ANBAS Corporation (Osaka, Japan). Nine-week-old male SD rats (n=6) that weighed approximately 300 g, were housed individually in a room kept at 24°C and illuminated for 12 h (8:00-20:00) every day. The rats were adapted to the environment for at least 1 week prior to the experiment and were divided into two groups. Food (MF, Oriental Yeast, Tokyo, Japan) and water were freely available. The animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of the ANBAS Corporation in accordance with the guidelines for animal experiments issued by the Science Council of Japan on June 1, 2006.

Protocol. The rats fasted for 3 h and were anesthetized with urethane (1 g/kg, intraperitoneally) prior to surgery.[63] LP14 and water were administered through a gastric cannula consisting of a polyethylene tube inserted into the stomach. After laparotomy, the distal ends of the sympathetic nerves innervating the interscapular BAT were exposed, ligated, and subsequently connected with a pair of silver wire electrodes. The body temperature was maintained at 35.0±0.5°C by using a heat pad and a thermistor inserted in the rectum. After stabilization period of 30 to 60 min, the electrical signals from the electrodes were collected, amplified, filtered, and monitored on an oscilloscope. Nerve activity was analyzed by conversion of the raw data into standard pulses by using a window discriminator. Baseline measurements of BAT-SNA were collected for 5 min before intragastric injection of LP14 (9.7×10^8 CFU/0.5 ml water) or water (0.5 ml). Lyophilized LP14 that was prepared in the same way as for the single-time administration study in humans was used after being dissolved in water. BAT-SNA was recorded for 90 min after the injection.

Statistical analysis. Due to the interindividual variability before administration, the percentage of deviation from the baseline (0 min) values was calculated for BAT-SNA. The data were expressed as means ± SEM. The Mann-Whitney U-test was used to compare basal levels in each group. ANOVA with repeated measures was performed to compare group responses of BAT-SNA. All statistical tests were two-sided. Statistical significance was set at $P<0.05$. The statistical analysis performed with SPSS 6.1 software (SPSS, Chicago, IL, USA).

Results

1. 4-week administration study

Pollen count and atmospheric temperature

Figure 1 shows the weekly averages of the Japanese cedar pollen count and atmospheric temperature. The Ministry of the Environment announced that the Japanese cedar pollen started to disperse on February 6 in Saitama Prefecture, which was the experiment site, while the amount of dispersion before February 1 was unknown.

Subjective allergy symptoms

The mean ocular SMS and mean nasal SMS for the LP14 group were lower than those for the placebo group during the intake period without any significant difference between the two groups (Table 2). No significant differences were observed in the medication score, but the dosage of medicine in the LP14 group was less than that of the placebo group and hardly increased during the intake period. The administration of LP14 was associated with an 86.2% reduction in the risk of aggravation of watery eyes (odds ratio, 0.138; 95% CI, 0.014 to 1.345, P=0.088) and a 73.1% reduction in the risk that obstacles in daily life increased (odds ratio, 0.269; 95% CI, 0.059 to 1.221, P=0.089).

Blood examination

To exclude the influence of medicine on allergy symptoms, The data from 11 subjects in each group who had not taken medicine within 1 week prior to blood examination were analyzed (Table 3). No significant differences between the placebo and LP14 groups were found in the pre-intake data. The distribution of data after excluding the subjects who had taken medicine was the same as that before exclusion. There were also no significant differences between the two groups in the data at intake cessation. Total IgE decreased significantly in both groups (LP14, P=0.046; placebo, P=0.041). However, only the LP14 group exhibited a significant decrease in anti-Japanese cedar pollen IgE after intake cessation (P=0.039). Eosinophil counts tended to increase in the placebo group (P=0.069), but were suppressed in the LP14

group. The IL-4 level tended to decrease in the placebo group (P=0.076). The IFN-γ levels of 20 subjects were below the detection limit of 0.1 IU/ml.

Blood pressure and pulse rate

There were no significant differences in blood pressure (Table 4). Pulse rate significantly decreased in both groups (LP14, P=0.024; placebo, P=0.031).

Body composition

There was no statistically significant change in the body composition of subjects (data not shown). Five subjects in each group had greater than 30% body fat. The body fat and muscle amounts in the placebo group decreased (Fig. 2). However, the subjects in the LP14 group had a greater reduction in body fat than the placebo group, and they also had an increased amount of muscle. As a result, the body fat percentage increased in the placebo group and decreased in the LP14 group. There were no significant differences in these data.

Basal body temperature

The subjects who had a steady menstrual cycle of 28±5 days were selected. There were five and eight subjects whose menstrual cycles were 27-33 days in the placebo and LP14 groups, respectively. Because some subjects lacked experience measuring their basal body temperature, data from the first 4 days after the start of the study were excluded. There was no significant difference in body temperature between the LP14 group and the placebo group (Fig. 3). However, the basal body temperature increased significantly only in the LP14 group over the intake period (LP14, P=0.021; placebo, P=0.427). The average increase in body temperature was 0.019±0.114°C in the placebo group, and 0.036±0.070°C in the LP14 group.

Condition of stool and abdomen

Table 5 shows the stool characteristics of all subjects. Frequency of defecation and fecal amount did not vary between the groups during the experimental period. The ratio

of appropriate fecal shape in the LP14 group after 4 weeks of intake tended to increase (P=0.056), and the frequency of solid stool and liquid stool decreased (data not shown).

Questionnaire after study

There were no significant differences in the self-reported alteration of body temperature and appetite from the questionnaire (Table 6). None of the subjects indicated that they continued to feel feverish. One subject in the placebo group and three subjects in the LP14 group responded that they felt warmer than usual.

2. Single-time administration study

Body temperature

Subjects whose body temperature differed by more than 1.0°C between LP14 and placebo on intake were excluded from the temperature analysis. Chest data were removed for two subjects and foot data were removed for other two subjects. The average increase in chest temperature was 0.10±0.32°C in the placebo group and 0.24±0.32°C in the LP14 group (Fig. 4a). There was a significant difference in average increase in chest temperature between the LP14 group and the placebo group (P=0.023). Chest temperature tended to increase from 45 to 90 min (P<0.10) and increased significantly from 135 to 165 min (135 min and 150 min, P=0.022; 165 min, P=0.0004) in response to LP14 intake as compared with the placebo intake. The maximum value of the difference between the chest temperature after LP14 intake and placebo intake was 0.24±0.24°C at 165 min. Foot temperature decreased in both groups without a significant difference (Fig. 4 b).

There was no significant correlation in the average amount of the increased chest temperature and other parameters (baseline body temperature, genetic polymorphisms, anthropometric measurements and body constitution and intake frequency of lactic acid bacterium). The parameters whose coefficients of determination with the average amount of the increased chest temperature were 0.1 or more were *ADRB3* polymorphism (r=0.498), body fat percentage (r=0.388), waist circumference (r=0.450) and intake frequency of lactic acid bacterium (r=-0.353) (Table 7). The chest

temperature of the subjects with *ADRB3* mutation was elevated by LP14 intake. The ratios of genetic polymorphism of *ADRB3* and *ADRB2* in this study were almost the same as the Japanese ratios reported by Yoshida *et al.*,[64-67] although there was some difference in the case of *UCP1*. In the present study, the frequency of the Trp64Arg allele in *ADRB3*, the A3826G allele in *UCP1* and the Arg16Gly allele in *ADRB2* was 0.20, 0.53 and 0.40, respectively. The frequency of these alleles was 0.23, 0.46 and 0.40 in the report by Yoshida *et al.*

There was no significant correlation in the average amount of the increased foot temperature and other parameters. The parameters whose coefficients of determination with the average amount of the increased foot temperature are 0.1 or more were chest circumference (r=-0.486), hip circumference (r=-0.339), normal temperature (r=0.382) and overall metabolism (r=0.375).

Blood pressure and pulse rate

The systolic blood pressure peaked 2 h after placebo intake and 2.7 h after LP14 intake (Fig. 5a). There was a significant difference in systolic blood pressure between the LP14 group and the placebo group at 2.7 h (P=0.017). The pulse rate decreased in both groups (Fig. 5c). However, the decrease tended to be smaller after LP14 intake as compared to placebo intake at 2.7 h (P=0.068).

U-K test

The total number of answers increased 1 h after intake of LP14 but decreased 1 h after intake of placebo, with a significant difference (P=0.028, Fig. 6a). The number of mistakes decreased gradually after LP14 intake but increased after placebo intake (Fig. 6b). There was a significant difference in the number of mistakes 2 h after intake (P=0.032).

SSS and SACL

There were no significant differences in sleepiness or stress scores between the LP14 and placebo groups (Fig. 7a, b). The arousal score increased 1 h after LP14 intake

and remained high until 3 h after intake (Fig. 7c). There tended to be a significant difference in arousal scores between LP14 and placebo 2 h after intake (P=0.080). The response to each item of arousal was analyzed. The scores after LP14 intake were significantly higher than the scores after placebo intake for two items ("not tired", P=0.022, 2 h after intake; "not alert", P=0.007, 3 h after intake) and tended to be higher for two items ("vigorous", P=0.067, 2 h after intake; "not sleepy", P=0.098, 3 h after intake).

Questionnaire after study

There were no differences between LP14 and placebo intake with respect to hunger and altered body temperature (Table 8). One subject correctly identified the intake samples. Two subjects incorrectly identified intake samples, and 18 subjects could not distinguish one sample from another. Therefore, blinding was confirmed to be effective.

3. Effect of LP14 on BAT-SNA in rats

The absolute values of BAT-SNA at 0 min, as determined by the mean values of the 5-min period before the administration of water and LP14, were 238±30 spikes/5 sec and 232±26 spikes/5 sec, respectively. Statistical significance was not found in the 0 min values between the two groups. Figure 8 shows the change in the 0 min BAT-SNA values after administration of LP14 and water. Administration of water did not affect the BAT-SNA until 40 min; water slightly enhanced the BAT-SNA after 40 min, with a peak value of 124.2% at 75 min. LP14 gradually enhanced the BAT-SNA after administration, with a peak value of 152.9% at 75 min. The statistical significance of the difference between the values of BAT-SNA 5-90 min after the administration of LP14 or water was analyzed by ANOVA, and the difference between the two groups was statistically significant (P=0.0005).

Discussion

In the 4-week administration study in humans, Japanese cedar pollen-specific IgE levels decreased significantly, and eosinophil counts tended to be suppressed in subjects taking LP14 when cedar pollen dispersal was heavy. LP14 tended to decrease the risk of aggravated watery eyes and the risk of increased obstacles in daily life. The Chapter 1 and the previous reports[7, 20)] has been reported about improvement in blood components and subjective symptoms of allergy by living LP14. The present study suggests that killed LP14 also has an antiallergic effect.

The reason why the IL-4 level in the placebo group tended to decrease is uncertain. The IFN-γ level of most subjects was below the detection limit, because the subjects did not have any inflammatory disorders. Nagata *et al.* reported that LP14 induced gene expression of *IL-12p35* in Peyer's patch cells and mesenteric lymph node cells of swine.[20)] IL-12 is an inducer of IFN-γ. However, IFN-γ in the LP14 group did not increase during the 4-week administration period in humans.

In a previous study,[7)] the pollen dispersal increased after the subjects had ingested the same amount of LP14 for 3 weeks, and a significant reduction in allergic symptoms was observed. In the present study, the pollen dispersal increased after the subjects had ingested LP14 for 1 week, and a significant reduction in allergic symptoms was not observed. To mediate an antiallergic effect, LP14 might have to be administered beforehand in a preventive manner.

Nagata *et al.* reported that the cedar pollen-specific IgE level significantly decreased after intake of 2.0×10^{10} CFU of LP14.[7)] But insignificantly decreased after intake of 8.7×10^{8} CFU in the Chapter 1.[20)] It was considered that one of the reasons for this difference might be the dose of LP14. Because the cedar pollen-specific IgE decreased significantly in the present study in which subjects received 1.8×10^{10} CFU of LP14, it is thought that an intake of more than 10^{10} CFU intake is necessary to exhibit a clear improvement in Japanese cedar pollinosis.

LP14 produces large amounts of EPS, when it cultured with glucose.[58)] In present study, LP14 was cultured in MRS medium with glucose. The culture medium was precipitated with ethanol and collected. Therefore, the LP14 sample contained EPS. It

was reported that most lactic acid bacteria and bifidobacteria produced one or two EPS of different molar mass *etc.*[68] There were few strains which produced three or more EPS. EPS produced by LP14 consisted of two neutral EPS and two acidic EPS.[69] EPS produced by lactic acid bacteria were reported to have immunomodulatory and protective effect. Only acidic EPS had strong activity in these reports. Stimulation of mouse splenocytes by acidic EPS of *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 increased IFN-γ production, and orally administrated acidic EPS augmented natural killer cell activity.[70] Acidic EPS of *Lactococcus lactis* ssp. *cremoris* KVS20 induced IFN-γ and IL-1α production in macrophages.[71] Both acidic EPS and neutral EPS produced by LP14 exerted immunostimulatory activity on both Peyer's patch cells and mesenteric lymph node cells of swine.[69] EPS of LP14 have specificity. LP14 including EPS was examined in humans for the first time in the present study. It is uncertain whether the antiallergic effect is increased by EPS.

In body composition of subjects with body fat percentage greater than 30% in the 4-week administration study, the amount of body fat and muscle in the placebo group decreased. However, the LP14 group experienced a larger reduction in body fat along with an increased amount of muscle. Therefore, LP14 reduced the body fat percentage. There was no difference in the self-reported appetite of the LP14 group and placebo group, although a diet survey was not performed. Frequency of defecation and fecal amount did not differ between the LP14 and placebo groups. Therefore, it is considered that the decrease in body fat percentage is not due to a change in appetite or fecal characteristics. There was no significant difference in body fat percentage. One possible reason for the lack of significant difference might be the limited number of subjects. Further investigation is necessary with a large number of subjects with a high percentage of body fat.

The body temperature increased in the 4-week administration study for subjects allergic to Japanese cedar pollen and in the single-time administration study for healthy subjects. There was no correlation between the average increase in chest temperature and allergy presence in the single-time administration study. These results suggest that allergy presence is unrelated to the body temperature elevation by LP14.

The basal body temperature in the LP14 group, which received 1.8×10^{10} CFU, was increased by 0.017°C as compared to the placebo group in the 4-week administration study. The chest temperature after intake of 1.6×10^{11} CFU of LP14 was elevated by 0.14°C as compared to after placebo intake in the single-time administration study. The pre-study was conducted by the following method: single-time intake of 1.2×10^{12} CFU of LP14 or placebo, 6-h measurement time, six subjects, double-blind, cross-over design (unpublished data). In the pre-study, the chest temperature peaked 4 h after intake, and the increase in chest temperature was at the same level as that after intake of 1.6×10^{11} CFU. Although the increase in body temperature in the single-time study was larger than that in the 4-week study, the difference might depend on not only a less intake of LP14 but also the timing of body temperature measurement. The basal temperature was measured before the subjects ingested LP14 every day, in a word, on the next day when the subjects ingested LP14. It was considered that the body temperature increase within a day by LP14 was repeated in the 4-week administration study. In the single-time administration study, the measurement time was adjusted to 3 h in consideration of the subjects' load in the pre-study. The body temperature elevation effect of the lactic acid bacterium has not been reported in humans, although there is a report of increased human body temperature after the administration of LPS of *E. coli*.[72, 73] Nagai *et al.*[74] reported body temperature elevation after lactic acid bacterium was fed to rats. The body temperature after the administration of LPS of *E. coli* was elevated 1.8-3.4°C from the baseline with chills, headache and nausea in human.[72, 73] The maximum value of the chest temperature after LP14 and placebo intake was 0.46°C and 0.22°C from the baseline without subjective symptoms. It is thought that the body temperature elevation caused by LP14 is at a safe level.

The β adrenergic system plays a key role in regulating energy balance through the stimulation of both thermogenesis and lipid mobilization in brown and white adipose tissues in humans and various animal models.[75, 76] It is suggested that a missense Trp64Arg mutation in the *ADRB3* gene is involved in obesity and insulin resistance.[64, 77] Kurokawa *et al.*[78] performed a meta-analysis of the relationship between an *ADRB3* variant and BMI, and they reported that the Trp64Arg variant of *ADRB3* was associated

with BMI in East Asians but not Europeans. In the present study, although there was no significant correlation among *ADRB3* polymorphism and other parameters, the parameters whose coefficients of determination with the average amount of increased chest temperature were high were *ADRB3* polymorphism, body fat percentage and waist circumference. The chest temperature of the subjects who had the Trp64Arg mutation in the *ADRB3* gene was elevated by LP14 intake. The decrease in body fat percentage in subjects with high body fat percentage in the 4-week administration study might have been observed because the body temperature of the subjects with *ADRB3* mutation and high body fat percentage was elevated to a greater extent. LP14 might have compensated for the β adrenergic system hypofunctioning caused by the *ADRB3* mutation. The intake frequency of lactic acid bacterium also correlated negatively with the average increase in chest temperature. There is a possibility that the subjects who had not so much been stimulated by the microorganism included in diet were more easily influenced by LP14.

The foot temperature fell by 4°C in both groups. The temperature of the experiment room was air-conditioned at 25±0.7°C. Because a peripheral temperature was easy to be subject to the influence of a room temperature, the foot temperature fell sharply in this study.

Although there was no significant correlation, the parameters whose coefficients of determination with the average amount of increased foot temperature were high were chest circumference (negative correlation), hip circumference (negative correlation), normal temperature and overall metabolism. There was a possibility that the foot temperature increased by LP14 in subjects whose body composition and body constitution were different from those of subjects with increased chest temperature by LP14.

The blood pressure and the pulse rate are reversed in circadian rhythm. In the single-time administration study, the systolic blood pressure significantly increased with LP14 intake, while the decrease of the pulse rate tended to be suppressed with LP14 intake. In the 4-week administration study, the blood pressure and pulse rate remained unchanged. It is considered that the blood pressure and pulse rate elevation caused by a

single dose of LP14 was resolved within a day like body temperature elevation by LP14. When sympathetic nerve innervating the vascular smooth muscle is activated, the blood vessels shrink in order to minimise heat loss, and blood pressure increases.[57] LP14 increased the blood pressure, but did not suppress heat loss.

In the single-time administration study, the placebo subjects significantly became fatigued compared with the LP14 subjects 2 h after intake. Subjects had a greater number of total answers and a decreased number of mistakes after LP14 intake as compared to subjects after placebo intake. The arousal score and the total number of answers increased 1 h after LP14 intake, while the total number of answers decreased after placebo intake. A slightly decreased arousal score and an increased number of mistakes were observed in the placebo intake group 2 h after intake, but the number of mistakes after LP14 intake kept decreasing. The arousal score, the total number of answers and the number of mistakes appeared to be synchronized. It was considered that the task performance improved because arousal had been elevated by LP14 intake.

The sympathetic autonomic system is involved in blood pressure, pulse rate and body temperature through the release of catecholamines from the adrenal medulla and sympathetic nerve endings,[79, 80] and it is involved in task performance through concentration and arousal.[81] The catecholamines in another pre-study of single-time intake of LP14 were measured. But they varied widely, and blood collection influenced the body temperature and feelings with an increased stress level (unpublished data). Therefore, catecholamines in humans were not analyzed in the present study. The effect of intragastric injection of LP14 on BAT-SNA in rats was examined. As the peripheral mechanism responsible for thermogenesis, the contribution of BAT has been suggested.[82] BAT is a tissue specified for metabolic heat production and has a significant role in cold- and diet-induced thermogenesis.[83, 84] BAT thermogenesis is principally dependent on the activation of UCP1, which uncouples oxidative phosphorylation in mitochondria to dissipate the electrochemical gradient as heat. The activity of UCP1 is controlled by the sympathetic nerves to BAT, mainly through the β-adrenergic mechanism. The activator of sympathetic nerves, ADRB3, also induces lipolysis in BAT,[85] and the produced fatty acids are used as a substrate for UCP1

thermogenesis.[86] The β3 agonist promotes lipolysis in white adipocytes, activates UCP1, changes the free fatty acid into heat, and finally decreases body fat.[87] LP14 enhanced the BAT-SNA. *Lactobacillus paracasei* ST11 injection also elevated sympathetic nerve activity, blood pressure and temperature in the abdominal cavity and BAT in rats, and reduced abdominal fat.[74] It is suggested that *L. paracasei* ST11 might affect the hypothalamic suprachiasmatic nucleus (SCN) of the brain and cause sympathetic elevation.

The involvement of BAT thermogenesis in fever was suggested first by Blatteis,[88] who demonstrated an endotoxin-induced and β-blocker-sensitive increase in BAT temperature in guinea pigs. Subsequently, it was reported that IL-1β/LPS injection increased guanosine diphosphate (GDP) binding to BAT mitochondria, a marker of UCP1 activity.[89-91] It was also shown that blood flow in BAT increased after IL-1β/LPS injection in rats.[89] Nagata *et al.* reported that LP14 induced gene expression of an endogenous pyrogen, *IL-1β*.[20] The effect of LP14 on decreased body fat percentage might relate to the immunostimulatory capacity of LP14. To prove our hypothesis, further research will be needed.

In conclusion, it is suggested that killed LP14 has an antiallergic effect, and an intake of more than 10^{10} CFU is needed to improve Japanese cedar pollinosis. LP14 induces thermogenesis in both short-term and long-term studies. The elevation in parameters related to the sympathetic nerve, including blood pressure, pulse rate, arousal and task performance, was observed. LP14 enhanced the BAT-SNA. Nagata *et al.* reported that LP14 induced gene expression of an endogenous pyrogen, *IL-1β*.[20] It was considered that LP14 decreased the body fat percentage by a mechanism in which LP14 induces *IL-1β*, activates the sympathetic nerve, and results in thermogenesis.

List of Tables and Figures

Table 1. Clinical Characteristics of the Subjects in 4-week Administration Study.

		Placebo	LP14
Number of subjects		16	17
Age (years)		22.8 ± 6.7	21.5 ± 1.8
Total IgE (IU/ml)		193.4 ± 263.1	298.1 ± 668.6
IgE specific for Japanese cedar pollen (UA/ml)		19.3 ± 28.1	23.0 ± 27.8
Eosinophil counts (/μl)		181.4 ± 90.3	155.3 ± 88.8
BMI (kg/m^2)		22.2 ± 3.4	21.0 ± 2.5
Body fat parcentage (%)		28.6 ± 6.7	28.2 ± 4.9
Severity of nasal symptoms at baseline (−)[a]		1.00 ± 0.82	0.88 ± 0.76
Severity of ocular symptoms at baseline (−)[a]		0.24 ± 0.40	0.15 ± 0.23
Number of subjects who answered	is regular	5	6
that physiology cycle	shifts a little	4	6

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (unpaired t-test or Mann-Whitney U-test). [a] Mean value in 4-week pre-intake period.

Table 2. Score of Allergic Symptom in 4-week Administration Study.

		Pre-intake[a]	Intake			
			1-week	2-week	3-week	4-week
Nasal SMS (-)	Placebo	1.09 ± 0.81	1.18 ± 0.87	1.76 ± 1.07	1.71 ± 1.06	1.96 ± 1.21
	LP14	0.89 ± 0.76	1.12 ± 0.90	1.46 ± 1.13	1.61 ± 1.17	1.63 ± 1.30
Ocular SMS (-)	Placebo	0.33 ± 0.46	0.50 ± 0.67	1.01 ± 1.02	1.18 ± 1.28	1.35 ± 1.43
	LP14	0.16 ± 0.23	0.44 ± 0.61	0.83 ± 0.83	0.69 ± 0.79	0.64 ± 0.63
Nasal symptom score (-)	Placebo	1.00 ± 0.83	1.05 ± 0.69	1.54 ± 0.90	1.41 ± 0.84	1.65 ± 0.98
	LP14	0.88 ± 0.77	1.10 ± 0.85	1.42 ± 1.12	1.53 ± 1.17	1.50 ± 1.26
Ocular symtom score(-)	Placebo	0.24 ± 0.41	0.38 ± 0.65	0.79 ± 0.84	0.88 ± 0.89	1.04 ± 0.99
	LP14	0.15 ± 0.22	0.42 ± 0.58	0.79 ± 0.81	0.61 ± 0.75	0.51 ± 0.54
Medication score (-)	Placebo	0.09 ± 0.26	0.13 ± 0.32	0.22 ± 0.35	0.30 ± 0.60	0.30 ± 0.66
	LP14	0.01 ± 0.03	0.02 ± 0.07	0.04 ± 0.17	0.08 ± 0.21	0.13 ± 0.26
Sneezing score (-)	Placebo	0.53 ± 0.55	0.70 ± 0.48	1.12 ± 0.87	1.03 ± 0.80	1.14 ± 0.97
	LP14	0.51 ± 0.56	0.96 ± 0.83	1.29 ± 1.01	1.31 ± 1.02	1.28 ± 1.11
Ruuny nose score (-)	Placebo	0.70 ± 0.62	0.90 ± 0.67	1.10 ± 0.73	1.09 ± 0.66	1.32 ± 0.84
	LP14	0.78 ± 0.81	0.89 ± 0.86	1.28 ± 1.08	1.34 ± 1.10	1.34 ± 1.18
Stuffy nose score (-)	Placebo	0.57 ± 0.81	0.62 ± 0.66	0.74 ± 0.85	0.75 ± 0.76	0.91 ± 0.91
	LP14	0.30 ± 0.58	0.33 ± 0.58	0.48 ± 0.63	0.49 ± 0.55	0.60 ± 0.70
Itchy eyes score (-)	Placebo	0.22 ± 0.39	0.35 ± 0.65	0.79 ± 0.84	0.88 ± 0.89	1.02 ± 1.00
	LP14	0.12 ± 0.20	0.42 ± 0.58	0.79 ± 0.81	0.61 ± 0.75	0.51 ± 0.54
Watery eyes score (-)	Placebo	0.07 ± 0.20	0.14 ± 0.33	0.29 ± 0.55	0.34 ± 0.49	0.47 ± 0.61
	LP14	0.04 ± 0.12	0.15 ± 0.36	0.25 ± 0.54	0.24 ± 0.49	0.19 ± 0.35
Score of obstacles in daily life (-)	Placebo	0.18 ± 0.31	0.37 ± 0.53	0.83 ± 0.78	0.93 ± 0.79	0.88 ± 0.80
	LP14	0.13 ± 0.33	0.26 ± 0.47	0.55 ± 0.75	0.59 ± 0.80	0.57 ± 0.86

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (Mann-Whitney U-test). No significant differences were observed between pre-intake and intake period in these measurements (Wilcoxon t-test). [a] Mean value in 4-week pre-intake period.

Table 3. Blood Components of Allergy Medicine-Free Subjects within 1 Week Prior to Blood Examination in 4-week Administration Study.

		Pre-intake	At intake cessation	P [a]
Total IgE (IU/ml)	Placebo	219.2 ± 275.5	187.8 ± 244.8	0.041 *
	LP14	374.8 ± 738.4	294.1 ± 497.9	0.046 *
IgE specific for Japanese cedar pollen (UA/ml)	Placebo	17.9 ± 26.9	17.4 ± 27.2	0.161
	LP14	25.2 ± 28.6	21.5 ± 23.6	0.039 *
Eosinophil counts (/ μ l)	Placebo	185.5 ± 88.6	220.0 ± 99.7	0.069
	LP14	151.8 ± 82.6	170.0 ± 84.2	0.626
IL-4 (pg/ml)	Placebo	14.8 ± 9.2	10.3 ± 9.4	0.076
	LP14	23.9 ± 34.1	15.1 ± 13.9	0.389

Data are expressed as means ± SD. There were 11 subjects in each group. No significant differences were observed between the two groups in these measurements (Mann-Whitney U-test). [a] Comparisons between pre-intake and intake cessation were made by Wilcoxon t-test. * P<0.05.

Table 4. Blood Pressure in 4-week Administration Study.

		Pre-intake	At intake cessation	P [a]
Systolic blood pressure (mmHg)	Placebo	109.7 ± 7.3	105.5 ± 8.8	0.166
	LP14	106.9 ± 12.0	104.0 ± 8.0	0.215
Diastolic blood pressure (mmHg)	Placebo	66.9 ± 5.7	65.0 ± 11.9	0.521
	LP14	62.9 ± 8.4	62.0 ± 5.7	0.559
Pulse rate (/min)	Placebo	72.2 ± 12.9	63.2 ± 7.1	0.031 *
	LP14	76.6 ± 14.6	65.4 ± 7.2	0.024 *

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (unpaired t-test). [a] Comparisons with pre-intake were made by paired t-test. * P<0.05.

Table 5. Score of Fecal Condition in 4-week Administration Study.

		Pre-intake[a]	Intake			
			1-week	2-week	3-week	4-week
Frequency of defecation (times/week)	Placebo	6.5 ± 3.2	6.9 ± 4.4	6.8 ± 3.4	7.6 ± 4.0	6.9 ± 3.3
	LP14	6.2 ± 2.5	6.1 ± 2.8	5.9 ± 3.3	6.8 ± 3.6	6.0 ± 3.0
Fecal amount (number of stools/week)[b]	Placebo	14.1 ± 9.7	16.2 ± 13.2	16.3 ± 13.2	17.7 ± 13.3	15.6 ± 10.7
	LP14	13.6 ± 6.9	13.1 ± 8.6	13.3 ± 9.0	16.8 ± 11.4	15.7 ± 9.4
Ratio of appropriate fecal shape (%)	Placebo	66.1 ± 32.2	73.9 ± 25.5	64.3 ± 35.6	73.4 ± 26.8	57.5 ± 33.4
	LP14	67.1 ± 24.8	67.0 ± 30.2	68.7 ± 35.5	81.2 ± 23.7	77.6 ± 29.2
Ratio of appropriate fecal color (%)	Placebo	20.6 ± 32.9	19.4 ± 32.3	14.1 ± 34.1	19.7 ± 34.4	14.6 ± 29.0
	LP14	30.1 ± 34.4	32.3 ± 36.9	26.4 ± 31.0	21.2 ± 32.6	14.3 ± 27.3
Ratio of appropriate fecal odor (%)	Placebo	68.8 ± 34.3	73.5 ± 33.4	70.8 ± 37.4	72.8 ± 37.0	73.6 ± 33.5
	LP14	75.0 ± 32.8	67.6 ± 38.6	70.9 ± 36.3	72.4 ± 37.2	69.8 ± 33.5
Ratio of appropriate sensation after defecation (%)	Placebo	76.8 ± 30.3	84.1 ± 27.1	82.0 ± 25.5	82.2 ± 27.0	80.7 ± 24.6
	LP14	80.7 ± 22.3	75.9 ± 28.8	83.2 ± 24.7	89.9 ± 18.5	82.8 ± 25.9
Ratio of appropriate abdominal condition (%)	Placebo	71.0 ± 32.3	77.7 ± 31.7	75.0 ± 35.6	78.6 ± 33.4	76.8 ± 31.2
	LP14	70.4 ± 30.6	76.5 ± 31.1	79.8 ± 24.3	79.8 ± 30.3	68.9 ± 32.4

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (Mann-Whitney U-test). No significant differences were observed between pre-intake and intake period in these measurements (Wilcoxon t-test). [a] Mean value in 4-week pre-intake period. [b] The fecal amount was based on the size of egg.

Table 6. Questionnaire after Study about Alteration in Body Temperature and Appetite in 4-week Administration Study.

Q1. The question how the subjects felt the body temperature change during a sample intake period ?

	Placebo	LP14
I continually felt feverish.	0	0
I felt warmer than usual.	1	3
I did not feel a change in body temperature.	15	14
I felt a little colder than usual.	0	0
I felt considerably colder than usual.	0	0
	P=0.994	

Q2. The question how the subjects felt the appetite during a sample intake period?

	Placebo	LP14
Increased	1	0
Increased a little	3	2
Not changed	11	15
Decreased a little	1	0
Decreased	0	0
	P=0.996	

Statistical analysis were performed by chi-square test with Yates correction for continuity for an m×n contingency table.

Table 7. Mean Values of Parameters whose Coefficients of Determination with the Average Increase of Body Temperature Were 0.1 or More in Each Quartile, According to Body Temperature Elevation in Single-time Administration Study.

	Average increase of chest temperature				R^2	r
	1st quartile (<−0.03° C)	2nd quartile (−0.03–0.10° C)	3rd quartile (0.10–0.25° C)	4th quartile (0.25<° C)		
ADRB3 (−)[a]	1.2 ± 0.4	1.5 ± 0.6	1.4 ± 0.5	1.6 ± 0.9	0.248	0.498
Body fat percentage (%)	24.1 ± 3.0	25.2 ± 3.5	29.4 ± 4.3	28.6 ± 5.8	0.151	0.388
Waist circumference (cm)	70.2 ± 3.6	75.0 ± 7.5	77.5 ± 9.3	79.8 ± 8.2	0.203	0.450
Intake frequency of lactic acid bacterium (−)[b]	2.4 ± 0.9	2.0 ± 1.2	1.2 ± 0.4	1.6 ± 0.9	0.125	−0.353

	Average increase of foot temperature				R^2	r
	1st quartile (<−0.57° C)	2nd quartile (−0.57–0.01° C)	3rd quartile (0.01–0.46° C)	4th quartile (0.46<° C)		
Chest circumference (cm)	94.4 ± 10.5	88.8 ± 11.1	84.8 ± 6.6	82.8 ± 3.6	0.236	−0.486
Hip circumference (cm)	94.0 ± 4.3	94.8 ± 14.1	82.8 ± 14.3	87.3 ± 5.2	0.115	−0.339
Normal temperature (−)[c]	1.4 ± 0.5	1.8 ± 0.5	1.6 ± 0.5	1.8 ± 0.4	0.146	0.382
Overall metabolism (−)[d]	1.8 ± 0.8	2.0 ± 0.8	2.4 ± 0.5	2.2 ± 0.8	0.141	0.375

Data are expressed as means ± SD. No significant correlations were observed between the average increase of body temperature and these measurements (Spearman's correlation coefficient or Pearson correlation coefficient). [a] *ADRB3* was scored as follows: “1-wild (no mutation)”, “2-hetero” and “3-homo”. [b] Intake frequency of lactic acid bacterium was scored as follows: “1-2 times or less/week”, “2-3-5 times/week” and “3-most every day”.[c] Normal temperature was scored as follows: “1-36°C or less”, “2-36-37°C” and “3-37°C or more”.[d] Overall metabolism was scored as follows: “1-bad”, “2-normal” and “3-good”based on the consciousness of the subjects.

Table 8. Questionnaire after Study about Alteration in Body Temperature and Appetite in Single-time Administration Study.

Q1. The question which sample the subjects felt warm during the study?

	Intake sample of the first day	
	Placebo	LP14
First day	5	2
Second day	2	2
Do not know	6	4

Q2. The question which sample the subjects felt hungry during the study?

	Intake sample of the first day	
	Placebo	LP14
First day	4	3
Second day	6	4
Do not know	3	1

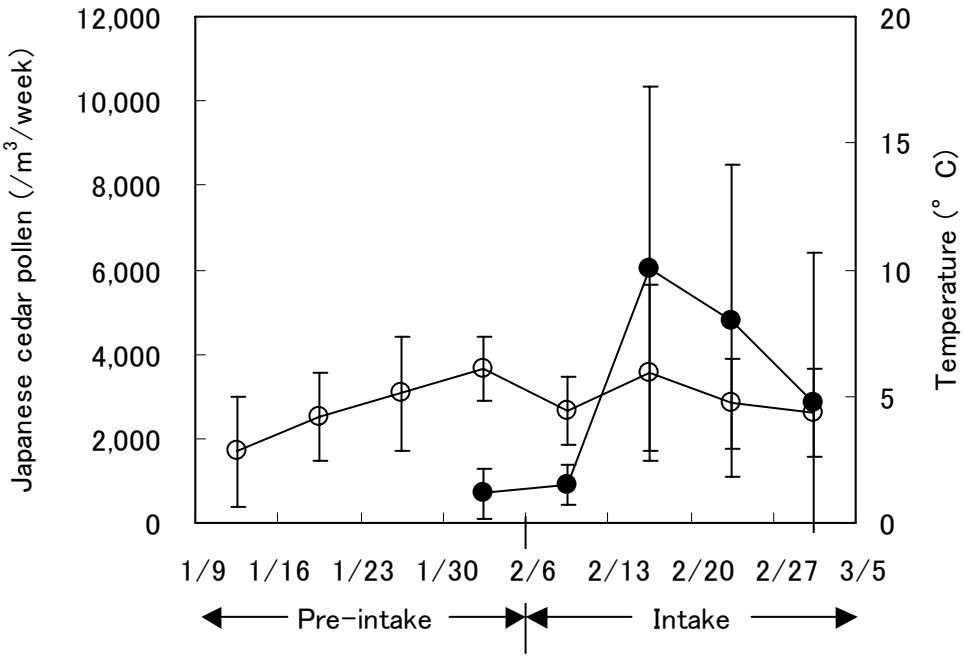

Fig. 1. Pollen Count and Atmospheric Temperature at the Experimental Site in 4-week Administration Study.

Weekly means ± SD of atmospheric temperature (○) and Japanese cedar pollen (●) are shown. There was no pollen data for January. The pollen dispersal started in February.

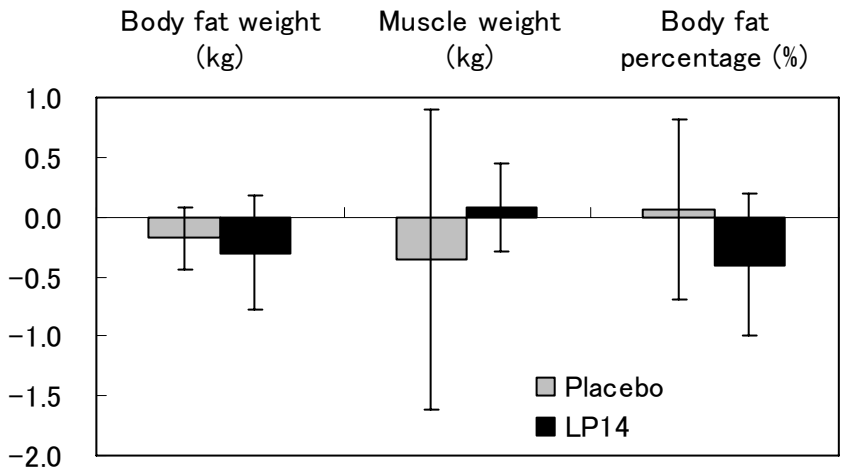

Fig. 2. Changes in Body Composition of Subjects with Body Fat Percentage Greater than 30% in 4-week Administration Study.

Data are expressed as means ± SD of ((Intake cessation)-(Pre-intake)). There were five subjects in each group. No significant differences were observed between the two groups in these measurements (unpaired t-test).

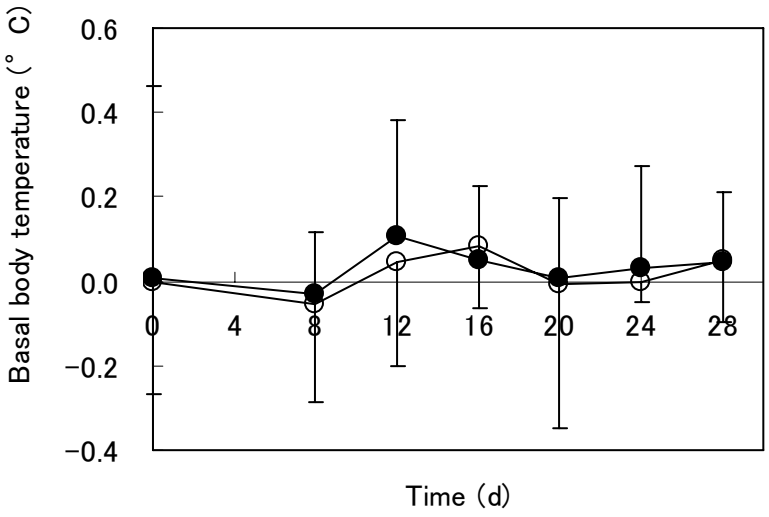

Fig. 3. Changes in Basal Body Temperature of the Subjects with a Steady Menstrual Cycle in 4-week Administration Study.

Data are expressed as 4-day means ± SD of ((Intake day) - (28 days prior to intake)) of five subjects in the placebo group (○) and eight subjects in the LP14 group (●). Because some subjects lacked experience in measuring their basal body temperature, the first 4 days of data were excluded. Basal body temperature increased significantly only in the LP14 group over the intake period, as determined by paired t-test (LP14, P=0.021; placebo, P=0.427).

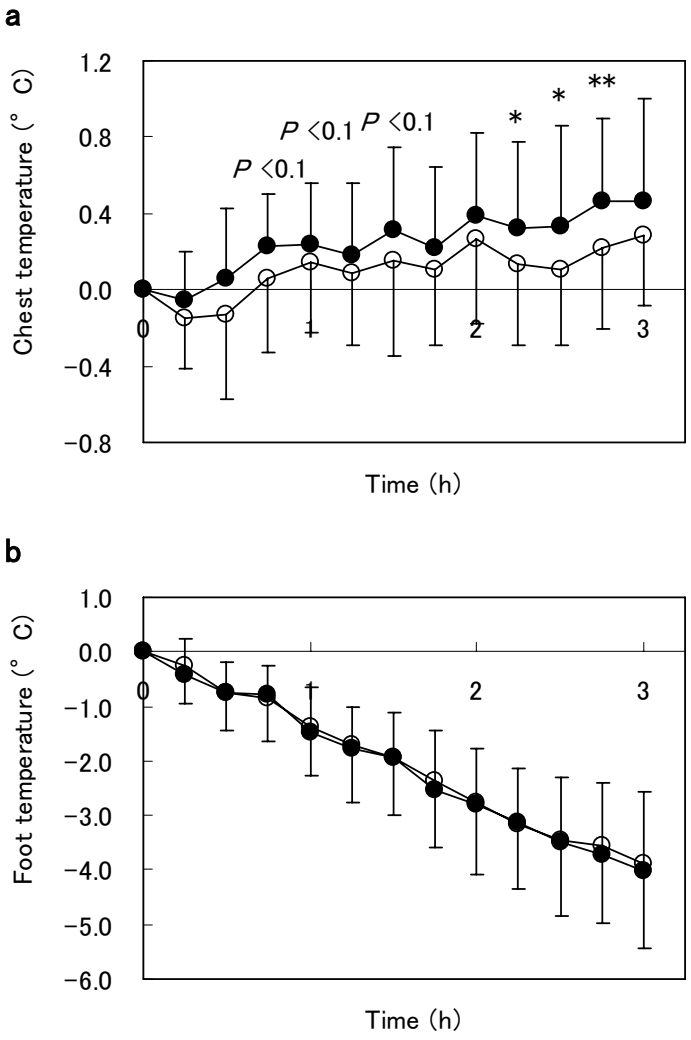

Fig. 4. Changes in Chest Temperature (a) and Foot Temperature (b) in Single-time Administration Study.

Data are expressed as means ± SD of ((Experimental period) - (Time point zero)) in the placebo group (○) and in the LP14 group (●). There was a significant difference between LP14 and placebo in the average increase of chest temperature (P=0.023). For comparisons between the placebo and LP14 groups, paired t-tests were performed. * P<0.05; ** P<0.01.

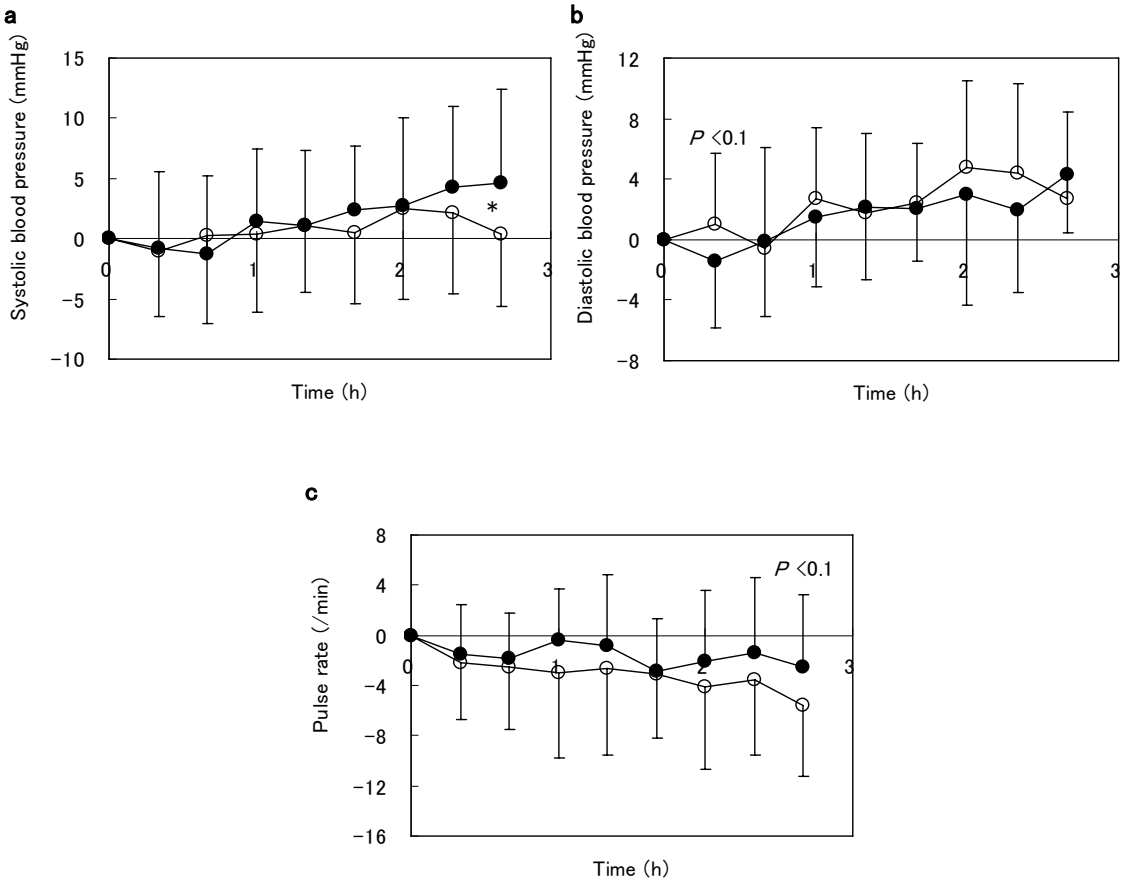

Fig. 5. Changes in Systolic Blood Pressure (a), Diastolic Blood Pressure (b) and Pulse Rate (c) in Single-time Administration Study.

Data are expressed as means ± SD of ((Experimental period) - (Time point zero)) in the placebo group (○) and in the LP14 group (●). For comparisons between the placebo and LP14 groups, paired t-tests were performed. * P<0.05.

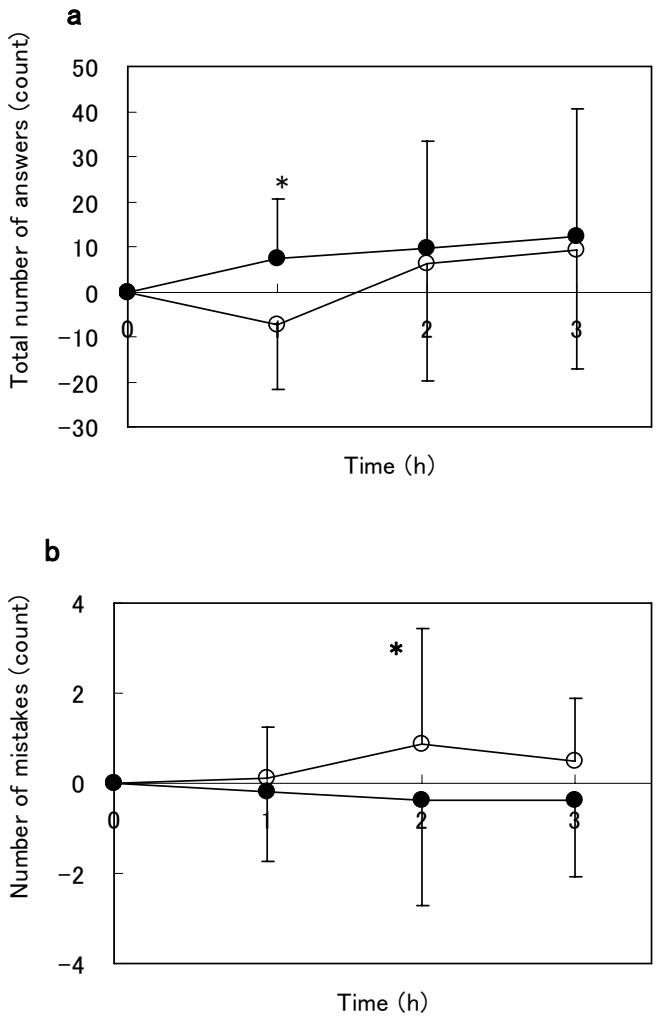

Fig. 6. Changes in Total Number of Answers (a) and Number of Mistakes (b) of Uchida-Kraepelin test in Single-time Administration Study.

Data are expressed as means ± SD of ((Experimental period) - (Time point zero)) in the placebo group (○) and in the LP14 group (●). For comparisons between the placebo and LP14 groups, paired t-tests were performed for the number of mistakes, and unpaired t-tests were performed for the total number of answers. * P<0.05.

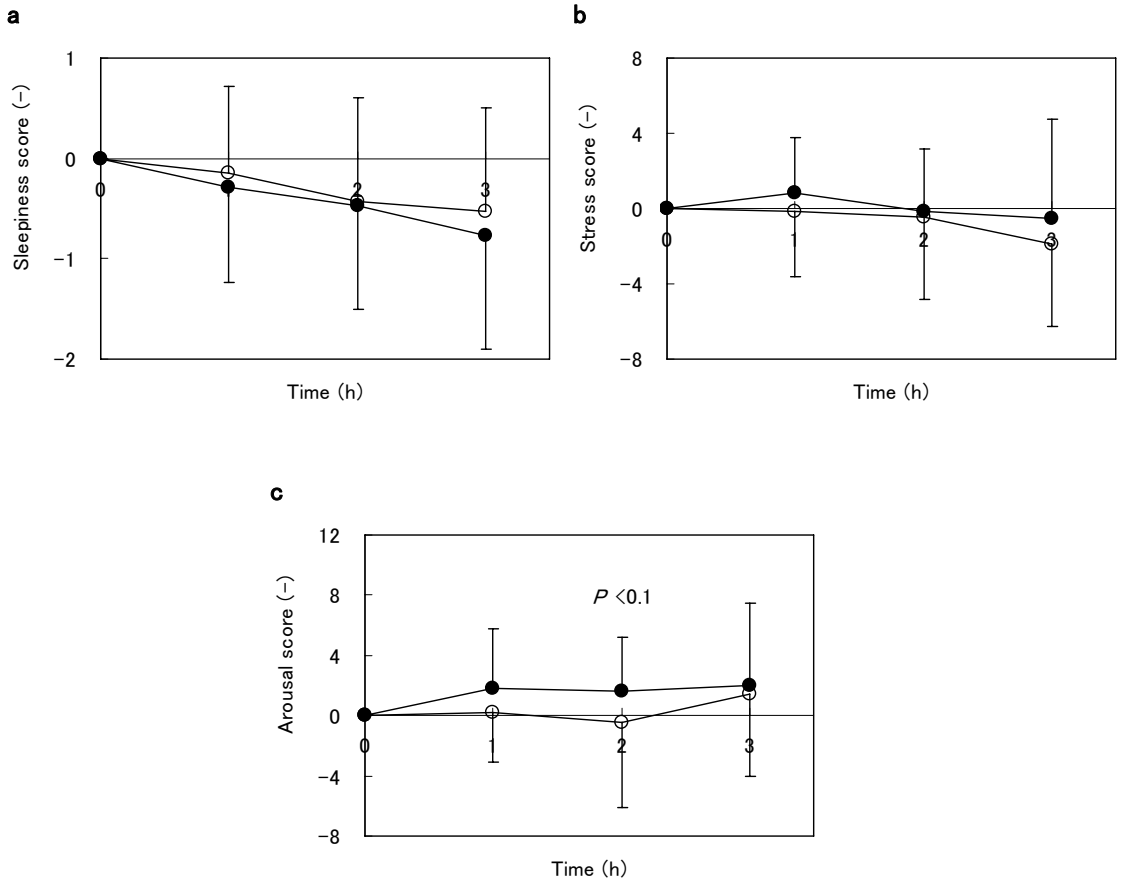

Fig. 7. Changes in Sleepiness Score (a), Stress Score (b) and Arousal Score (c) in Single-time Administration Study.

Data are expressed as means ± SD of ((Experimental period) - (Time point zero)) in the placebo group (○) and in the LP14 group (●). For comparisons between the placebo and LP14 groups, Wilcoxon t-tests were performed. * P<0.05.

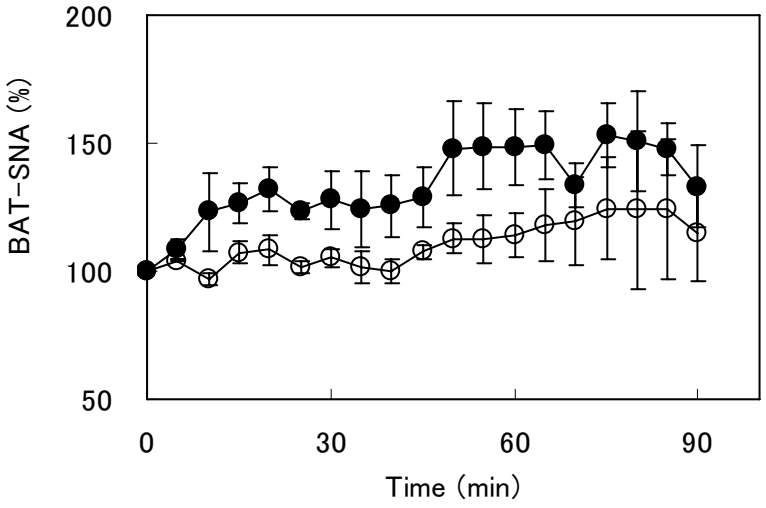

Fig. 8. Changes in BAT-SNA in Rats.

Data are expressed as means ± SEM of percentage of values at baseline in the water group (○) and in the LP14 group (●). There was a significant difference between values from 5-90 min after intragastric injection of water or LP14, as determined by ANOVA (P=0.0005).

Chapter 4: Influences of the Intake of a Processed Food Containing LP14 on Several Clinical Parameters

Introduction

The Chapter 1, 3 and the previous reports[7, 20] suggested that LP14 had an antiallergic effect, a reducing effect on the body fat percentage, and a rising effect on the body temperature. Powder of LP14 was employed for the test sample in a series of examinations. LP14 is known to be effective as a fermenting starter for pickles. In general, fermenting improves food preservability and creates food flavors. In addition, fermented foods are expected to make the intake of lactic acid bacteria palatable. A novel processed food through fermentation with LP14 was developed.

LP14 is known to have the suppressing effect on pollinosis prevailing among Japanese people to the extent of 26.5%.[1] Rice, a Japanese staple food, was selected as media for the culture of LP14. Jellies packed with fermented rice powder were prepared. The Chapter 1, 3 and the previous reports[7, 20] suggested that pollinosis was prevented by the intake of over 10^{10} CFU of LP14, and the effect was also observed in the examination of killed LP14. Furthermore, not only LP14 cell but also EPS of LP14 was found to have an immunostimulatory effect.[69] To obtain a rice jelly containing 10^{11} CFU of LP14, LP14 cultured in MRS medium was precipitated by ethanol and concentrated into the suspension containing EPS of LP14. The suspension was applied to adjust the number of LP14 in the rice jelly. Gelling agent A (San-Ei Gen F.F.I., Osaka, Japan), resistant against heat and acid, was used to attain a desirable gel texture. The jelly was sterilized after being packed.

The effects of the intake of this rice jelly on a body fat and allergy were examined. The performances of the jelly were observed when treated with artificial digestive juices *in vitro*. The number of cells of LP14 released from the jelly was counted after digestion.

Experimental methods

1. Intake of rice jelly

Study design. This study was conducted under the design being randomized, placebo-controlled and double-blinded. After a pre-intake period of 1 week (from November 20 to 26, 2009), the intervention group received one LP14 jelly (2.5×10^{11} CFU/50 g), and the placebo group received one placebo jelly (that did not contain LP14) every day for 12 weeks (from November 27 to February 26, 2010). Subjects were instructed to keep a diary of medication, appetite and stool and abdominal conditions throughout the experimental period. Blood samples, body composition and blood pressure data were examined three times (pre-intake, November 20; 7th week of intake, January 22; cessation of intake, February 26). Then, the subjects answered mood questionnaire.

The studies were conducted in accordance with the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of Kagawa Nutrition University. Written informed consent was offered from all subjects.

Subjects. A total of 21 healthy female college students (age range, 18-28 years) were randomized into an intervention group (n=11) and a placebo group (n=10). No significant differences in baseline of clinical characteristics were found between the placebo group and the intervention group (Table 1).

Preparation of active and placebo sample.

Bacterial suspension: After being pre-cultured, LP14 as a fermenting starter was cultured in MRS medium at the concentration of 1% at 30°C for 24 h. The culture medium was pasteurized at 100°C for 20 min. After being cooled, ethanol was added up to a final concentration of 70% and the mixture was kept at 4°C for 48 h. The precipitate formed was collected and suspended in distilled water. It was reprecipitated in 70% v/v of ethanol and centrifuged at 13,000×*g* for 20 min at 4°C. The precipitate obtained was resuspended in distilled water. The suspension, 4.2 L, was obtained from 96 L of MRS medium. This suspension contained 3.5×10^{10} CFU/ml of LP14. The sample for placebo

was prepared by the same method without inoculation.

Starter: The mixture composed of rice powder, sugar, KH_2PO_4, K_2HPO_4, sake lees extract and water were heated at 80°C for 15 min, and autoclaved at 121°C for 15 min. After being cooled, LP14 culture was added to the mixture at 1%, being cultured at 30°C for 24 h (pH 4.4). The starter was adjusted to pH 7.0 with 2N-NaOH. Water was used for the placebo.

Fermented rice material: The mixture composed of rice powder, sugar, sake lees extract and water was heated at 80°C for 15 min. After being cooled to 40°C, the starter was added to the mixture, being cultured at 30°C for 24 h (3.1×10^8 CFU/ml, pH 3.4). The material for placebo lacked the starter and its pH was adjusted with lactic acid. Twenty five kg of fermented rice material was manufactured.

Rice jelly: The mixture composed of fermented rice material, bacterial suspension, sugar syrup, gelling agent A (carrageenin23.4%, gellan gum 9.1%, locust bean gum 6.2%, xanthan gum 6.2%, Trisodium citrate 8%, calcium lactate 8%; San-Ei Gen F.F.I., Osaka, Japan), trisodium citrate, calcium lactate, flavor and water was heated at 100°C. Caramel was added to the jelly of placebo to adjust the hue. Fifty g of the mixture was charged into a plastic container, being sterilized at 70-75°C for 20 min, and cooled down. The jelly for the intervention group contained 2.5×10^{11} CFU/50g of LP14. Nine hundred and fifty jellies (47.5 kg) were prepared. The rice jelly is shown in Fig. 1. One jelly (50 g) contained 6.8 g of carbohydrate; 27 kcal.

Personal recordings. The subjects recorded medication use. Subjects selected one of five statements to best describe their state of appetite. Each subject recorded stool and abdominal conditions throughout the experimental period (Chapter 1-Table 4).

Measurements. Subjects were not allowed to eat or drink (except water) after 21:00 in the evening the day before to the blood collection. The levels of total IgE, eosinophil count, CRP, neutrophil rate, eosinophil rate, basophil rate, monocyte rate, and lymphocyte rate were measured. All blood tests were performed by SRL. (Tokyo, Japan).

Body weight, body fat weight, lean body weight and BMI were measured by the use of a body composition analyzer (InBody 3.0, BioSpace, Seoul, Korea). Blood pressure and pulse rate were measured with an upper arm automatic blood pressure monitor (HEM-7115, Omron Healthcare, Kyoto, Japan).

Mood questionnaire. Each subject completed a questionnaire concerning state anxiety (20 items), stress (17 items), arousal (13 items), tension (9 items), depression (15 items), anger-hostility (12 items), fatigue (7 items), confusion (7 items), vigour (8 items), friendliness (7 items) (Table 2). The question paper used for Subjective Indexes and Mental task Programs for Laboratory Experiment (S.I.M.P.L.E.) was developed by Miyake *et al.*[92] This mood evaluation was arranged by 92 items except for duplication of a same or similar item from a total of 115 items of three kinds of questionnaires, SACL, State Trait Anxiety Inventory (STAI, only state anxiety), Profiles of Mood States (POMS). SACL and STAI are four-point scale and POMS is five-point scale. The mood evaluation was unified into a four-point scale. Each index value calculated by the original computing method.

Questionnaire after study. Subjects were asked if there was a difference in appetite and the alteration in body temperature between the test sample and the placebo. The effectiveness for blinding was proved by asking the subjects to indicate which sample they believed they had received (test sample, placebo, or do not know) after 12 weeks of intake.

Statistical analysis. The data was expressed as means ± SD. In the clinical tests, the Shapiro-Wilk test was applied to the null hypothesis. Total IgE, eosinophil counts and CRP were distributed in non-normality. Thus, a nonparametric analysis was applied to these data and categorical data (personal recording and questionnaire). The non-normal data in the intervention group and the placebo one were compared with the Mann-Whitney U-test. In the case of normal data, the unpaired t-test was used. The Wilcoxon t-test was used for paired data analysis to compare the intake with the

pre-intake for non-normal data. The paired t-test was used for the normal data. Two-way ANOVA was performed for the personal recordings. For the questionnaire after study, statistically significant differences were assessed by using the chi-square test with Yates correction for continuity for an m×n contingency table. All statistical tests were two-sided. Statistical significance was set at $P<0.05$. The statistical analysis was performed with SPSS 6.1 software (SPSS, Chicago, IL, USA) and ystat2006 (Igakutosho-shuppan, Tokyo, Japan).

2. Digestion of rice jelly

Materials. Samples were as follows; 1) rice jelly, 2) fermented rice material according to the same production method as described in the administration study, 3) rice jelly without the gelling agent, 4) rice jelly gelled with gelatin, 5) placebo rice jelly, 6) LP14 suspension and 7) jelly gelled by various gelling agents (gelling agent A+0.1% calcium lactate, κ-carrageenin, ι-carrageenin, gellan gum+0.1% calcium lactate, xanthan gum+locust bean gum (1:1) and agar) with LP14 suspension which was added 10% sugar syrup, 0.9% gelling agents and water, followed by heating at 100°C, and cooling. Xanthan gum and locust bean gum formed gel on being mixed. The LP14 suspension of the jelly made with various gelling agents was prepared with or without ethanol precipitation of LP14 culture. These jelly samples were prepared the day before each experimental test.

The enzyme mixture was prepared on the day of use. For 18 samples, 3.0 g pancreatin (Sigma, St Louis, MS, USA) was weighed into each of 6 centrifuge tubes and 20 ml water was added to each. The pancreatin was suspended by a mixer vortex and then mixed for 10 min on a magnetic stirrer. The tubes were centrifuged at 1,500×*g* for 10 min; 15 ml of the cloudy supernate from each tube (90 ml total) was removed into a flask and 4 ml amyloglucosidase (Sigma) was added and mixed well.

Protocol. Digesting conditions were in accordance with the method of Englyst *et al.*[93] Samples were homogenized for 30 sec. They were put into 50-ml polypropylene centrifuge tubes (containing <0.6 g carbohydrate), and water was added so that the

whole weight was adjusted to 5.0 g. The jellies were weighed 3.5 g with 1.7×10^{10} CFU of LP14. The fermented rice material was weighed 1.25 g with 2.5×10^{8} CFU of LP14. LP14 suspension was 0.5 ml with 1.8×10^{10} CFU. Ten ml of freshly prepared pepsin solution (5 g pepsin/l in 0.05 mol HCl/l) was added. After being mixed with vortex, the test tubes were placed in a water bath at 37°C for 30 min. Five ml of 0.5 mol/l of sodium acetate (previously equilibrated to 37°C) was added to each tube to hold buffer action at pH 5.2. Five glass balls (1.5-cm diameter) were thrown into the tubes, and were shaken gently to disperse the contents and then placed in the 37°C water bath to be equilibrated for a few minutes. During shaking, the glass balls disrupted mechanically the physical structure of the samples. The sample in each tube was put into 1.5-ml polyethylene tubes at each process by 0.2 ml, and cooled down in ice water.

The above sample tube was added 5 ml of enzyme mixture. The tube was immediately capped and the content were stirred gently before it was kept standing in the shaking water bath at 37°C. Each tube was removed from the bath 1, 2, 4, 24 h after the enzyme mixture was added and 0.2 ml of the contents was put into 1.5-ml polyethylene tubes. The tubes were cooled in ice water and were subjected to a microscopic observation (×400).

3. Cell count of LP14 released from jelly by digestive process

Materials. The jellies for test were made of LP14 suspension with gelling agents A or gellan gum. The mixture of 10% sugar syrup, 0.9% gelling agents, 0.1% calcium lactate, LP14 suspension with ethanol precipitation, and water was heated at 100°C, and cooled. The jelly sample was prepared the day before experiment.

Protocol. Each jelly prepared varied in particle size; 1) sieved at 150 μm, 1 mm and 4 mm, 2) homogenized for 30 sec, 3) crushed by Stomachere for 30 sec. The jellies were weighed 7.0 g containing 3.5×10^{10} CFU of LP14. The jellies were digested by the similar method as described above. The suspension after 4 h of intestinal juice digestion was put into the sterilized filter bag, PYXON-20 (opening of sieve 60 μm, Elmex, Tokyo, Japan). The filtrated cells of LP14 were counted on a hemacytometer.

Results

1. Administration study of rice jelly

Body composition

No statistically significant change was found in the body composition of subjects (Table 3).

Blood examination

The result of blood examination was shown in Table 4. Total serum cholesterol of the placebo group increased significantly after 12 weeks of intake (P=0.033). HDL-cholesterol tended to increase after 7 weeks of intake in the LP14 group (P=0.053) and increased significantly in both groups after 12 weeks of intake (LP14, P=0.003; placebo, P=0.014).

Seven subjects were found to be allergic in the placebo group and five in the LP14 group. Those allergic subjects were not habitual users of medication. Total IgE decreased significantly in the LP14 group after 7 weeks of intake (P=0.028) and in both groups after 12 weeks of intake (LP14, P=0.013; placebo, P=0.022). Eosinophil counts decreased significantly in the placebo group after 7 weeks and 12 weeks of intake (P=0.026, P=0.013). Eosinophil rate decreased significantly in the placebo group after 7 weeks and 12 weeks of intake (P=0.041, P=0.019). Monocyte rate tended to increase in the LP14 groups after 7 weeks and 12 weeks of intake (P=0.069, P=0.099). There was no significant difference in granulocyte-lymphocyte rate.

There were no significant differences in blood pressure and pulse rate.

Personal recordings

There were significant differences between the LP14 and the placebo group in the data of pre-intake period. Then, those data were expressed as (Intake)-(Pre-intake)). The result of personal recordings was shown in Table 5. Frequency of defecation and fecal amount did not vary between the groups during the intake period. Sensation after defecation and abdominal condition of the LP14 group were significantly lower than the placebo group (P=0.003, P=0.012), though these changes were temporary.

The score of appetite in the LP14 group was significantly higher than that in the placebo group (P=0.042).

Mood questionnaire

After 7 weeks of intake, the score of state anxiety and stress increased significantly in the LP14 group (P=0.034, P=0.026, Table 6). The score of depression in the placebo group increased significantly (P=0.021). The score of friendliness in the placebo group decreased significantly (P=0.042). After 12 weeks of intake, the score of vigour in the LP14 group increased significantly (P=0.019).

Questionnaire after study

There were no significant differences between the LP14 group and the placebo one with respect to appetite and the alteration in body temperature (Table 7). Three subjects in the placebo group correctly identified the placebo samples. Four subjects in the LP14 group correctly identified the LP14 samples. Therefore, blinding was proved to be effective.

2. Digestion of rice jelly

Microscopy of rice jelly

Figure 2a shows the microphotograph of the digesting process of the rice jelly. The pictures revealed that the cell of LP14 was a small dot and the starch of fermented rice material was a large grain, respectively.

Amoeba-like gel without any motion was observed in all the microphotographs of the rice jelly formed by the use of gelling agent A. Most cells of LP14 were buried in the amoeba-like gel matrix. The gelatin rice jelly was digested by pepsin and liquefied. Starch was digested after 1-2 h of treatment, and the particle size of starch reduced and its number decreased. After 4 h of digestion, most of the particles of starch disappeared.

Microscopy of jelly gelled by various gelling agents

The jellies made of gellan gum and xanthan gum+locust bean gum had elasticity

like rice cake and did not get fine after homogenization. The jellies of κ-carrageenin and ι-carrageenin became liquefied after homogenization. The jelly of agar got fine after homogenization.

In the gellan gum jelly prepared by ethanol precipitation, any amoeba-like gel was not observed in water suspension treatment (Fig. 2b). Amoeba-like gel was found in the sample after gastric juice treatment at the pH of 1.7. At the pH of 5.2 after gastric juice was neutralized, the amoeba-like gel became somewhat loose. However, amoeba-like gel existed also after intestinal juice treatment. The change of jelly made from gelling agent A resembled that of gellan gum. The change of jelly of xanthan gum+locust bean gum also resembled that of gellan gum, but the amoeba-like gel was looser than that of gellan gum.

There were also amoeba-like gels in the jellies of κ-carrageenin or ι-carrageenin after gastric juice treatment. But the amoeba-like gel disappeared after neutralizing gastric juice, resulting in fluid LP14. There was no great difference in change of the jellies of κ-carrageenin or ι-carrageenin. The gel of ι-carrageenin became partially filamentous by the vortex stirring after gastric juice treatment.

Also in the jelly of agar, LP14 adhered to gel matrix strongly after gastric juice treatment. However, LP14 gradually got out of gel matrix during intestinal juice treatment. After 24 h of intestinal juice treatment, there were found gels without LP14.

LP14 without ethanol precipitation adhered to gel matrix more strongly than LP14 with ethanol precipitation except for agar (Fig. 2b, c).

3. Cell count of LP14 released from jelly by digestive process

Amoeba-like gel was not observed in the filtrate. The number of LP14 released from jelly and its rate to total LP14 were shown in Table 8 and Fig. 3. The form of the jelly made of gellan gum hardly altered by homogenization or Stomachere crushing. The number of cell of LP14 liberated from gellan gum jelly after digestion was 2-3% after homogenization, Stomachere crushing and 4 mm-sieving. The proportion of cells of LP14 liberated from gellan gum jelly of 150 μm-sieving was 12% in number.

The proportion of cells of LP14 liberated from the jelly made of gelling agent A

after digestion were 44% to homogenization and 14% to Stomachere crushing, respectively. The rate of liberated cells of LP14 from gelling agent A jelly were 10% at 4 mm-sieving and 46% at 150 μm-sieving, respectively.

Discussion

The study of intake of the rice jelly focused on the reduction of body fat percentage. However, the body fat percentage was not found to reduce by the intake of LP14. The score of appetite in the LP14 group was significantly higher than that in the placebo group. There was no difference in fecal amount between the LP14 and the placebo group.

Total cholesterol increased significantly in the placebo group after 12 weeks of intake (LP14 group, +3.9mg/dl; placebo group, +10.3mg/dl). Although HDL-cholesterol increased significantly in both groups after 12 weeks of intake, there was no difference in the amount of rises (LP14 group, +5.1mg/dl; placebo group, +7.2mg/dl). Although there was no significant change, the amount of change of LDL-cholesterol was -4.4 mg/dl by LP14 group, and was +5.0 mg/dl in the placebo group. The serum cholesterol level depends on season, rising in winter.[94] Therefore, LP14 possibly adjusted cholesterol rise. In Chapter 1, the intake of LP14 of six weeks did not affect the serum lipid composition. Serum total choresterol in mice fed diet of ordinary level of fat for 11 weeks was not different between LP14 and the vehicle administered mice.[52] In mice fed high fat diet, however, LP14 administered mice tended to lower total choresterol in comparison with the vehicle administered mice. It was considered that it took a long time feeding to reduce cholesterol level by LP14 intake.

Total IgE and eosinophil counts were employed for the indicator of antiallergic effect. Although the total IgE decreased significantly in LP14 group after 7 weeks of intake, it decreased significantly in both group after 12 weeks of intake. Eosinophil counts decreased significantly in the placebo group after 7 weeks of intake and 12 weeks of intake. No antiallergic effect was observed in the intake of LP14.

When a sympathetic nerve predominates, adrenalin is secreted, increasing granular leukocyte with adrenergic receptor.[95] When parasympathetic nerve predominates, acetylcholine is secreted, increasing lymphocyte with cholinergic receptor. Because sympathetic nerve activated in the Chapter 3 study, the ratio of granular leukocyte to lymphocyte was measured in the rice jelly administration study. However, there was no significant difference between the LP14 group and the placebo one. Blood pressure and

pulse rate did not alter. In mood questionnaire, the score of state anxiety, stress and vigour in LP14 group increased significantly, while the score of depression in the placebo group increased significantly. The score of friendliness in the placebo group decreased significantly. It was considered that arousal in LP14 group increased in mood questionnaire.

Although the rice jelly administration study was performed in a large numbers of cells of LP14 (2.5×10^{11} CFU/50 g) and for the long period of intake, no effects were observed on the body composition or allergy. In the present study, the effect of LP14 was considered to be suppressed by the presence of food components or the matrix of gell. Ding *et al.* [96] reported that gastrointestinal tract tolerance improved by gelling probiotic bacteria by various gelling agents (arginate, guar gum, xanthan gum, locust bean gum and carrageenan) because of indigestibility of gelling agents. And it was suggested that gel protected the bacterial cells from the adverse environment. LP14 was recognized by TLR2 (unpublished data). LP14 was considered to affect human immune cells of an intestinal tract. Therefore, LP14 buried in gel matrix could not affect humans. The performances of the jelly were observed when treated with artificial digestive juices *in vitro*.

In consequence, it was observed that the amoeba-like gel wrapping LP14 was formed by the action of strong acid of gastric juice. The amoeba-like gel made of carrageenan dissolved by neutralizing gastric juice. In the jellies of gelling agent A, gellan gum and xanthan gum+locust bean gum, the amoeba-like gel held its shape even after neutralizing gastric juice. The change of the jellies of gelling agent A, gellan gum and xanthan gum+locust bean gum by the digestive process resembled from each other. Gellan gum and xanthan gum contain glucuronic acid. In this experiment, xanthan gum was mixed with locust bean gum by 1:1. Gelling agent A contains 9.1% gellan gum and xanthan gum 6.2%. Glucuronic acid might participate in adhesion to LP14. The LP14 suspension obtained by the precipitation with ethanol contained EPS released out of the cell of LP14. The LP14 cell lacking EPS without ethanol precipitation appeared to adhere with the gel more strongly except for agar.

The number of cells of LP14 released from gellan gum jelly and that from gelling

agent A jelly were counted after digestion. The cell of LP14 released from gellan gum jelly which homogenized, crushed by the use of Stomachere and was sieved at 4 mm was 2-3%. That from gellan gum jelly sieved at 150 μm was 12%. It was considered that most cells of LP14 in the jelly made of 0.9% gellan gum might be excreted. LP14 decreased IgE level by the intake of 10^{10} CFU. When cells of LP14 were liberated less than 4% from the rice jelly, it was considered that the number of cell of LP14 was insufficient to reduce the level of IgE. The jelly after being swallowed was considered to be crushed to a similar extent with Stomachere processing. About 85% of LP14 in the jelly made of gelling agent A was considered to be excreted.

The reports from Pubmed data were listed on Table 9, concerning to immune function of carrageenan, gellan gum, xanthan gum and locust bean gum contained in gelling agent A. No report was found as to gellan gum and locust bean gum related to immunoreaction of the gelling agent itself. Ishizaka *et al.*[97] reported that xanthan gum induced both a significant increase of DNA synthesis in mouse splenic B cells and thymocytes as well as polyclonal IgM and IgG antibody responses in B cells. Takeuchi *et al.*[98] reported that oral administration of xanthan gum in mice significantly retarded tumor growth and the effect was dependent on TLR4. Carragheenan was used as an enteritis guidance agent in Rodentia, and there were many reports about immunity-related carrageenan. The studies on rats, guinea pigs and monkeys indicated that degraded carrageenan (poligeenan) might cause ulcerations in the gastro-intestinal tract and gastro-intestinal cancer.[99] Poligeenan is produced from carrageenan when exposed to high temperatures and acidity or to digestive enzyme of Rodentia. Other report indicated that carrageenan induced inflammation in human intestinal epithelial cells in tissue culture through a Bcl10-mediated pathway that led to activation of NF-κB and IL-8.[100] The Joint FAO/WHO expert committee on food additives states that, “based on the information available, it is inadvisable to use carrageenan in infant formulas”.[101] In the rice jelly, the content of LP14 and that of carrageenan was 0.34 g and 0.11 g, respectively. The carrageenan was considered to compete with LP14 in immunoreaction, inhibiting the effect of LP14. Some reports suggested that gelation improved the viability of probiotic bacteria to help deliver viable bacteria to the host’s

gastrointestinal tract, and might contribute to effects of probiotics bacterium.[96, 102, 103] They had not considered a possibility that the effect would be inhibited by gelation. In order to develop the immunological effect of LP14, it is necessary to examine the kind of gelling agent (also including not using). The food components are also required to be examined concerning to the immunological effect of LP14.

In conclusion, the rice jelly was ineffective to body composition or allergy in spite of increased intake amount of LP14. It was considered that most cells of LP14 in the rice jelly was buried in gel matrix, leading to excretion. Further experiment is required in using culture cell in order to develop the immune effective LP14 products made of gelling agents. The problems became clear about processing LP 14 into foods.

List of Tables and Figures

Table 1. Clinical Characteristics of the Subjects in Rice Jelly Administration Study.

	Placebo	LP14
Number of subjects	10	11
Age (years)	20.1 ± 2.7	20.0 ± 1.0
BMI (kg/m^2)	22.1 ± 3.2	21.4 ± 1.2
Body fat parcentage (%)	28.4 ± 4.7	28.2 ± 3.5
Eosinophil counts (/ μ l)	228.0 ± 120.4	160.0 ± 130.8
Total IgE (IU/ml)	196.8 ± 202.5	127.9 ± 135.2
Total cholesterol (mg/dl)	173.4 ± 31.6	191.8 ± 36.0
HDL-cholesterol (mg/dl)	61.4 ± 8.3	64.0 ± 13.7
LDL-cholesterol (mg/dl)	97.4 ± 30.3	112.4 ± 28.3
Triglyceride (mg/dl)	66.3 ± 29.5	65.9 ± 28.8

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (unpaired t-test or Mann-Whitney U-test).

Table 2. Question Item for Mood Questionnaire.

State anxiety
calm
secure
tense
strained
at ease
upset
worry over misfortunes
satisfied
frightened
comfortable
self-confident
nervous
jittery
indecisive
relaxed
content
worried
confused
steady
pleasant

Stress
distressed
calm
worried
satisfied
content
tense
uneasy
indecisive
at ease
frightened
secure
dejected
nervous
jittery
comfortable
strained
relaxed

Arousal
sleepy
lively
tired
drousy
activated
passive
vigorous
active
calm
efficient
energetic
sluggish
confused

Tension
panicky
nervous
not calm
indecisive
relaxed
anxious
restless
tense
shaky

Depression
hopeless
discouraged
worthless
blue
sad
sorry for things doing
unhappy
lonely
miserable
gloomy
desperate
helpless
unworthy
terrified
guilty

Anger-hostility
resentful
annoyed
spiteful
peeved
jittery
angry
ready to fight
bad-tempered
rebellious
deceived
furious
bitter

Fatigue
tired
listless
bushed
exausted
sluggish
weary
worn out

Confusion
muddled
unable to concentrate
confused
forgetful
bewildered
efficient
uncertain about things

Vigour
cheerful
vigorous
active
lively
alert
full of pep
carefree
vigorous

Friendliness
friendly
clear-headed
considerate
heplful
sympathetic
trusting
good natured

Table 3. Body Composition in Rice Jelly Administration Study.

		Pre-intake	Intake			
			7-week	*P* [a]	12-week	*P* [a]
Fat mass (kg)	Placebo	16.3 ± 5.8	16.4 ± 5.7	0.705	16.2 ± 5.8	0.564
	LP14	15.4 ± 2.4	15.0 ± 3.3	0.358	15.5 ± 3.2	0.651
Muscle mass (kg)	Placebo	37.7 ± 5.7	37.7 ± 5.9	0.963	38.3 ± 5.8	0.078
	LP14	36.8 ± 2.9	37.1 ± 2.5	0.499	37.1 ± 2.4	0.270
Fat-free mass (kg)	Placebo	40.0 ± 6.0	40.0 ± 6.1	0.946	40.7 ± 6.1	0.084
	LP14	39.1 ± 3.0	39.3 ± 2.6	0.515	39.4 ± 2.5	0.270
Body weight (kg)	Placebo	56.4 ± 11.3	56.5 ± 11.3	0.771	56.8 ± 11.5	0.368
	LP14	54.4 ± 3.8	54.3 ± 4.7	0.875	55.0 ± 4.2	0.358
Body fat percentage (%)	Placebo	28.4 ± 4.7	28.5 ± 4.6	0.747	27.8 ± 4.7	0.058
	LP14	28.2 ± 3.5	27.3 ± 4.3	0.191	28.1 ± 4.3	0.855
BMI (kg/m^2)	Placebo	22.1 ± 3.2	22.1 ± 3.3	0.668	22.3 ± 3.3	0.326
	LP14	21.4 ± 1.2	21.4 ± 1.6	0.885	21.7 ± 1.3	0.387

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (unpaired t-test). [a] Comparisons with pre-intake were made by paired t-test.

Table 4. Blood Components in Rice Jelly Administration Study.

		Pre-intake	intake period			
			7-week	P [a]	12-week	P [a]
Neutrophil rate (%)	Placebo	54.5 ± 12.0	56.2 ± 12.1	0.635	58.5 ± 10.9	0.176
	LP14	54.4 ± 11.6	56.9 ± 10.1	0.335	55.7 ± 10.4	0.650
Eosinophil rate (%)	Placebo	3.50 ± 1.58	2.60 ± 0.97	0.041 *	2.60 ± 0.84	0.019 *
	LP14	2.18 ± 1.17	2.18 ± 1.54	1.000	2.64 ± 1.80	0.242
Basophil rate (%)	Placebo	0.40 ± 0.52	0.70 ± 0.48	0.081	0.70 ± 0.48	0.193
	LP14	0.36 ± 0.50	0.55 ± 0.52	0.167	0.64 ± 0.50	0.082
Granulocyte rate (%)	Placebo	58.4 ± 12.4	59.5 ± 12.3	0.753	61.8 ± 10.9	0.213
	LP14	56.9 ± 11.9	59.6 ± 10.6	0.300	59.0 ± 10.7	0.460
Monocyte rate (%)	Placebo	5.20 ± 0.42	4.80 ± 0.79	0.223	5.10 ± 0.57	0.678
	LP14	4.73 ± 1.49	5.55 ± 1.44	0.068	6.00 ± 2.49	0.099
Lymphocyte rate (%)	Placebo	36.4 ± 12.5	35.7 ± 12.1	0.832	33.1 ± 10.5	0.210
	LP14	38.4 ± 12.0	34.8 ± 10.2	0.183	35.0 ± 9.4	0.265
Granulocyte/lymphocyte (-)	Placebo	1.92 ± 1.08	2.02 ± 1.25	0.735	2.34 ± 1.79	0.257
	LP14	1.73 ± 0.88	1.89 ± 0.71	0.386	1.88 ± 0.84	0.559
Total cholesterol (mg/dl)	Placebo	173.4 ± 31.6	172.1 ± 33.3	0.876	183.7 ± 28.6	0.033 *
	LP14	191.8 ± 36.0	190.5 ± 30.5	0.793	195.7 ± 39.2	0.526
HDL-cholesterol (mg/dl)	Placebo	61.4 ± 8.3	61.8 ± 11.2	0.892	68.6 ± 7.8	0.014 *
	LP14	64.0 ± 13.7	66.7 ± 13.0	0.053	69.1 ± 12.8	0.003 **
LDL-cholesterol (mg/dl)	Placebo	97.4 ± 30.3	96.0 ± 30.6	0.838	102.4 ± 27.4	0.198
	LP14	112.4 ± 28.3	106.8 ± 23.1	0.258	108.0 ± 30.7	0.374
Triglyceride (mg/dl)	Placebo	66.3 ± 29.5	70.9 ± 50.2	0.672	58.1 ± 22.1	0.204
	LP14	65.9 ± 28.8	77.6 ± 31.2	0.148	94.3 ± 72.2	0.181
Free fatty acid (μEq/l)	Placebo	430.2 ± 168.9	548.7 ± 264.6	0.235	486.0 ± 157.5	0.449
	LP14	437.2 ± 121.6	600.5 ± 399.9	0.227	450.7 ± 174.3	0.822
CRP (mg/dl)	Placebo	0.036 ± 0.032	0.034 ± 0.024	0.780	0.034 ± 0.029	1.000
	LP14	0.033 ± 0.029	0.031 ± 0.020	1.000	0.034 ± 0.019	1.000
Total IgE (IU/ml)	Placebo	196.8 ± 202.5	188.8 ± 185.6	0.799	175.1 ± 178.6	0.022 *
	LP14	127.9 ± 135.2	107.7 ± 106.1	0.028 *	101.0 ± 101.0	0.013 *
Eosinophil counts (/μl)	Placebo	228.0 ± 120.4	157.0 ± 71.7	0.026 *	169.0 ± 87.4	0.013 *
	LP14	160.0 ± 130.8	143.6 ± 93.4	0.799	165.5 ± 129.3	0.930
Systolic blood pressure (mmHg)	Placebo	102.1 ± 12.3	99.9 ± 12.1	0.371	100.6 ± 10.1	0.455
	LP14	103.7 ± 8.6	101.8 ± 7.5	0.460	101.2 ± 11.2	0.368
Diastolic blood pressure (mmHg)	Placebo	64.7 ± 8.1	63.2 ± 7.9	0.210	64.4 ± 8.1	0.736
	LP14	67.1 ± 7.3	64.7 ± 7.8	0.414	65.5 ± 8.9	0.578
Pulse rate (/min)	Placebo	72.7 ± 9.6	72.0 ± 11.3	0.855	74.5 ± 10.1	0.227
	LP14	77.0 ± 15.3	75.2 ± 9.1	0.637	75.2 ± 11.8	0.698

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (unpaired t-test or Mann-Whitney U-test). [a] Comparisons with pre-intake were made by paired t-test or Wilcoxon t-test. * P<0.05; ** P<0.01.

Table 5. Change in Personal Recordings about Conditions of Stool and Abdomen and Appetite in Rice Jelly Administration Study.

	Intake						
	1-week	2-week	3-week	4-week	5-week	6-week	7-week
Frequency of defecation (times/week)	0.5 ± 2.1	0.5 ± 3.2	1.1 ± 2.5	−0.9 ± 3.4	−0.1 ± 2.6	−0.4 ± 2.6	0.5 ± 2.6
	0.3 ± 2.2	−0.5 ± 3.5	−0.7 ± 2.8	−0.2 ± 2.7	−0.4 ± 2.7	−0.1 ± 2.2	−0.9 ± 1.9
Fecal amount (number of stools/week)[a]	0.5 ± 5.5	1.1 ± 5.3	3.0 ± 7.1	−0.5 ± 6.2	0.5 ± 5.0	−0.9 ± 8.2	4.2 ± 12.0
	1.1 ± 3.6	0.3 ± 5.7	−1.7 ± 3.9	1.1 ± 5.4	1.2 ± 5.1	1.5 ± 5.9	0.8 ± 4.5
Ratio of appropriate fecal shape (%)	9.3 ± 19.2	7.1 ± 30.3	7.5 ± 36.1	26.0 ± 44.1	13.0 ± 33.2	12.2 ± 34.8	8.1 ± 42.7
	−0.2 ± 33.9	7.1 ± 23.2	−9.9 ± 31.4	5.9 ± 34.3	18.6 ± 23.8	14.1 ± 28.7	3.1 ± 39.8
Ratio of appropriate fecal color (%)	−0.5 ± 21.2	−7.2 ± 16.0	−4.4 ± 19.2	−8.2 ± 15.0	−5.2 ± 16.1	6.2 ± 33.5	0.7 ± 8.2
	7.4 ± 18.5	3.9 ± 43.0	−9.3 ± 31.3	−12.7 ± 26.5	2.5 ± 39.6	1.6 ± 33.9	3.1 ± 24.3
Ratio of appropriate fecal odor (%)	−1.4 ± 19.8	−4.3 ± 23.4	−18.7 ± 28.7	4.4 ± 40.6	−6.3 ± 34.3	−11.7 ± 31.4	−2.5 ± 24.0
	−8.2 ± 27.6	1.4 ± 37.5	−1.4 ± 22.2	1.1 ± 28.4	−24.7 ± 42.7	−11.2 ± 37.4	−12.3 ± 31.1
Ratio of appropriate sensation after defecation (%)	3.1 ± 24.2	20.0 ± 28.3	10.1 ± 31.4	23.1 ± 39.1	20.3 ± 21.5	13.2 ± 26.2	13.1 ± 28.7
	2.0 ± 37.9	0.2 ± 34.0	−13.3 ± 32.4	−13.5 ± 44.2	−1.7 ± 37.0	5.6 ± 28.2	4.3 ± 26.1
Ratio of appropriate abdominal condition (%)	−7.8 ± 35.8	2.6 ± 31.2	1.3 ± 19.6	7.8 ± 24.2	11.7 ± 28.4	1.3 ± 30.3	6.5 ± 30.2
	7.8 ± 33.4	−14.3 ± 41.9	−1.3 ± 32.9	1.3 ± 28.9	2.6 ± 27.0	−10.4 ± 30.7	−15.6 ± 37.0
Appetite score (−)	0.2 ± 0.4	0.0 ± 0.4	0.2 ± 0.3	0.1 ± 0.4	0.3 ± 0.6	0.0 ± 0.4	0.2 ± 0.4
	0.2 ± 0.6	0.1 ± 0.8	−0.2 ± 0.7	0.1 ± 0.7	0.5 ± 0.6	0.3 ± 0.5	0.4 ± 0.6

	Intake					*P* [a]		
	8-week	9-week	10-week	11-week	12-week	Intervention	Time	Interaction
Frequency of defecation (times/week)	0.7 ± 3.9	0.1 ± 2.2	0.5 ± 3.1	0.2 ± 2.7	−0.3 ± 2.6	0.202	0.997	0.959
	0.0 ± 2.7	0.3 ± 2.2	−0.4 ± 2.3	−0.2 ± 2.7	−0.1 ± 4.1			
Fecal amount (number of stools/week)[a]	−0.5 ± 7.7	0.7 ± 6.0	0.2 ± 7.9	0.7 ± 7.8	0.8 ± 6.5	0.587	0.995	0.706
	2.0 ± 5.2	3.5 ± 5.4	2.0 ± 6.1	1.5 ± 6.9	1.8 ± 6.4			
Ratio of appropriate fecal shape (%)	7.8 ± 36.2	2.0 ± 45.8	10.0 ± 31.9	14.9 ± 34.2	8.5 ± 38.8	0.285	0.854	0.983
	−5.6 ± 48.9	6.5 ± 37.4	4.9 ± 39.4	15.6 ± 44.4	9.3 ± 34.7			
Ratio of appropriate fecal color (%)	−4.9 ± 8.6	1.0 ± 14.8	−4.0 ± 13.6	−7.0 ± 16.1	0.2 ± 15.8	0.871	0.750	0.937
	−7.4 ± 19.8	−13.0 ± 37.6	−5.9 ± 20.5	−4.1 ± 36.1	−5.4 ± 26.4			
Ratio of appropriate fecal odor (%)	−14.6 ± 37.4	−11.6 ± 27.1	−7.6 ± 22.8	−16.2 ± 37.8	−5.1 ± 30.8	0.472	0.826	0.806
	−1.5 ± 31.4	−7.9 ± 33.2	3.0 ± 30.0	−3.2 ± 41.2	3.2 ± 25.3			
Ratio of appropriate sensation after defecation (%)	5.0 ± 41.8	10.4 ± 27.0	7.4 ± 27.8	13.0 ± 20.3	17.8 ± 29.9	0.003 **	0.961	0.741
	0.5 ± 30.9	3.8 ± 22.7	9.0 ± 41.4	11.9 ± 34.7	6.9 ± 38.3			
Ratio of appropriate abdominal condition (%)	−2.6 ± 31.2	−2.6 ± 32.5	2.6 ± 27.7	7.8 ± 41.6	7.8 ± 22.5	0.012 *	0.932	0.879
	−13.0 ± 30.3	−14.3 ± 42.9	−3.9 ± 25.6	−11.7 ± 46.9	−13.0 ± 35.8			
Appetite score (−)	0.0 ± 0.4	0.2 ± 0.5	0.1 ± 0.3	0.1 ± 0.2	0.0 ± 0.5	0.042 *	0.593	0.830
	0.4 ± 0.7	0.5 ± 0.9	0.2 ± 0.6	0.3 ± 0.5	0.3 ± 0.6			

Data are expressed as means ± SD. [a] Statistical analysis were performed by Two-way ANOVA. * $P<0.05$; ** $P<0.01$.

Table 6. Scores of Mood Questionnaire in Rice Jelly Administration Study.

	Score range		Pre-intake	Intake			
				7-week	P [a]	12-week	P [a]
State anxiety	0-60	Placebo	20.4 ± 8.7	25.1 ± 10.8	0.059	24.7 ± 9.6	0.217
		LP14	24.4 ± 7.6	27.4 ± 7.5	0.034 *	21.9 ± 14.1	0.626
Stress	0-17	Placebo	5.5 ± 2.6	7.2 ± 3.1	0.051	6.6 ± 2.6	0.325
		LP14	6.4 ± 1.8	7.9 ± 2.2	0.026 *	6.1 ± 4.2	0.799
Arousal	0-13	Placebo	5.4 ± 1.7	4.5 ± 1.9	0.142	5.2 ± 1.8	0.724
		LP14	5.8 ± 1.1	5.4 ± 1.2	0.366	6.2 ± 1.6	0.217
Tension	0-36	Placebo	10.7 ± 8.0	13.2 ± 6.3	0.185	11.8 ± 6.0	0.325
		LP14	14.2 ± 4.0	15.9 ± 6.3	0.217	13.1 ± 9.6	0.725
Depression	0-60	Placebo	10.2 ± 7.3	18.2 ± 11.7	0.021 *	11.4 ± 8.8	0.482
		LP14	12.4 ± 7.0	13.6 ± 9.4	0.724	12.2 ± 10.0	0.725
Anger-hostility	0-48	Placebo	6.7 ± 6.1	11.4 ± 10.1	0.182	5.8 ± 5.3	0.296
		LP14	7.0 ± 6.8	9.1 ± 12.6	0.648	8.2 ± 8.7	0.454
Fatigue	0-28	Placebo	10.5 ± 5.7	13.6 ± 6.9	0.358	9.6 ± 3.9	1.000
		LP14	7.3 ± 3.6	9.7 ± 5.2	0.155	9.8 ± 6.6	0.271
Confusion	0-28	Placebo	9.5 ± 5.5	11.8 ± 4.9	0.059	9.9 ± 5.6	0.454
		LP14	10.2 ± 4.7	12.0 ± 4.6	0.244	9.9 ± 4.2	0.879
Vigour	0-32	Placebo	11.2 ± 7.0	9.2 ± 8.2	0.296	9.6 ± 7.1	0.659
		LP14	10.4 ± 4.8	11.6 ± 5.3	0.398	14.5 ± 7.1	0.019 *
Friendliness	0-28	Placebo	15.3 ± 4.7	13.1 ± 5.6	0.042 *	13.1 ± 4.5	0.069
		LP14	15.0 ± 3.4	14.3 ± 3.1	0.538	15.4 ± 5.2	1.000

Data are expressed as means ± SD. No significant differences were observed between the two groups in these measurements (Mann-Whitney U-test). [a] Comparisons with pre-intake were made by Wilcoxon t-test. * P<0.05.

Table 7. Questionnaire after Study about Alteration in Body Temperature, Appetite and Blinding in Rice Jelly Administration Study.

Q1. The question how the subjects felt the body temperature change during a sample intake period?

	Placebo	LP14
I continually felt feverish.	0	0
I felt warmer than usual.	1	1
I did not feel a change in body temperature.	10	10
I felt a little colder than usual.	0	0
I felt considerably colder than usual.	0	0
	P=0.968	

Q2. The question how the subjects felt the appetite during a sample intake period?

	Placebo	LP14
Increased	0	1
Increased a little	3	3
Not changed	8	7
Decreased a little	0	0
Decreased	0	0
	P=0.997	

Q3. The question which sample the subjects believed they had received?

	Placebo	LP14
Test sample	2	4
Placebo	3	2
Do not know	6	5
Accuracy rate (%)	27.3	36.4
Wrong rate (%)	18.2	18.2
	P=0.882	

Statistical analysis were performed by chi-square test with Yates correction for continuity for an m×n contingency table.

Table 8. The Number of LP14 Released from Jelly by Digestive Process.

log (cells)		Gellan gum	Gelling agent A
Stomachere processing		8.7 ± 0.02	9.7 ± 0.13
Homogenization		8.8 ± 0.01	10.2 ± 0.02
	4 mm	9.0 ± 0.03	9.6 ± 0.05
Sieve	1 mm	9.4 ± 0.02	9.9 ± 0.01
	150 μm	9.6 ± 0.02	10.2 ± 0.01
			Total: 10.5 log (cells)

Each value represents means ± SD of triplicate determinations.

Table 9. Search Results in Pubmed of Gelling Agent × Immunity Keyword.

	Carrageenan 7446	Gellan gum 279	Locust bean gum 318	Xanthan gum 574
Immune	344	2	1	4
TLR	7	0	0	1
Cytokine	528	0	0	3
Antitumor	76	0	0	1
Inflamation	3036	1	1	3
Lymphocyte	392	1	2	2

(February 25, 2011)

Fig. 1. Rice Jelly.

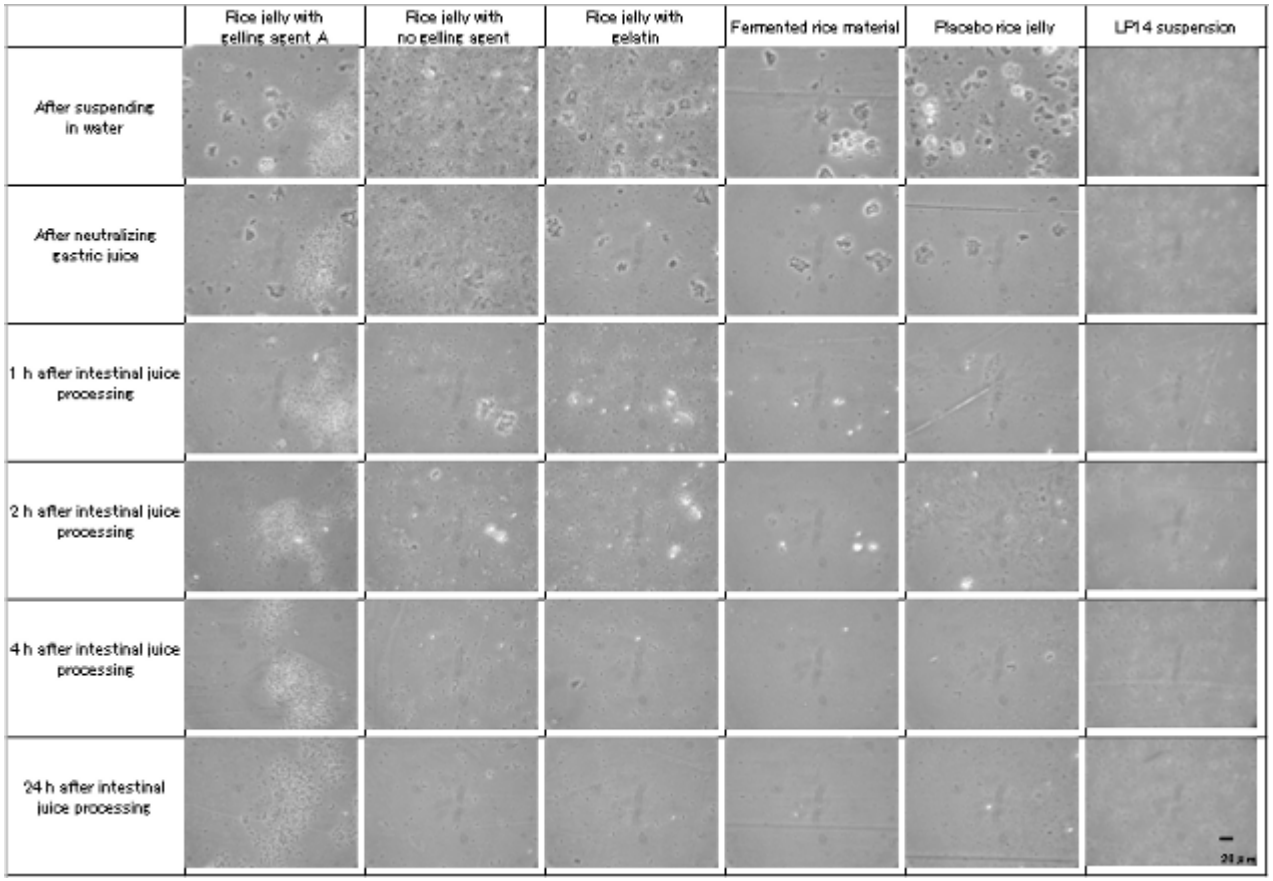

Fig. 2a Microphotographs of the Digestive Process of Rice Jellies (×400).

About “after suspending in water” and “after neutralizing gastric juice”, their jelly concentration was equally adjusted to that “after intestinal juice processing” with water. Fermented rice material was mixed at the rate contained in rice jelly.

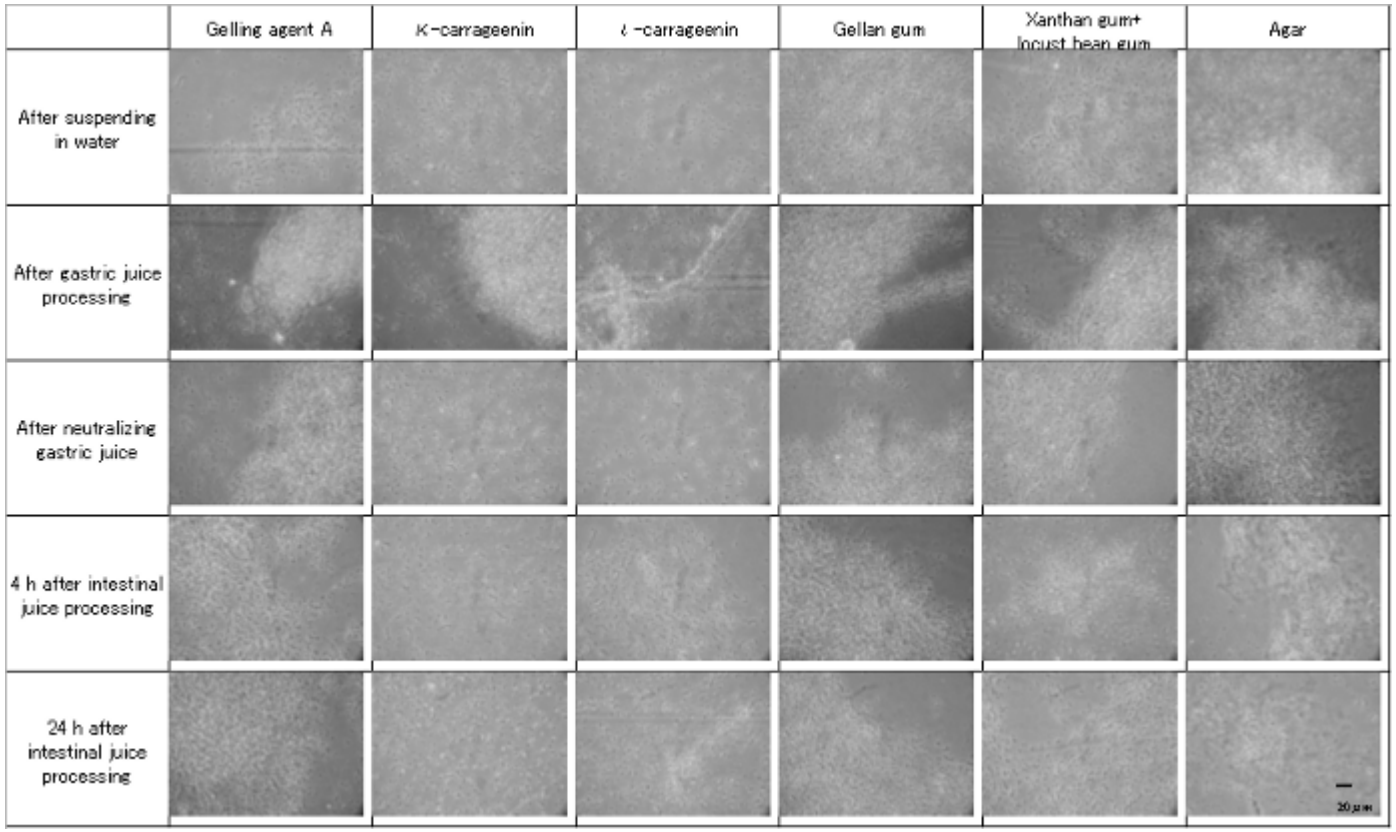

Fig. 2b Microphotographs of the Digestive Process of Jellies which Gelled LP14 Suspension with Ethanol Precipitation (×400).

About “after suspending in water” and “after neutralizing gastric juice”, their jelly concentration was equally adjusted to that “after intestinal juice processing” with water. “After gastric juice processing” was not diluted.

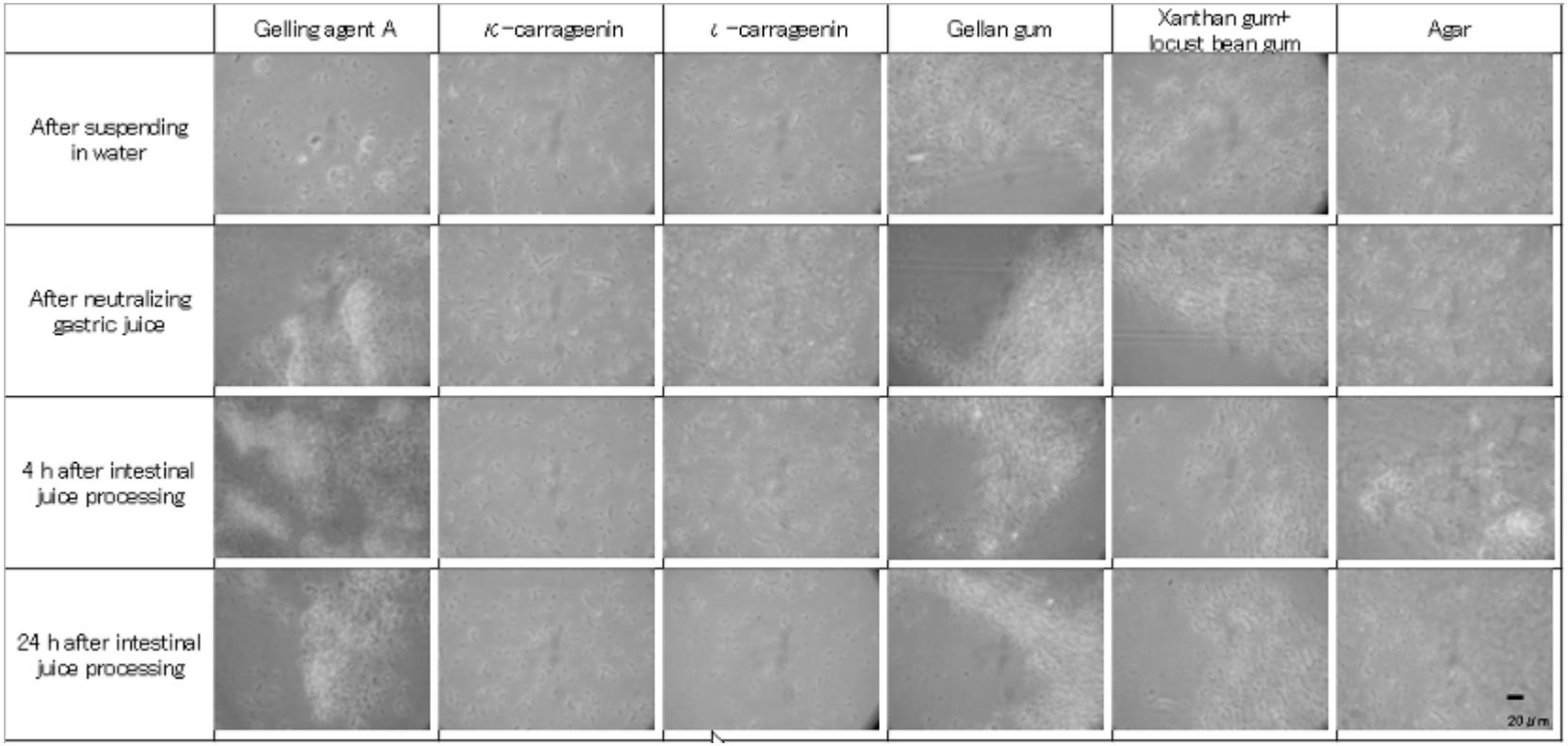

Fig. 2c Microphotographs of the Digestive Process of Jellies which Gelled LP14 Suspension without Ethanol Precipitation (×400).

About “after suspending in water” and “after neutralizing gastric juice”, their jelly concentration was equally adjusted to that “after intestinal juice processing” with water.

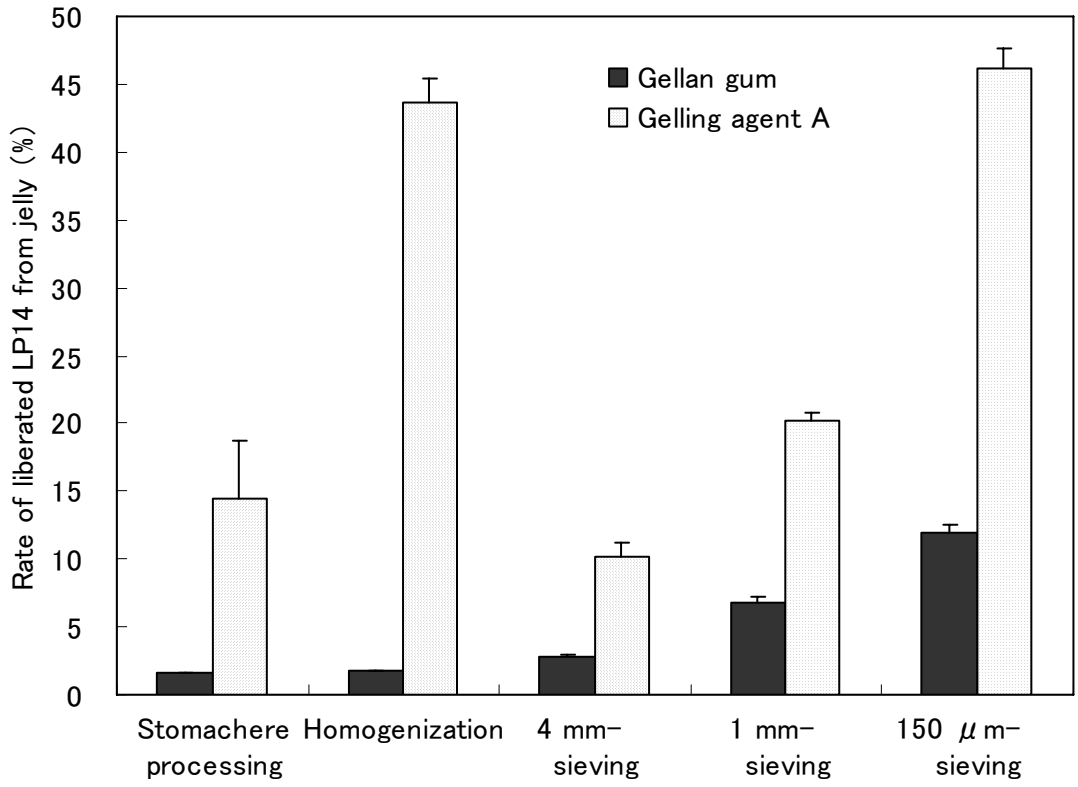

Fig. 3. Number of LP14 Liberated from Jelly after Digestive Process.

Each value represents means ± SD of triplicate determinations.

Conclusion

Lactobacillus plantarum No.14 (LP14) was isolated from pickled shallots, a traditional Japanese food, and has long been used in food processing. A placebo-controlled, double-blind study was conducted in 2005.[7] It was suggested that LP14 was useful lactic acid bacteria which improved both allergy and overweight. LP14 was the first reported lactic acid bacterium that decreased body fat percentage in humans.

These effects of LP14 were investigated further in this work. The administration studies were conducted on allergy in spring and autumn. The gastrointestinal tolerance of LP14 was evaluated *in vitro.* Body fat percentage was assumed to decrease because LP14 induced thermogenesis, and the effect of LP14 on human body temperature was measured in two administration studies. The effect of intragastric injection of LP14 on sympathetic nerve activity innervating the brown adipose tissue (BAT-SNA) of rats was examined. A novel fermented food including LP14 was developed based on above research: a kind of jelly packing fermented rice powder employing a gelling agent. The effects of the intake of the rice jelly on a body fat, allergy were examined. In addition, the properties of the jelly were observed in artificial digestive juices *in vitro*. The number of cells of LP14 released from the jelly was counted after digestion.

Chapter 1: Improvements in Seasonal Allergic Disease with LP 14

Two randomized, placebo-controlled, double-blind studies were conducted in female students with seasonal allergic diseases in spring and autumn. For subjects who took 8.7×10^8 CFU of living LP14 cultured in Rogosa medium that fructose was used as carbon source, a significant improvement in ocular symptom-medication score was observed in the spring study. In the placebo group, the T helper type 1 (Th1)/T helper type 2 (Th2) ratio tended to decrease after a 6-week intake period, while in the LP14 group, the percentage of Th1 cells significantly increased. Post-intake eosinophil counts significantly increased in comparison to those at intake cessation in the placebo group, but it appeared to be suppressed in the LP14 group. There were no changes in fecal microflora. In the autumn study, there were no significant differences, but the nasal and

the ocular symptom score in the LP14 group were lower than those in placebo group.

These studies indicate the clinical effects of LP14 on seasonal allergic diseases. LP14 might behave as an effective counter-regulator of Th2-skewed immunity. Some lactic acid bacteria are thought to be one of the tools for establishing a Th1 predominance through changes in the composition of the host's intestinal microflora by their affects or by their direct action towards gut-associated lymphoid tissue (GALT). No significant change in fecal microflora was observed in the LP14 group. The underlying mechanism might be explained by direct action on the host systemic immune system.

Chapter 2: Gastrointestinal Transit Tolerance of LP14

Transit tolerance was determined at 37°C against simulated gastric juices at pH values of 2.5, 3.0 and 3.5, and against simulated small intestinal juices containing 0%, 0.2% or 0.4% oxgall. LP14 obtained from the culture with glucose had high gastrointestinal transit tolerance, but that with fructose had low tolerance. Hence the amounts of exopolysaccharide (EPS) from LP14 cultured in various carbon sources were compared. The EPS levels were 146.5± 8.1 mg/l (culture) with glucose, and 20.1±17.0 mg/l (culture) with fructose. When EPS was removed by centrifugation, the simulated gastric tolerance of LP14 cultured with glucose decreased markedly, but that with fructose did not decrease.

These results suggested that the simulated gastrointestinal transit tolerance of LP14 varied with the carbon source of fermentation. The gastrointestinal transit tolerance of LP14 related to EPS contents. Because the living LP14 was considered to insufficient tolerance for gastrointestinal transit under the conditions of the sample preparation in Chapter 1, it might affect humans also in the state of being killed.

Chapter 3: Thermogenesis by LP14

The effect of LP14 on human body temperature was measured in 4-week administration study and single-administration study. The 4-week administration study had a randomized, placebo-controlled, double-blind design. The intervention group

received one killed LP14 capsule (1.8×10^{10} CFU). The basal body temperature increased significantly only in the LP14 group over the intake period. The average increase in body temperature was 0.019±0.114°C in the placebo group, and 0.036±0.070°C in the LP14 group. The single-administration study had a randomized, double-blind, cross-over design. The sample capsules were filled with 0.2 g (1.6×10^{11} CFU) of killed LP14. The chest temperature, blood pressure and task performance were significantly higher after LP14 ingestion than after placebo ingestion, and the arousal score tended to be higher. The average increase in chest temperature was 0.10±0.32°C in the placebo group and 0.24±0.32°C in the LP14 group. The effect of LP14 was examined on BAT-SNA in rats. Killed LP14 (9.7×10^{8} CFU) or water was administered through a gastric cannula. LP14 enhanced the BAT-SNA in rats.

These results demonstrated that killed LP14 induced thermogenesis in human. Nagata *et al.* reported that LP14 induced gene expression of an endogenous pyrogen, *IL-1β*.[20] It was considered that LP14 decreased the body fat percentage by a mechanism in which LP14 induces *IL-1β*, activates the sympathetic nerve, and results in thermogenesis.

Chapter 4: Influences of the Intake of a Processed Food Containing LP14 on Several Clinical Parameters

The intervention study was conducted under the design being randomized, placebo-controlled and double-blinded. The intervention group received one rice jelly (LP14, 2.5×10^{11} CFU/50 g), and the placebo group received one placebo jelly every day for 12 weeks. No effects of LP14 were observed on the body composition or allergy, which was considered to be suppressed by the presence of food components or the matrix of gel. The properties of the jelly were observed in artificial digestive juices under a microscope (×400). The amoeba-like gel was recognized to wrap most of LP14 in rice jelly. Amoeba-like gel wrapping LP14 remained after intestinal juice digestion. The number of cells of LP14 released from the jelly was counted after digestion. The jelly after being swallowed was considered to be crushed to a same extent in the particle size with Stomachere processing. In the jelly crushed by Stomachere, 14 % of LP14 was

liberated after digestion.

In conclusion, the rice jelly was ineffective to body composition or allergy in spite of increased intake amount of LP14. It was considered that most cells of LP14 in the rice jelly were buried in gel matrix, leading to excretion. LP14 was considered to affect human immune cells of an intestinal tract. Therefore, LP14 buried in gel matrix could not affect humans. The problem became clear about processing LP 14 into foods.

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