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Improving Effect of Acetic Acid Bacteria (*Gluconacetobacter hansenii* GK-1) on sIgA and Physical Conditions in Healthy People: Double-Blinded Placebo-Controlled Study

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Abstract

It is known that the consumption of bacteria such as lactobacilli and bifidobacteria has beneficial effects on human immune function. Most of them are Gram-positive bacteria, and there are few reports on Gram-negative bacteria. In this study, we evaluated the effects of intake of *Gluconacetobacter hansenii* GK-1 (GK-1), Gram-negative acetic acid bacteria, for 12 weeks on physical condition and immune indices. We conducted a randomized, double-blind, placebo-controlled, parallel-group study in 100 healthy adults. The subjects were randomized into the GK-1 and the placebo groups. The diary-administered physical condition survey was conducted during the study period. The evaluation of salivary sIgA levels, NK-cell activity, and serum IFN-γ levels and quality of life survey was conducted before, in 6 weeks, and 12 weeks after the start of ingestion. Based on the physical condition survey, the cumulative onset-days of symptoms were significantly suppressed in the GK-1 group compared to the placebo group regarding the evaluation of 13 symptoms related to immunity, every 3 weeks. Additionally, salivary sIgA levels per hour were significantly increased in the GK-1 group compared with the placebo group at 6 and 12 weeks. Despite no significant differences in the NK-cell activity, serum IFN-γ levels or quality of life survey between the groups. Serum IFN-γ levels in the GK-1 group were significantly elevated at 12 weeks after the start of ingestion compared with those before ingestion. In conclusion, intake of GK-1 was shown to increase salivary sIgA levels and improve physical condition. This suggested that oral intake of GK-1 may help maintain the immune system.

Keywords

Acetic Acid Bacteria, Gram-Negative Bacteria, *Gluconacetobacter hansenii* GK-1 (GK-1), Functional Food, Immune

1. Introduction

Recently, the maintenance of immunity using probiotics [1] has been noticed; moreover, there have been many reports on lactic acid bacteria [2] [3], bifidobacteria [4], and natto bacteria [5] etc. Probiotics also act on killed bacteria, and it has been assumed the peptidoglycan and nucleotides in the bacterial cells stimulate intestinal immune cells to help maintain immunity [6]. Lactic acid and natto bacteria are commonly found in fermented foods, such as yogurt and natto, but acetic acid bacteria can also be found in the same fermentation bacteria. Acetic acid bacteria are gram-negative bacteria that are widely found in nature as indigenous bacteria and have been used for fermentation production of vinegar for a long time. These bacteria can be found in rice vinegar, Caspian Sea yogurt, natadecoco, and other foods, and they represent microorganisms with food experience [7]. Lactic acid bacteria and bifidobacteria are Gram-positive bacteria. However acetic acid bacteria are gram-negative bacteria, which differ in properties from gram-positive bacteria, including lipopolysaccharide (LPS) [8].

In an animal experiment using pollinosis model, it has been confirmed that acetic acid bacteria have an allergy depressing effect, and it was indicated that LPS's action through Toll-like receptor (TLR)-4 is implicated in this effect [9]. *Gluconacetobacter hansenii* GK-1 (GK-1), a type of acetic acid bacteria, has also been shown to reduce nasal dis-comfort in humans caused by pollen and house dust [10] [11]. Furthermore, GK-1 has been shown to improve the activity of blood natural killer (NK)-cell and synthesis of immunoglobulin (Ig)A and interferon (IFN)-α in a cellular study; moreover, it is expected to have an immunostimulatory function [12]. There have been several reports on the effects of gram-positive bacteria, such as lactobacilli, on human body condition and immune indicators; however, there have been few reports on gram-negative bacteria.

Therefore, we conducted a randomized, double-blind, placebo-controlled, parallel-group study over 12 weeks in healthy Japanese adults to investigate the effect of continuous oral GK-1 ingestion on subjective assessment of physical condition and individual immune indicators.

2. Material and Methods

2.1. Study Design

The study was conducted under the supervision of physicians between December 2020 and May 2021 at EP Mediate Co., Ltd., a clinical laboratory. Subjects were randomly divided into two groups using a random number table, placebo

and GK-1 groups, in a 12 week, randomized, double-blind, placebo-controlled, parallel-group study. The allocation personnel not involved in the laboratory tests were randomly assigned to two groups using a random number table to ensure the absence of bias in sex, age, or body mass index (BMI) in the pretest. The allocation information was securely stored from the allocation until the end of the study. Moreover, subjects and interventions and outcome evaluators were all blinded. One capsule was consumed daily for 12 weeks in both the placebo and GK-1 groups. A diary was recorded every day during the ingestion period. Blood tests, saliva tests, MOS 36-Item Short-Form Health Survey (SF-36) [13] [14], POMS2 short version for adults [15], Oguri-Shirakawa-Azumi sleep inventory MA version (OSA-MA) [16] and PSQI [17] [18] as quality of life (QOL) surveys, and physician interviews were performed before, in 6 weeks, and 12 weeks after the ingestion. The study was conducted after obtaining approval from the Ethics Review Committee of Medical station Clinic in accordance with the spirit of the Declaration of Helsinki (approval number: 20000022, approval date: 10 December 2020). Also, the study protocol was pre-registered in the Clinical Trials Registration System (UMIN-CTR) (UMIN 000042948).

2.2. Test Foods and Intakes

The acetic acid bacteria used in this study were *Gluconacetobacter hansenii* GK-1 (Kewpie Co., Ltd, Tokyo, Japan). "GK-1 capsule" containing 1.5×10^{10} GK-1 per grain was used as the test food, and "GK-1 unformulated capsule" was used as a placebo. Placebo contents comprised dextrin (Matsuya Chemical Co., Ltd., Tokyo, Japan) that did not influence the immunity or physical condition. Test foods and placebo represented capsule shaped supplements manufactured by Aliment Industries, Ltd. (Yamanashi, Japan), and the taste, appearance, and nutrient composition were adjusted to be equivalent. **Table 1** demonstrates the nutritional content of each test food.

2.3. Subjects

The subjects comprised 100 healthy Japanese adult males and females aged 20 to 64 years who were aware of their susceptibility to colds, had low scores according to POMS2 short version for adults [15] and PSQI [17] [18], and were judged as not sick by a doctor. The main exclusion criteria consisted of those who

Table 1. Nutrition facts of each test sample (/1 capsule).

		Placebo	GK-1
Energy	(kcal)	1.0	1.0
Protein	(g)	<0.1	<0.1
Fat	(g)	<0.1	<0.1
Carbohydrates	(g)	0.2	0.2
Salt equivalent	(g)	0.0	0.0

routinely consumed foods with high acetic acid bacteria and lactobacilli content,those who consumed medicinal products or healthy foods with a potential effect on the immunity, those who were allergic to drugs and foods, those who engaged in day/night shifts or physical work, those with strenuous exercise habits, those with serious illness or disease under treatment, those who were possibly pregnant, pregnant, or breastfeeding, and those who were deemed inappropriate as a subject by the investigator. The number of subjects included 100 in two groups of 50, based on the number of reported cases in human studies examining the effects of Lactobacillus intake on immune function [19] and a report comparing the immunoreactivity of *Lactobacillus* and *Acetobacillus* in cell studies [12]. The investigator conducted the study after fully explaining the purpose and content of the study to the subjects and obtaining written informed consent based on the subjects' free will.

2.4. Outcomes

A diary-administered physical condition survey was distributed as the primary endpoint. Immune indicators (salivary secretory immunoglobulin (sIg)A levels, NK-cell activity, and serum IFN-γ levels) were measured as secondary endpoints. Additionally, SF-36 [13] [14], POMS2 short version for adults [15], OSA-MA [16], PSQI [17] [18]) was conducted as QOL surveys.

2.4.1. Diary-Based Physical Condition Survey

A physical condition survey was administered daily in a diary form during the intake period. Questionnaire items comprised 1) runny nose, 2) plugged nose, 3) sneezing, 4) sore throat, 5) cough, 6) hoarseness, 7) general malaise, 8) arthralgia, 9) phlegm accumulation, 10) chills, 11) fever, 12) feverishness, 13) fatigue, and 1)-13) cumulative onsetdays were compared and assessed between the two groups for each item. Subjects reported at least one of the 1)-13) symptoms on the day of onset, and cumulative onset-days every 3 weeks were compared between the two groups to assess overall clinical manifestations. The 9)-13) symptoms comprehensively assessed systemic subjective symptoms. The 1)-8) symptoms comprehensively assessed subjective symptoms at specific sites. Responses were documented using an 8-point Likert scale questionnaire ranging from normal (0) and slight (1) to severe (7).

Additionally, the presence or absence of changes in physical condition was evaluated daily during the ingestion period in two phases: "with change" and "without change."

2.4.2. Immunological Indicators (Salivary sIgA Levels, NK-Cell Activity, and Serum IFN-γ Levels)

The salivary examination was conducted before, in 6 weeks, and 12 weeks after the ingestion. Salivary sIgA levels were measured by enzyme-linked immunoassay (ELISA) method (SALIVARY SECRETORY IgA, SALIMETRICS LLC, USA) and combined with the volume of saliva secreted per unit of time to calculate the

level of salivary sIgA per unit of time. Blood NK-cell activity was evaluated by ^{51}Cr release method, and serum IFN-γ was also measured by the ELISA method (AuthentiKine™ Human IFN-γ Measure ELISA Kit. PROTEINTECH Group Inc, Japan *et al*.).

2.4.3. QOL Survey

QOL survey, in 6 weeks, and 12 weeks after the ingestion was performed. The short version for SF-36, POMS2 short version for adults, OSA-MA, and PSQI were used for the evaluation.

2.5. Safety Assessment

Anthropometric (height (pretest only), weight, and BMI) and physical assessments (systolic blood pressure, diastolic blood pressure, and pulse) were performed before and 12 weeks after the ingestion. Additionally, hematology tests (white blood cell count, red blood cell count, hemoglobin, hematocrit, and platelet count), blood biochemistry (total protein, albumin, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LD), γ-glutamyl transpeptidase (GTP), total cholesterol, triglycerides, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, urea nitrogen, creatinine, uric acid, Na, K, Cl, blood glucose, glycated hemoglobin (HbA1c; pretest only), and urinalysis (protein, glucose, and occult blood) were performed under fasting for more than 8 hours. Blood tests and urinalysis measurements were commissioned to BE-EL, Inc. (Saitama, Japan).

2.6. Statistical Analysis

Among the diary-administered physical condition surveys, the aggregation of the cumulative number of days was performed for the 13-item symptoms in the two categories of no symptom (normal, slight: 0-1) and symptom+ (mild, moderate, severe: 2-7), and the χ^2 test was performed. The cumulative number of days with and without changes in physical condition was tabulated, and the χ^2 test was performed. Salivary tests (salivary sIgA levels and salivary secretions per hour), NK-cell activity, and serum IFN-γ levels were compared between the GK-1 and the placebo group at each test time point by unpaired t-test. Moreover, in each group, pre-, 6 weeks post-, and 12 weeks post-ingestion measures were evaluated using the Dunnet test. SF-36, POMS2 short version for adults, OSA-MA, and PSQI compared the GK-1 and the placebo group treated measures at each time point by Mann-Whitney's U-test. In the safety assessment, pre- and 12-week post-ingestion measurements were compared by unpaired t-test. The test results are presented as the mean ± standard error (SE) of the mean, and a hazard ratio of less than 5% was judged to be significantly different. Statistical analysis was performed using computer software IBM SPSS Statistics 27.0 (manufactured by Nippon IM Co., Ltd.).

3. Results

3.1. Subjects Characteristics

During the study period, two individuals dropped out for personal reasons. Moreover, 3 people who were not healthy subjects (2 subjects who were judged to have degree 2 obesity according to the criteria of the Japanese Society of Obesity [20] and 1 subject who had repeated positive urine occult blood before and after the ingestion, in whom disease suspicion could not be denied) were excluded, and 95 individuals were evaluated as the analysis objects. Figure 1 demonstrates the selection flow of patients for analysis, and Table 2 presents the patients' backgrounds. There were no differences in both the placebo and GK-1 groups in each item, such as gender and age. Moreover, the participants' sample intake rate was 99.8%.

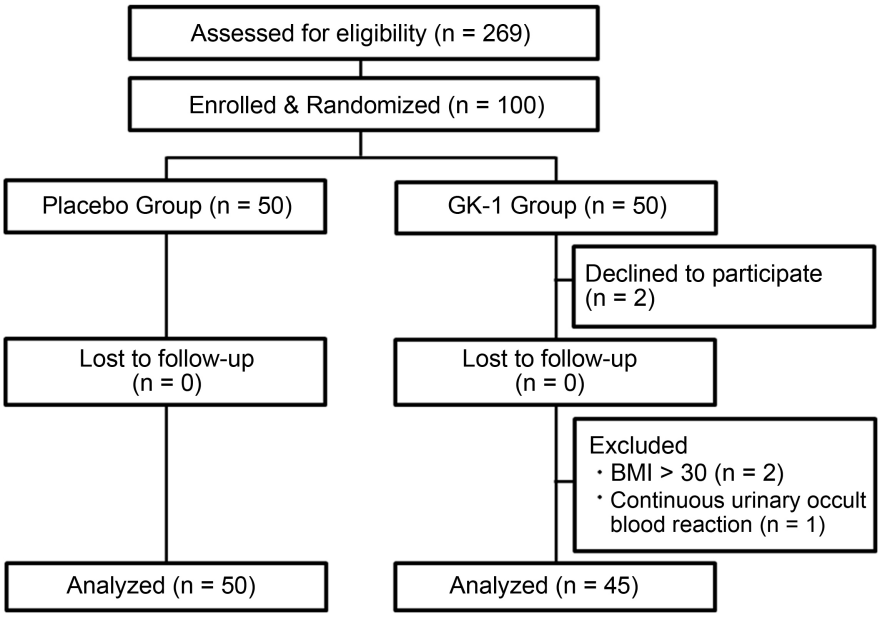

Figure 1. Example flowchart of selecting study subjects.

Table 2. Background values of the subjects analyzed.

	Placebo group	GK-1 group	*p*-value
Number of subjects (Male: Female)	50 (23:27)	48 (19:26)	0.713
Age (y)	45.1 ± 1.5	44.6 ± 1.7	0.823
Height (cm)	165.2 ± 1.2	165.9 ± 1.4	0.695
Body weight (kg)	59.2 ± 1.4	60.5 ± 1.9	0.561
BMI	21.6 ± 0.4	21.8 ± 0.4	0.807
Systolic blood pressure (mmHg)	117.7 ± 2.3	116.4 ± 2.2	0.690
Diastolic blood pressure (mmHg)	72.7 ± 1.7	71.5 ± 1.6	0.609

Values are represented as mean ± SE. p-values were analyzed as unpaired t-test vs. placebo group.

3.2. Diary-Based Physical Condition Survey

Table 3 demonstrates the cumulative number of days for each symptom from the start of the ingestion to 12 weeks after the ingestion. The cumulative on-set-days in runny nose, nasal stuffiness, cough, general malaise, and fatigue were

Table 3. Cumulative number of days of each symptoms with physical condition questionnaire.

	Group	No symptom (Normal, Slight)	Symptom+ (Mild, Moderate, Severe)	Chi-square test *p*-value
Runny nose	Placebo	4051	147	0.005**
	GK-1	3690	92	
Plugged nose	Placebo	4109	89	<0.001**
	GK-1	3748	34	
Sneezing	Placebo	4108	90	0.169
	GK-1	3717	65	
Sore throat	Placebo	4164	34	0.858
	GK-1	3750	32	
Cough	Placebo	4141	57	0.040*
	GK-1	3749	33	
Hoarseness	Placebo	4190	8	0.834
	GK-1	3774	8	
General malaise	Placebo	3977	221	<0.001**
	GK-1	3711	71	
Arthralgia	Placebo	4175	23	0.965
	GK-1	3761	21	
Phlegm accumulation	Placebo	4173	25	<0.001**
	GK-1	3705	77	
Chill	Placebo	4172	26	0.931
	GK-1	3758	24	
Fever	Placebo	4194	4	0.016*
	GK-1	3769	13	
Feverishness	Placebo	4184	14	0.008**
	GK-1	3753	29	
Fatigue	Placebo	3562	636	<0.001**
	GK-1	3498	284	

In the placebo group, n value was 4198 (47 subjects × 84 days and 3 subjects × 76 - 91 days because of missing values and excess days). In the placebo group, n value was 3782 (41 subjects × 84 days and 4 subjects × 81 - 91 days because of missing values and excess days). *$p < 0.05$, **$p < 0.01$ by Chi-square test.

significantly lower in the GK-1 group compared with the placebo group ($p < 0.01$, $p < 0.001$, $p < 0.05$, $p < 0.001$, and $p < 0.001$). However, the GK-1 group had significantly higher cumulative onset-days than the placebo group in phlegm accumulation, fever, and feverish ($p < 0.001$, $p < 0.05$, and $p < 0.01$). Considering the other five items, there was no significant difference between the two groups.

Table 4 demonstrates the cumulative incidence of overall clinical symptoms every 3 weeks. In weeks 7 - 9 and weeks 10 - 12, the GK-1 group had a significantly lower number of cumulative onset-day of the overall clinical condition than the placebo group ($p < 0.001$).

Table 5 shows the cumulative incidence of systemic subjective symptoms

Table 4. The cumulative number of days of overall clinical symptoms per 3 weeks.

Week	Group	Symptom, −	Symptom, +	Chi-square test *p*-value
1 - 3	Placebo	875	175	0.543
	GK-1	797	148	
4 - 6	Placebo	836	214	0.065
	GK-1	783	162	
7 - 9	Placebo	834	216	<0.001**
	GK-1	843	102	
10 - 12	Placebo	843	198	<0.001**
	GK-1	868	71	

In weeks 1 - 3, 4 - 6, and 7 - 9, n value was 1050 (50 subjects × 21 days) in the placebo group and 945 (45 subjects × 21 days) in the GK-1 group. The missing values for weeks 10 - 12 were n = 1041 for the placebo group and n = 939 for the GK-1 group. **$p < 0.01$ by chi-square test.

Table 5. Cumulative number of days of onset of systemic subjective symptoms every 3 weeks.

Week	Group	Symptom, −	Symptom, +	Chi-square test *p*-value
1 - 3	Placebo	893	157	0.014*
	GK-1	839	106	
4 - 6	Placebo	904	146	0.282
	GK-1	829	126	
7 - 9	Placebo	864	186	<0.001**
	GK-1	899	46	
10 - 12	Placebo	862	179	<0.001**
	GK-1	898	41	

In weeks 1 - 3, 4 - 6, and 7 - 9, n value was 1050 (50 subjects × 21 days) in the placebo group and 945 (45 subjects × 21 days) in the GK-1group. The missing values for weeks 10 - 12 were n = 1041 for the placebo group and n = 939 for the GK-1 group. *$p < 0.05$, **$p < 0.01$ by chi-square test.

every 3 weeks up to 3 weeks after the ingestion. In weeks 1 - 3, weeks 7 - 9, and weeks 10 - 12, the GK-1 group had a significantly lower number of cumulative onset-days of overall subjective symptoms than the placebo group ($p < 0.001$).

Table 6 presents the cumulative onset-days of subjective symptoms at specific sites every 3 weeks. In weeks 1 - 3, weeks 7 - 9, and weeks 10 - 12, the placebo group had a significantly lower number of cumulative days of subjective symptoms at particular sites than the GK-1 group ($p = 0.001$), whereas the GK-1 group had a significantly lower number of cumulative onset-days than the placebo group ($p = 0.008$) in weeks 10 - 12.

The cumulative number of days with and without changes in physical condition was significantly lower in the GK-1 group than in the placebo group (Table 7, $p < 0.001$).

3.3. Immunological Indicators (Salivary sIgA Levels, NK-Cell Activity, and Serum IFN-γ Levels)

Table 8 demonstrates the values of immune indicators before, in 6 weeks after, and 12 weeks after the ingestion. Considering the salivary sIgA level per hour,

Table 6. Cumulative number of days of subjective symptoms at specific sites per 3 weeks.

Week	Group	Symptom, −	Symptom, +	Chi-square test *p*-value
1 - 3	Placebo	1016	34	0.001**
	GK-1	885	60	
4 - 6	Placebo	954	96	0.654
	GK-1	864	81	
7 - 9	Placebo	993	57	0.149
	GK-1	879	66	
10 - 12	Placebo	975	66	0.008*
	GK-1	904	35	

In weeks 1 - 3, 4 - 6, and 7 - 9, n value was 1050 (50 subjects × 21 days) in the placebo group and 945 (45 subjects × 21 days) in the GK-1group. The missing values for weeks 10 - 12 were n = 1041 for the placebo group and n = 939 for the GK-1 group. *$p < 0.05$, **$p < 0.01$ by chi-square test.

Table 7. Changes in physical condition.

Group	Not changed	Changed	Chi-square test *p*-value
Placebo	3947	251	<0.001**
GK-1	3665	117	

In the placebo group, n value was 4198 (47 subjects × 84 days and 3 subjects × 76 - 91 days because of missing values and excess days). In the GK-1 group, n value was 3782 (41 subjects × 84 days and 4 subjects × 81 - 91 days because of missing values and excess days). **$p < 0.01$ by chi-square test.

Table 8. Changes in immune index.

	Group	Baseline	*p*-value[a]	After 6 weeks	*p*-value[b]	*p*-value[a]	After 12 weeks	*p*-value[b]	*p*-value[a]
Saliva flow rate (mL/min)	Placebo	0.95 ± 0.03	0.697	0.97 ± 0.04	>0.050	0.479	0.96 ± 0.03	>0.050	0.223
	GK-1	0.97 ± 0.03		1.01 ± 0.03	>0.050		1.01 ± 0.03	>0.050	
SIgA concentration (μg/mL)	Placebo	188.8 ± 20.2	0.367	195.2 ± 18.6	>0.050	0.219	211.4 ± 20.2	>0.050	0.192
	GK-1	213.1 ± 17.2		228.5 ± 19.5	>0.050		249.7 ± 21.0	0.010†	
SIgA secretion rate (μg/min)	Placebo	162.3 ± 11.8	0.057	170.9 ± 11.5	>0.050	0.012*	190.9 ± 15.0	0.015†	0.027*
	GK-1	198.6 ± 15.0		223.0 ± 17.4	>0.050		245.9 ± 19.7	0.005††	
NK-cellactivity	Placebo	59.8 ± 2.4	0.766	62.4 ± 2.4	>0.050	0.680	62.6 ± 2.4	>0.050	0.983
	GK-1	58.8 ± 2.7		63.8 ± 2.4	0.002††		62.6 ± 3.0	0.024†	
selm IFN-γ levels	Placebo	170.7 ± 30.4	0.015*	215.0 ± 42.3	>0.050	0.084	168.5 ± 32.4	>0.050	0.741
	GK-1	85.5 ± 15.7		128.3 ± 25.6	>0.050		154.1 ± 28.4	0.007††	

Values are represented as mean ± SE. a p-value was analyzed with unpaired t-test vs. placebo group. *$p < 0.05$ vs. placebo group. b p-value was analyzed with Dunnet test vs. baseline. †$p < 0.05$, ††$p < 0.01$ vs. baseline.

the GK-1 group showed significantly higher levels than the placebo group 6 and 12 weeks after the ingestion ($p < 0.05$, $p < 0.05$). It was also significantly higher in both the GK-1 and placebo groups at 12 weeks after the ingestion than that before the ingestion ($p < 0.05$, $p < 0.05$).

No significant differences in NK-cell activity or serum IFN-γ levels were observed between the two groups. However, considering the NK-cell activity, the GK-1 group showed significantly higher values at 6 and 12 weeks after the ingestion compared with that before the ingestion ($p < 0.01$, $p < 0.05$), and considering serum IFN-γ levels, the GK-1 group showed significantly higher values at 12 weeks after the ingestion compared with that before the ingestion ($p < 0.01$).

3.4. QOL Survey

The results of the QOL survey before, in 6 weeks, and 12 weeks after the consumption showed no significant differences between the groups.

3.5. Safety Evaluation

There were no adverse events attributable to the study food intake during the study. Although significant changes were observed in some parameters, including physical examination, hematology, blood chemistry, and urinalysis, the changes were within the reference range, and the investigator judged the 12-week continuous inges-tion of acetic acid bacteria to be safe. Table 9 demonstrates the change in laboratory values before and 12 weeks after the ingestion.

4. Discussion

The GK-1 has been reported to have allergy-controlling effects in cellular and human studies [10] [11] [12]. Moreover, the cellular studies that assessed the

Table 9. Safety evaluation.

Item		Reference values			Group		Baseline			After 12 weeks			
WBC ($\times 10^4/\mu L$)	M:	3500	-	9700	Placebo	n = 50	5320	±	191	5056	±	195	
	F:	3500	-	9700	GK-1	n = 45	5309	±	156	5049	±	164	
RBC ($\times 10^4/\mu L$)	M:	438	-	577	Placebo	n = 50	457	±	6	460	±	7	
	F:	376	-	516	GK-1	n = 45	457	±	6	460	±	7	
Hb (g/dL)	M:	13.6	-	18.3	Placebo	n = 50	14.1	±	0.2	13.9	±	0.2	
	F:	11.2	-	15.2	GK-1	n = 45	14.0	±	0.2	13.9	±	0.2	
Ht (%)	M:	40.4	-	51.9	Placebo	n = 50	43.2	±	0.5	43.5	±	0.6	
	F:	34.3	-	45.2	GK-1	n = 45	43.2	±	0.5	43.5	±	0.6	
PLT ($\times 10^4/\mu L$)	M:	14.0	-	37.9	Placebo	n = 50	26.5	±	1.0	26.9	±	1.1	
	F:	14.0	-	37.9	GK-1	n = 45	27.3	±	0.8	27.4	±	0.9	
TP (g/dL)	M:	6.5	-	8.2	Placebo	n = 50	7.06	±	0.05	7.10	±	0.06	
	F:	6.5	-	8.2	GK-1	n = 45	7.15	±	0.05	7.18	±	0.07	
ALB (g/dL)	M:	3.7	-	5.5	Placebo	n = 50	4.39	±	0.03	4.38	±	0.04	
	F:	3.7	-	5.5	GK-1	n = 45	4.42	±	0.04	4.40	±	0.04	
T-Bil (mg/dL)	M:	0.3	-	1.2	Placebo	n = 50	0.73	±	0.04	0.73	±	0.03	
	F:	0.3	-	1.2	GK-1	n = 45	0.74	±	0.04	0.74	±	0.04	
D-Bil (mg/dL)	M:	0.0	-	0.4	Placebo	n = 50	0.236	±	0.014	0.228	±	0.013	
	F:	0.0	-	0.4	GK-1	n = 45	0.240	±	0.013	0.227	±	0.013	
I-Bil (mg/dL)	M:	0.0	-	0.8	Placebo	n = 50	0.490	±	0.026	0.500	±	0.020	
	F:	0.0	-	0.8	GK-1	n = 45	0.502	±	0.027	0.516	±	0.025	
ALP (U/L)	M:	38	-	113	Placebo	n = 50	61	±	2	63	±	2	
	F:	38	-	113	GK-1	n = 45	60	±	2	62	±	3	
AST (U/L)	M:	10	-	40	Placebo	n = 50	21.0	±	0.7	20.7	±	0.9	
	F:	10	-	40	GK-1	n = 45	21.1	±	0.8	20.8	±	0.8	
ALT (U/L)	M:	5	-	45	Placebo	n = 50	17	±	1	17	±	1	
	F:	5	-	45	GK-1	n = 45	18	±	1	19	±	1	
LD (U/L)	M:	120	-	245	Placebo	n = 50	162	±	4	154	±	4	*
	F:	120	-	245	GK-1	n = 45	169	±	5	162	±	5	*
γ-GTP (U/L)	M:	0	-	79	Placebo	n = 50	22.6	±	1.7	23.1	±	2.0	
	F:	0	-	48	GK-1	n = 45	21.1	±	1.9	21.9	±	2.0	
T-Cho (mg/dL)	M:	150	-	219	Placebo	n = 50	201	±	4	205	±	4	
	F:	150	-	219	GK-1	n = 45	205	±	5	212	±	6	*
TG (mg/dL)	M:	50	-	149	Placebo	n = 50	73.0	±	5.4	75.1	±	5.9	
	F:	50	-	149	GK-1	n = 45	76.3	±	8.5	90.1	±	12.5	
HDL-Cho (mg/dL)	M:	40	-	80	Placebo	n = 50	67.1	±	2.3	69.7	±	2.3	*
	F:	40	-	90	GK-1	n = 45	67.9	±	2.5	69.9	±	2.8	

Continued

LDL-Cho (mg/dL)	M:	70	-	139	Placebo	n = 50	116	±	4	115	±	4
	F:	70	-	139	GK-1	n = 45	117	±	4	118	±	4
BUN (mg/dL)	M:	8.0	-	20.0	Placebo	n = 50	13.4	±	0.5	13.5	±	0.5
	F:	8.0	-	20.0	GK-1	n = 45	12.6	±	0.5	12.9	±	0.5
CRE (mg/dL)	M:	0.65	-	1.09	Placebo	n = 50	0.702	±	0.021	0.735	±	0.020*
	F:	0.46	-	0.82	GK-1	n = 45	0.675	±	0.022	0.692	±	0.022
UA (mg/dL)	M:	3.6	-	7.0	Placebo	n = 50	5.29	±	0.19	5.21	±	0.20
	F:	2.7	-	7.0	GK-1	n = 45	5.05	±	0.20	5.07	±	0.21
Na (mEq/L)	M:	135	-	145	Placebo	n = 50	141	±	0	141	±	0
	F:	135	-	145	GK-1	n = 45	141	±	0	141	±	0
K (mEq/L)	M:	3.5	-	5.0	Placebo	n = 50	4.42	±	0.04	4.39	±	0.04
	F:	3.5	-	5.0	GK-1	n = 45	4.36	±	0.04	4.45	±	0.05
Cl (mEq/L)	M:	98	-	108	Placebo	n = 50	104	±	0	104	±	0
	F:	98	-	108	GK-1	n = 45	104	±	0	104	±	0
GLU (mg/dL)	M:	70	-	109	Placebo	n = 50	89.2	±	1.0	89.3	±	1.1
	F:	70	-	109	GK-1	n = 45	88.9	±	0.9	90.1	±	1.2

Values are mean ± SE, M: Male reference value, F: Female reference value. *$p < 0.05$ vs. baseline, by unpaired t-test.

effects on TLR-4, NK activity using human natural killer-like cell line KHYG-1 cells (JRCB), IgA and IFN-α production using human peripheral blood mononuclear cells have been confirmed to improve several immune indicators [12]. GK-1 is expected to help maintain and promote immunity. In this study, we evaluated the effects of 12-week continuous oral ingestion of GK-1 (1.5×10^{10} cells/day) on subjective assessment of physical condition and individual immune indicators in healthy Japanese adults.

Many infections are acquired on mucosal surfaces, such as the oral cavity [21]. Moreover sIgA has been discovered to bind to pathogens on human mucosal surfaces, hereby actively preventing infections and promoting immune function by ginhibiting viral functions [22]; therefore, people with lower sIgA levels have been reported to be more susceptible to infectious diseases [23]. In addition, decreased levels of sIgA have been reported to be associated with an increased exhaustion [24]. The ingestion of acetic acid bacteria was considered to promote sIgA release and suppress the changes in the physical condition, including the systemic manifestations, such as fatigue, etc. Although the reason for the significant increase in the cumulative onset-days of the GK-1 group in sputum, fever, and feverish was unclear, these items were compared with five items of the physical condition questionnaire in which the effect of acetic acid bacteria was observed, and the occurrence frequency of onset-days was low on its own, suggesting that accidental factors had a significant impact. In a comparison of cumulative onset days every 3 weeks, it was shown that continuous intake for more

than 7 - 9 weeks improves overall body condition. In comparing cumulative onset days every 3 weeks, continued consumption of GK-1 for more than 7 - 9 weeks was shown to improve overall body condition. This effect continues at 10 - 12 weeks. Therefore, it was shown that it is desirable to consume GK-1 continuously for more than 7 - 9 weeks.

GK-1 stimulating the intestinal immune system is a possible mechanism by which salivary sIgA levels increase. LPSs in GK-1 have been reported to bind to TLR-4, which is also found in the intestinal epithelium and promote IL-6 production from dendritic cells to enhance IgA production [25]. Furthermore, pDCs play an important role in the intestinal immune system upstream of the immune maintenance system, and pDC knockout mice have confirmed that pDCs are involved in promoting the production of IFN-alphaand other proteins, including sIgA [26]. Although CpG-DNA sequences are known to activate pSCs via TLR9 responsiveness [27], the presence of CpG-DNA sequences in analysis of the genetic sequence of the acetic acid bacterium GK-1 has been confirmed (date not shown). Moreover, acetic acid bacteria have been reported to promote the production of IFN-alpha produced by pDCs in cellular assays [12], indicating that pDCs may be activated by acetic acid bacteria. These results suggest that the LPS-and CpG-DNA sequences of acetic acid bacteria enhanced IgA-production by acting on TLR-4 and TLR-9. In this study, it was confirmed that blood NK-cell and IFN-γ activities were significantly elevated in the acetic acid bacteria group at 12 weeks after ingestion compared with those before ingestion, and alongwith IgA, the involvement of pDCs via the LPS and CpG-DNA sequences of acetic acid bacteria may be considered as the mechanism [28] [29].

Lactic acid bacteria and bifidobacteria are described as fermentation bacteria maintaining immunity differently from acetic acid bacteria, and lactic acid bacteria have been reported to maintain immunity at an intake of about 1 - 10×10^{10} cells/day. Yamamoto *et al*. reported that ingestion of approximately 3×10^{11} cells/day of *Lactobacillus delbrueckii ssp. bulgaricus* OLL1073R-1 for 12 consecutive weeks increased salivary sIgA levels [30]. Kotani *et al*. demonstrated that ingestion of 4 × 109 cells/day of *Lactobacillus pentosus* B240 for 12 consecutive weeks increased salivary sIgA levels by approximately 60 μg/5 minutes [31]. In this study, 1.5×10^{10} cells/day of *Gluconacetobacter hansenii* GK-1 for 12 consecutive weeks increased salivary sIgA secretory levels by approximately 47 μg/min. When compared to sIgA secreted levels per minute, acetic acid bacteria provided about four times higher results than B240 strains, suggesting a function that is not inferior to that of lactic acid bacteria, although with differences in intakes. However, there are variations due to the environment of each examination, and it is generally not comparable, thus, requiring future studies.

Different factors, such as age [32] [33], nutritional status [34], daily mood [35], and exercise [36], have been reported to affect sIgA secreted volume, which is an effector of mucosal immunity. It has also been reported that a decrease in sIgA secreted volume means lower mucosal immunity [37]. When stratified analyses were conducted into groups with higher and lower levels of salivary sI-

gA per unit of time, no significant differences were found in the group with higher initial levels of salivary sIgA per hour ($p = 0.252$, 0 weeks vs. 12 weeks), whereas the group with lower initial levels of salivary sIgA per hour was confirmed to have increased sIgA secretion after 12 weeks of ingestion as related to the initial levels ($p < 0.01$, 0 week vs. 12 weeks, data not shown). It is considered that GK-1 ingestion increases sIgA secretion and returns it to that of a healthy human condition. The physical immune maintenance system is nearly constant regardless of race in healthy adults [38], so it is assumed that GK-1 ingestion can have a similar function in non-Japanese races as the subjects of this study.

The limitations of this study include the limited generalizability of the results since only adults were investigated, whereas the effects on children and sick individuals are unknown. Moreover, there is still insufficient evidence on the possible effects on pDCs and IFN-*α*. Further studies on these issues are also expected to clarify the efficacy and mechanisms of immune maintenance function caused by GK-1 acetic acid bacterial ingestion.

5. Conclusion

In this study, it was observed that 12 weeks of continuous oral ingestion of GK-1, gram-negativeacetic acid bacteria, significantly increased salivary sIgA levels and significantly suppressed the development of overall subjective assessment of physical conditions. These results suggest that continuous ingestion of GK-1 regulates immune function and is effective in maintaining a healthy physical condition.

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Conflicts of Interest

S.Y., M.O., M.K., Y.O., S.S., D.K., R.M. and Y.M. are employees of Kewpie Corporation. The remaining authors have no other conflicts of interest to report in this work.

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