

***Lactobacillus gasseri* SBT2055 stimulates immunoglobulin production and innate immunity after influenza vaccination in healthy adult volunteers**

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ABSTRACT

Background: *Lactobacillus gasseri* strain SBT2055 (LG2055) is a human intestine-originating probiotic bacterium and potent probiotic known to exert various health promotion effects, including prevention of abdominal adiposity in rats and humans. A recent finding in mice has suggested that oral administration of LG2055 induces a protective effect against influenza A virus infection. In this context, evidence for the efficacy of LG2055 using a human clinical trial was imminently required.

Methods: To confirm this in humans, a randomized, double-blind, placebo-controlled, parallel-group study in healthy adult volunteers was conducted to examine the effect of drinkable yogurt (DY) containing LG2055 on influenza, with vaccine-specific antibody responses as the primary objective and innate immune responses as the secondary objective. Subjects were asked to consume 100 g/day of DY with LG2055 (LG2055 group; n = 94) or without LG2055 (placebo group; n = 94) for 16 weeks. After 4 weeks, all subjects received a trivalent influenza vaccine.

Results: We found that the intake of LG2055 DY increased hemagglutination inhibition titers against influenza viruses A/H1N1 and B and the rate of seroprotection against influenza B after vaccination as compared with the intake of placebo DY by healthy volunteers. In support of this result, we confirmed that total IgG and IgA levels in plasma and sIgA production in saliva were also higher in the LG2055 group than in the placebo group. Furthermore, the intake of LG2055 DY enhanced natural killer cell activity and myxovirus resistance A gene expression, which is one of the antiviral genes stimulated by type I or type III Interferons in peripheral blood mononuclear cells.

Conclusions: These results strongly indicate that LG2055 activates both the innate and adaptive human immune responses, suggesting the potential to prevent influenza virus infections by providing specific probiotics as complementary foods.

Keywords: human clinical trial, innate immunity, influenza virus, *Lactobacillus gasseri*, vaccine

INTRODUCTION:

Influenza virus is a major cause of respiratory tract infection. Influenza epidemics occur almost every winter, and the social and economic damage caused by influenza pandemics is substantial. The prevention of influenza virus infections is an important public health challenge. Effective therapies to prevent and treat influenza virus infections could reduce morbidity and economic losses due to this illness. It is important to find safe alternatives to reduce the risk of influenza virus infection.

The human immune response is composed of “innate immunity,” otherwise known as the nonspecific immune response that functions as the body’s first line of defense, and “adaptive immunity,” referred to the acquired immune response or specific immunity. Type I interferon (IFN) production and natural killer (NK) cell activity are representative innate cytokine and innate lymphoid cells that play roles in innate immunity against virus-infected cells. Vaccination, which is induced by adaptive immune mechanisms such as antigen-specific antibody and cytotoxic T cells, is the most effective way to prevent influenza virus infections. Influenza vaccination is recommended not only to protect against influenza virus infection, but also to reduce the risk of severe disease such as influenza encephalitis and pneumonia. Influenza virus infections are highly dangerous to specific populations such as pregnant women, diabetes

patients, infants, and the elderly [1, 2]. Furthermore, these immune responses are weakened by specific lifestyle attributes, such as obesity [3], stress [4, 5], and smoking cigarettes [6]. Therefore, maintaining robust innate and adaptive immunity is thought to be important for the prevention of the severe health impacts of influenza.

Probiotics are defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts [7]. Probiotic bacteria, mainly belonging to the class of lactic acid bacteria (LAB), are known to be beneficial in human and animal health. In particular, lactobacilli are characterized by the production of lactic acid and are commonly applied to many vegetable, meat, and dairy fermentations. These bacteria can affect the composition and activity of the gut microbiota. Currently, there is a general consensus that orally administered probiotic bacteria contribute to immune homeostasis by altering the microbial balance or by interacting with the host immune system [8, 9, 10]. However, not all probiotic strains have the ability to modulate the immune system in humans, even when immunomodulatory functions have been confirmed by in vitro or mouse model studies [11, 12]. A randomized controlled clinical trial must be conducted to confirm the effectiveness of a probiotic strain on immune function. One of the suggested methods for assessing immune function in healthy subjects is by vaccine challenge. The vaccine challenge model is included in the guidelines on scientific requirements for health claims related to gut and immune function from the European Food Safety Authority [13].

Lactobacillus gasseri strain SBT2055 (LG2055) is a human intestine-originating probiotic bacterium with properties including bile tolerance [14], the ability to establish in the intestine and lower the fecal *Staphylococcus* population and p-cresol concentration [15, 16], a cholesterol lowering effect in humans with mild hypercholesterolemia [17], and the ability to prevent abdominal adiposity in rats [18, 19] and humans [20, 21]. Recently, oral administration of LG2055 was found to confer a protective effect against influenza A virus infection through the induction of antiviral genes by type I IFN signaling [22] and IgA production in the mouse small intestine [23]. These findings raise the possibility that administration of LG2055 may enhance both innate and adaptive immunity.

In this study, a randomized, double-blind, placebo-controlled, parallel-group study in healthy adult volunteers was conducted to examine the effect of oral administration of drinkable yogurt (DY) containing LG2055 on influenza vaccine-specific antibody responses as the primary objective and innate immune responses as the secondary objective.

SUBJECTS AND METHODS:

Subjects. We recruited healthy Japanese men and women aged 20 years or older who were living in Ebetsu City and the neighboring communities in Hokkaido, Japan. Volunteers were excluded from participation in this study if they had met any of the following exclusion criteria: 1. frequent intake of yogurt containing LG2055 from Megmilk Snow Brand Co., Ltd., 2. any current relevant infections such as influenza, 3. vaccination against influenza virus 12 week before study entry, 4. current use of immunomodulating medicine (antibiotics, immunosuppressive medicine, anti-inflammatory medicine), Chinese herb, supplements (such as lactic acid bacteria preparation, mushroom, east, seaweed), 5. current use of any medicine for diarrhea or constipation, 6. history of significant illness, 7. pregnancy or lactating, 8. heavy smoking (more than 20 cigarettes/day) and/or excessive alcohol consumption (more than 20 g alcohol/day), and 9. history of severe allergic reaction to food, medication, and vaccine. Participants were asked to stop the intake of any foods and supplements containing lactic acid bacteria. The sample size was determined based on the data of our previous clinical test using LG2055 and *Bifidobacterium longum* SBT2928 [24]. To demonstrate differences between groups at 5% significance level, a sample size of at least 80 subjects in each group would be required. Therefore, in this study we set the number of subjects to 100 in each group.

Test Samples. *Lactobacillus gasseri* SBT2055 (LG2055) was originally isolated by Milk Science Research Institute, Megmilk Snow Brand Co., Ltd. (Tokyo, Japan) and deposited in the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Tsukuba, Ibaraki 305-8566, Japan). This strain was selected on the basis of its human origin, nonpathogenic status, resistance to intestinal acid and bile, and previously proven to survive transiently through the GI tract in human studies and to be free of side effects [15]. Two types of drinkable yogurt (DY) were prepared: an active DY containing LG2055 and a control DY lacking LG2055. The active DY was prepared with lactic acid bacteria starter cultures (*Streptococcus thermophilus*) commonly used for conventional yogurt production and viable cells of LG2055. A DY mixture consisting of approximately 10% skim milk powder and a small amount of flavoring, polysaccharide from soy beans, pectin as a stabilizer, and sucralose as an artificial sweetener, was inoculated with the yogurt starter cultures and LG2055 cells, and then fermented. Active DY contained a minimum of 1×10^9 cfu/100 g of LG2055. The control DY was prepared in the same manner except that the LG2055 cells were not added. The active and

control DYs were identical in energy (36 kcal), protein (3.3g), fat (0g), carbohydrate (5.5g), sodium (41mg), and calcium (120mg) content per 100g; they were also indistinguishable from flavors. The test DYs were kept in cold storage and delivered weekly.

Study Design. The intervention was performed in a placebo-controlled, randomized, double-blind clinical trial during November 2014 to March 2015. Subjects were randomly divided into two groups, an active DY (LG2055) group (n=100) and a control DY (placebo) group (n=100). All of the subjects consumed 100g/day of either the active DY or control DY for 16 weeks. We advised the volunteers not to change their daily lifestyle, including their meals, exercise and alcohol consumption during this study. A blood sample was collected (W0) before intake of the first study drink. After a prevaccination period of 4 weeks, all of the subjects were administered the trivalent influenza vaccine (A/California/7/2009(X-179A)(H1N1)pdm09, A/New York/39/2012(X-233A)(H3N2), B/Massachusetts/2/2012(BX-51B)). Blood samples were collected at Week 4 (W4) at the time of vaccination, Week 7, 11, 16 (W7, W11, W16). All blood samples were analyzed the Hemagglutination inhibition (HI) antibody titer; additionally, the immune biomarkers of the blood samples at W0, W7, and W16 were also analyzed.

Ethics Committee. Ethical clearance was obtained from the ethics committee of the Hokkaido Information University before the study was initiated (certificate number: 2014-09). Informed consent was obtained after the contents of this study were explained. Any undesirable events affecting a subject's health during the course of the study were defined as adverse events. If a subject asked to be released from the study, the protocol was immediately terminated for that subject. This study was conducted in accordance with the Declaration of Helsinki. This study was registered at the UMIN Clinical Trial Registry (UMIN000019264).

Immunogenicity Assessment. Hemagglutination inhibition (HI) tests were performed using hemagglutinating antigens and reference antisera for hemagglutination-inhibition tests influenza virus type A and type B (DENKA SEIKEN, Japan), according to standard procedures at a clinical laboratory testing company (SRL, Japan). Immunogenicity was evaluated in three ways: geometric mean HI Ab titers to each influenza antigen; seroprotection rate, defined as the proportion of subjects with HI titers $\geq 1:40$; and seroconversion rate, defined as the proportion of subjects with a ≥ 4 -fold increase in HI titers at baseline and post-vaccination. These biomarkers

were measured at a clinical laboratory testing company (Sapporo Clinical Laboratory Inc., Sapporo, Japan).

Hematological and Biochemical Analyses. Hematological analyses were carried out with all blood samples (WBC, RBC, Hb, Ht, Plt). Plasma biochemical analyses were carried out with blood samples of W0, W7, and W16, including total IgG, total IgA, liver function test (AST, ALT, ρ -GTP, ALP, LDH), renal function test (BUN, CRE, UA), serum lipids (T-Cho, LDL-Cho, HDL-Cho, TG), CRP, and blood glucose. These biomarkers were measured at a clinical laboratory testing company (Sapporo Clinical Laboratory Inc., Sapporo, Japan).

NK Cell Activity. With the blood samples of W0, W7, and W16, the natural killer (NK) cell activity was measured. Blood was briefly withdrawn using EDTA-containing vacutainers from the subjects. Chromium-51 (^{51}Cr) release assays were used to measure NK cell activity (PerkinElmer, Waltham, MA). Target cells were labeled with ^{51}Cr , and the label was then released from the target cells by cytolysis. The label could be isolated by centrifuging the samples and collecting the supernatants. The supernatants from the centrifugation could either be counted directly by a gamma counter or mixed with a scintillation cocktail in a microplate (or dried on a LumaPlateTM) and counted by a liquid scintillation counter. All measurements were performed by a clinical laboratory (SRL, Tokyo, Japan)

Saliva Collection and Secretory IgA Detection. Saliva samples were collected at W0, W7, and W16. Saliva were collected using cotton swabs and Salivette devices (Salimetrics, State College, PA). Subjects were instructed to refrigerate the sample tubes after collection and to submit them to the laboratory on the morning of the study day. The sample tubes were immediately centrifuged at $1500\times g$ for 10 min to collect the saliva from the swab. Saliva samples were stored at -80°C until analysis. Secretory IgA (sIgA) concentration in saliva was measured with ELISA using a commercial kit (Salimetrics, Carlsbad, CA). Data were expressed in saliva flow rate ($\mu\text{g}/\text{min}$), sIgA concentration ($\mu\text{g}/\text{ml}$), and sIgA secretion rate ($\mu\text{g}/\text{min}$). The sIgA secretion rate was calculated by multiplying the sIgA concentration ($\mu\text{g}/\text{ml}$) by the saliva flow rate ($\mu\text{g}/\text{min}$).

Preparation of PBMC and Gene Expression Analysis. Twenty-five subjects were randomly selected from LG2055 and placebo group respectively. Peripheral blood mononuclear cells

(PBMC) in blood samples of W0, W7, and W16 were isolated by density gradient centrifugation over Ficoll-Paque. Total RNA was extracted from PBMC (1×10^7 cells) using RNeasy Mini Kit (QIAGEN Inc., CA). First-strand cDNA was synthesized from total RNA by using Prime Script RT Mastermix (TAKARA BIO Inc., Japan) according to the manufacture's protocol. Quantitative reverse transcription (qRT)-PCR analysis was carried out using SYBR premix EX tag II (TAKARA BIO Inc.) and TKARA TP800 thermal cycler dice real time system. The transcriptional levels of MxA gene at each time point was normalized to β -actin level and presented as changes from base line (W0). The sequences of primers used were as follows: MxA (forward) 5'-TTCAGCACCTGATGGCCTATC-3', (reverse) 5'-CCGTACGTCTGGAGCATGAAG-3' as previously described [25], and β -actin (forward) 5'-TGCCGACAGGATGCAGAAG-3', (reverse) 5'-CTCAGGAGGAGCAATGATCTTGA-3' as previously described [26].

Questionnaire. All subjects were notified to report any influenza-like symptoms (fever, headache, cough, nasal symptoms, diarrhea, vomiting, and loss of appetite) in the entire study. When subjects were suspected to be infected by influenza viruses, they were immediately checked by a rapid influenza immunoassay kit and confirmed by physicians.

Statistical Analysis. All data were collected and analyzed independently of the investigators, who did not have access to the data or to its analysis until the latter had been completed. The between-group comparisons in geometric mean of the HI titer were assessed by Mann-Whitney's U test. The seroprotection rate and the seroconversion rate between placebo group and LG2055 group were assessed by the chi-square test. Total IgG and IgA in plasma, sIgA in saliva, NK cell activity, MxA gene expression in PBMC, within-group comparisons between W0 and each subsequent time point (W7 and W16) were carried out using repeated-measures ANOVA, followed by Bonferroni multiple comparisons. The interaction between the group and the consumption period of time (group-by-time interaction) were carried out using repeated-measures ANOVA. The between-group comparisons in actual values and the amount of change from baseline were assessed by Student's *t*-test. Statistical analyses were performed using SPSS Statistic 19 (IBM, NY). A significance level of $p < 0.05$ was considered to indicate significance.

RESULTS:

Baseline and Demographic Data. Participants were recruited from residents of Ebetsu City and neighboring communities. The flow of participant involvement through the trial is shown in Figure 1. Subjects who provided consent (n=320) were assessed for eligibility, and a total of 200 subjects were enrolled in this study. All enrolled subjects were randomized to one of the two intervention groups (placebo group: n=100 or LG2055 group: n=100). During the course of this study, 10 subjects discontinued the trial because of poor compliance (n=7), pregnancy (n=2), or bone fracture (n=1). Two subjects were excluded from the evaluable subjects because of too frequent administration of supplements containing LAB (n=1) or regular use of an antibiotic (n=1). The final number of evaluable subjects was 188 (placebo group: n = 94 and LG2055 group: n=94). We found no significant difference between the placebo and LG2055 groups regarding age, gender, body mass index (BMI), body fat, or intake rate (Table 1).

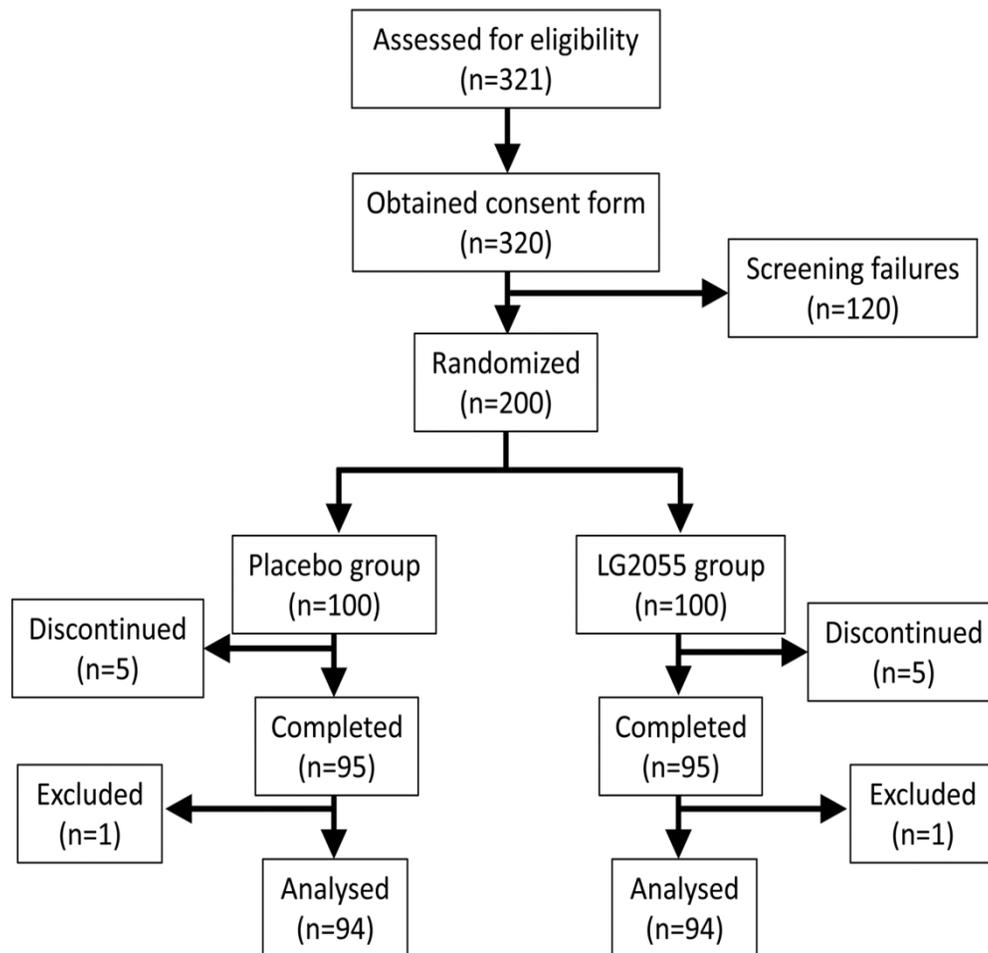


Figure 1. Disposition of the subjects. Values are expressed as the number of participants.

Table 1. Characteristics of the subjects in the placebo and LG2055 groups

	Placebo		LG2055	
	(n=94)		(n=94)	
	Mean	SD	Mean	SD
Sex				
Male, n(%)	17 (18.1)		16 (17.0)	
Female, n(%)	77 (81.9)		78 (83.0)	
Age (years)	49.6	12.5	49.4	12.7
Height (cm)	159.1	6.3	159.8	7.2
Weight (kg)	54.5	8.8	56.0	10.9
BMI (kg/cm ²)	21.5	3.0	21.9	3.7
Body fat (%)	26.6	6.4	28.0	7.4
Intake rate (%)	98.7	3.7	98.6	2.6

Values shown are mean and standard deviation (SD). n = number of subjects.

Serum Biochemical Data and Vital Signs. We found no abnormal biochemical data for liver or renal functions, blood glucose, blood lipid, or CRP (C-reactive protein) that may have indicated any adverse effect in the placebo and LG2055 groups during this trial. Vital signs (blood pressure, pulse, and body temperature) raised no safety concerns.

Vaccine-specific Antibody Responses. The geometric mean of the HI antibody titers before (W0 and W4) and after vaccination (W7, W11, and W16) are shown in Table 2. HI titers against A/H1N1 at W7 and W11, and B at W4 and W11, were significantly higher in each LG2055 group compared with the corresponding placebo group. There were no differences in HI titers against A/H3N2 between the placebo group and the LG2055 group. The seroprotection rate (HI titer $\geq 1:40$) against B at W11 was significantly higher in the LG2055 group than in the placebo group (Table 3). There were no differences in the seroconversion rate (HI titer ≥ 4 -fold increase) between the placebo group and the LG2055 group.

Table 2. Change in the HI antibody titers to Influenza Vaccine during the study period

Antigen	Group	HI antibody titers				
		W0	W4 vaccination	W7	W11	W16
A/H1N1	Placebo	15.1 (12.2–18.7)	15.3 (12.4–18.9)	84.2 (67.6–104.9)	63.7 (51.4–79.4)	53.1 (42.1–67.0)
	LG2055	20.6 (16.4–25.8)	21.1 (16.6–26.7)	115.7 * (91.1–146.8)	87.5 * (70.2–109.1)	66.5 (53.0–83.5)
A/H3N2	Placebo	41.5 (31.8–54.1)	34.3 (26.2–44.8)	132.1 (102.0–171.1)	145.4 (112.1–188.7)	142.0 (109.7–183.9)
	LG2055	43.4 (33.7–55.9)	34.0 (26.2–44.2)	138.1 (108.2–176.2)	151.9 (117.7–196.0)	133.1 (103.9–170.5)
B	Placebo	13.1 (11.3–15.3)	12.9 (10.9–15.1)	28.1 (24.4–32.4)	27.3 (23.8–31.3)	26.0 (22.6–29.82)
	LG2055	15.0 (12.7–17.7)	16.9 * (14.3–20.0)	33.3 (28.2–39.2)	34.0 * (29.2–39.5)	29.1 (24.8–34.2)

The numbers is the geometric mean of the HI antibody titers, and 95% confidence interval in parentheses. *, $p < 0.05$, Mann-Whitney U test between-group comparisons.

Table 3. Change in the seroprotection rate and seroconversion rate to Influenza Vaccine during the study period

Antigen	Group	Seroprotection rate (%)					Seroconversion rate (%)		
		W0	W4 vaccination	W7	W11	W16	W7	W11	W16
A/H1N1	Placebo	25.5	28.7	87.2	75.5	69.9	53.2	42.6	36.6
	LG2055	37.2	35.1	87.2	83.9	77.7	51.1	46.2	38.3
A/H3N2	Placebo	68.1	60.6	89.4	90.4	90.3	47.9	54.3	51.6
	LG2055	68.1	60.6	90.4	91.4	87.2	48.9	50.5	45.7
B	Placebo	16.0	20.2	44.7	43.6	45.2	14.9	12.8	15.1
	LG2055	23.4	26.6	56.4	58.1*	46.8	22.3	22.6	14.9

Data are presented as percentages. *, $p < 0.05$, the chi-square test between-group comparisons.

Total IgG Concentration in Plasma. In total, IgG concentrations in plasma of all subjects (Table 4), group-by-time interaction and group-effect were statistically significant by repeated-measure ANOVA. In a between-group comparison, the IgG concentration in the LG2055 group was significantly higher at W7 and W16 compared to the placebo group. In

within-group comparisons, the LG2055 group at W7 and W16 revealed significant increases in IgG concentration from W0. The placebo group, by contrast, showed no significant changes at any time point. In order to analyze the data in detail, subjects were subdivided into a high IgG concentration layer and low IgG concentration layer, according to whether the IgG concentration before DY administration (W0) was above the median (1209 mg/dl) or not (Table 4). In the low IgG concentration layer, the group-by-time interaction and group-effect was statistically significant. In a between-group comparison, the IgG concentration in the LG2055 group was significantly higher at W7 and W16 than in the placebo group. In within-group comparisons, the LG2055 group at W7 and W16, and the placebo group at W16, revealed significant increases from W0 in each group. In the high IgG concentration layer, the group-by-time interaction and group-effect were not statistically significant. The LG2055 group showed significant increases in the amount of the change in total IgG concentration from the baseline (W0) to both W7 and W16 in all subjects and in the low IgG concentration layer as compared with the placebo group. There was no increase seen in the high IgG concentration layer (Figure 2).

Table 4. Change in the total IgG concentration in plasma during the study period

Subjects	Groups	n=	W0	W7	W16	Group-by-time Group
Total subjects	Placebo	94	1201.5 (26.2)	1204.7 (25.6)	1227.2 (24.8)	#
	LG2055	94	1256.0 (27.4)	1304.3 (26.2) *	1317.8 (25.3) ###	*** †
Low IgG layer (<1209)	Placebo	49	1003.4 (21.8)	1044.4 (26.2)	1052.5 (24.2)	** ##
	LG2055	45	1038.2 (20.3)	1144.4 (28.4) ***	1163.6 (25.3) ***	*** †
High IgG layer (>1209)	Placebo	45	1417.3 (20.7)	1379.1 (27.4)	1413.5 (22.0)	
	LG2055	49	1456.0 (26.6)	1451.2 (30.6)	1462.4 (30.8)	

Data are presented as total subjects, which is divided into “low IgG layer” subjects with less than median value (1209 mg/dl), and “high IgG layer” subjects with the median value and more than this value. Data are presented as mean ± standard error (mg/dl). #, p < 0.05, ##, p < 0.01, t test, between group comparisons. *, p < 0.05, **, p < 0.01, ***, p < 0.001, within-group comparisons between W0 and each subsequent time point (W7 and W16) using by Bonferroni multiple comparisons.

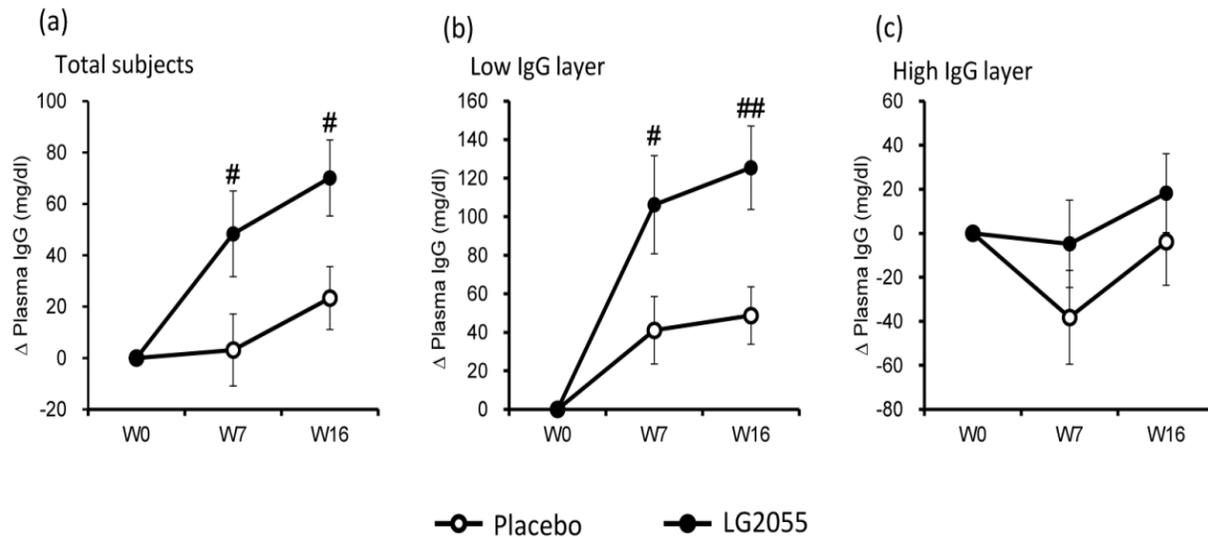


Figure 2. Change in total IgG levels in plasma from the baseline (W0). (a) Data represents total subjects (placebo: n=94, LG2055: n=94), (b) low IgG layer subjects with less than the median value (1209 mg/dl) (placebo: n=49, LG2055: n=45), and (c) high layer subjects with more than the median value (placebo: n=45, LG2055: n=49). Data are presented as mean \pm standard error for the LG2055 group (closed circle) and placebo group (open circle), #: $p < 0.05$, ##: $p < 0.01$, t test, between-group comparisons.

Total IgA in Plasma and secretory IgA in Saliva. The group-by-time interaction of total IgA concentration in plasma (Figure 3(a)) was found statistically significant by repeated-measures ANOVA ($p < 0.05$). In within-group comparisons, the LG2055 group at W7 and W16 demonstrated significant increase from W0. The placebo group, by contrast, showed no significant changes at any time point. In the between-group comparison for the amount of change from the baseline (Figure 3(b)), the LG2055 group showed significantly higher IgA levels at W7 and a tendency toward higher levels at W16 than the placebo group. In within-group comparisons in the secretory IgA (sIgA) secretion rate in saliva (Figure 3(c)), the placebo group at W7 and W16 revealed significant decrease or a tendency to decrease from W0, but the LG2055 group did not show any significant changes. In the between-group comparison for the amount of change in sIgA secretion rate in saliva from the baseline (Figure 3(d)), the LG2055 group showed a tendency to higher levels at W7 than the placebo group.

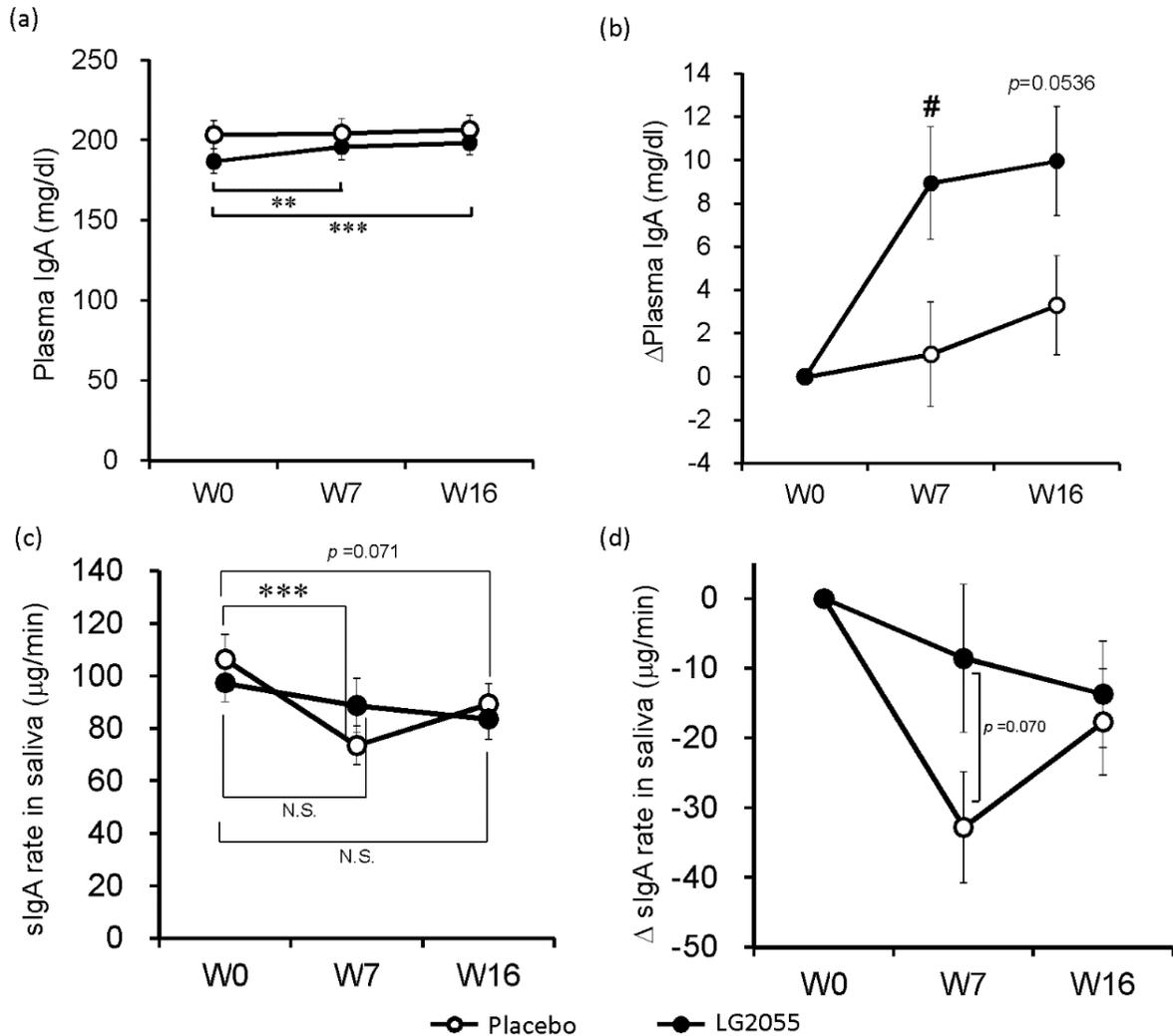


Figure 3. Total IgA in plasma and secretory IgA in saliva. (a) Total IgA concentration in plasma. (b) Changes in the plasma total IgA concentration from the baseline (W0). (c) Secretory IgA (sIgA) secretion rate in saliva ($\mu\text{g}/\text{min}$). (d) Changes in the sIgA secretion rate in saliva from the baseline (W0). Data are presented as mean \pm standard error for the LG2055 group (closed circle) and placebo group (open circle). #: $p < 0.05$, ##: $p < 0.01$, between-group comparisons by t test. **: $p < 0.01$, ***: $p < 0.001$, within-group comparisons between W0 and each subsequent time point (W7 and W16) using Bonferroni multiple comparisons test.

NK Cell Activity. In NK cell activities in all subjects, the group-by-time interaction and the group-effect were found to be statistically significant ($p < 0.05$) by repeated-measures ANOVA (Figure 4(a)). In the between-group comparison, NK activity in the placebo group was significantly higher at W0 than the LG2055 group. In within-group comparisons, the LG2055 group at W7 revealed a significant increase in NK activity from W0. In contrast, the placebo

group at W16 showed a significant decrease in NK activity from W0. In amount of a change in the NK cell activity from the baseline (W0) in all subjects (Figure 4(d)), the LG2055 group demonstrated significantly higher NK activity at W7 than the placebo group. Subjects were subdivided into a high NK cell activity layer and low NK cell activity layer, according to whether the NK cell activity before DY administration (W0) was above the median (32%) or not. In the low NK cell activity layer, the group-by-time interaction and group-effect were statistically significant ($p < 0.05$) (Figure 4(b)). In the between-group comparison, NK activity of the placebo group was significantly higher at W0 than in the LG2055 group. In within-group comparisons, the LG2055 group and the placebo group at W7 each revealed significant increases from W0. This increase was marked in the LG2055 group. In the amount of the change in NK cell activity from the baseline (W0) in the low NK cell activity layer, the LG2055 group was significantly higher at W7 and showed a tendency toward higher levels at W16 than the placebo group (Figure 4(e)). In the high NK cell activity layer, the group-by-time interaction and group-effect were not statistically significant (Figure 4(c)). In within-group comparisons, the LG2055 group and the placebo group at W16 both revealed significant decreases from W0 (Figure 4(f)).

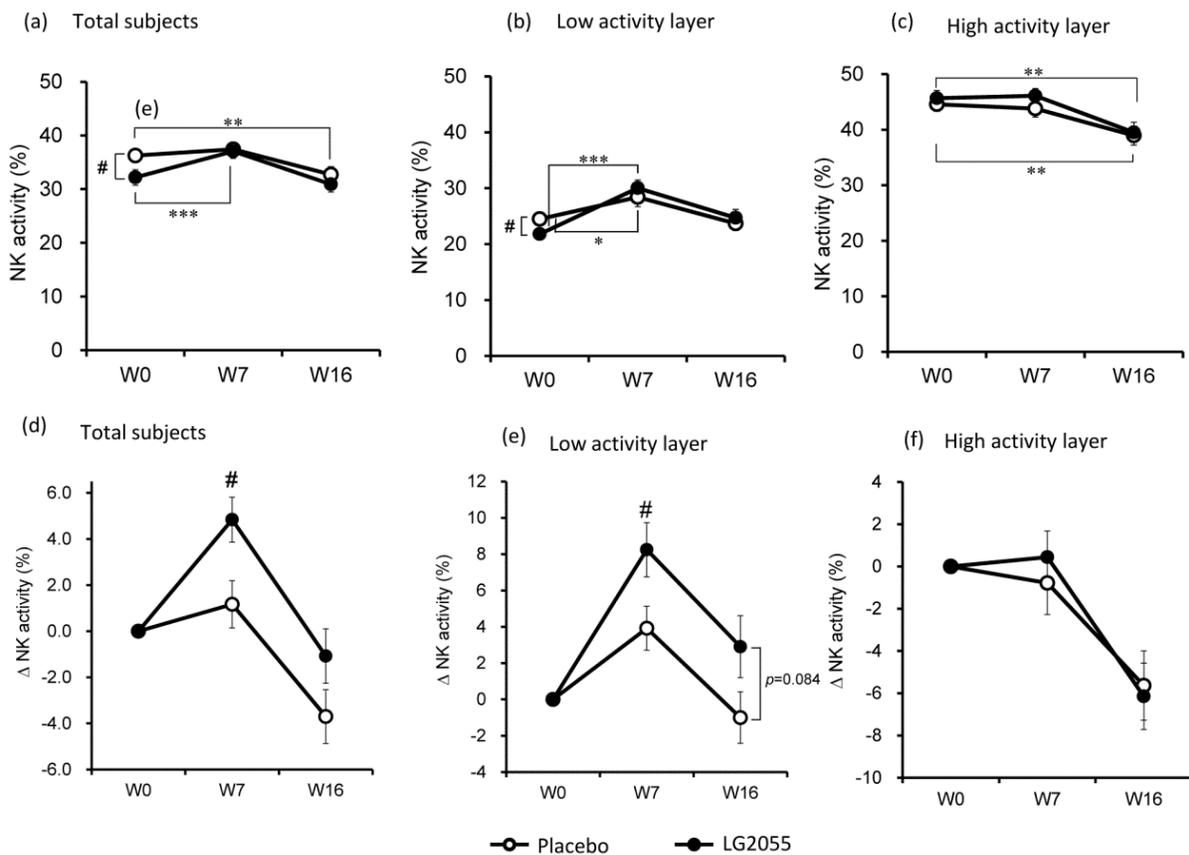


Figure 4. Changes in NK cell activity during the study period. Data are presented as (a)(d) “total

subjects” (placebo: n = 94, LG2055: n=94), (b)(e) “low activity layer” subjects with less than the median value of NK cell activity at W0 (34%) (placebo: n=39, LG2055: n=53), (c)(f) “high activity layer” subjects with the median value or greater than the median value (placebo: n=55, LG2055: n=41). Data are presented as actual values (a)(b)(c) and the amount of the change from the baseline (d)(e)(f). Data are presented as mean \pm standard error for the LG2055 group (closed circle) and placebo group (open circle), #: $p < 0.05$, between-group comparisons by t test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, within-group comparisons between W0 and each subsequent time point (W7 and W16) using Bonferroni multiple comparisons test.

Myxovirus resistance protein A Gene Expression in PBMC. To evaluate antiviral gene expression in PBMC, expression levels of the Myxovirus resistance protein A (MxA) gene were determined by qRT-PCR using total RNA prepared from PBMC. Total RNA extractions from PBMC of all subjects could not be performed because of limitations in manpower. Consequently, total RNA samples were prepared from 38 subjects randomly selected from each group. One sample in the LG2055 group failed to yield total RNA, so the sample number in the LG2055 group was 37. Expression of the MxA gene is shown in Figure 5. The group-by-time interaction and group-effect were found to be statistically significant by repeated-measures ANOVA ($p < 0.01$ and $p < 0.05$, respectively). In the between-group comparison, the LG2055 group showed significantly higher level of MxA gene expression at W16 than in the placebo group. In within-group comparisons, the LG2055 group at W7 and W16 showed significant increases from W0. The placebo group showed no significant changes at any time point.

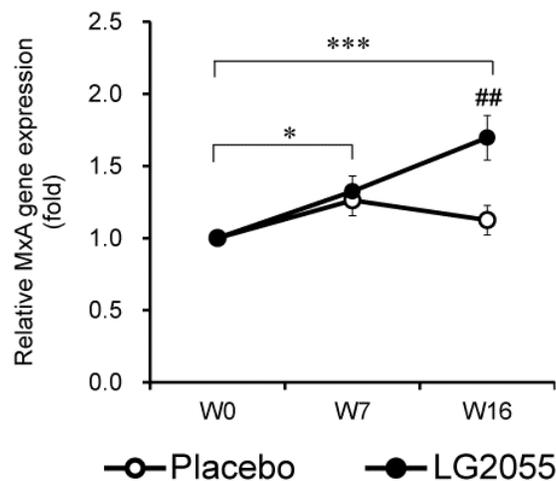


Figure 5. Myxovirus resistance protein A (MxA) gene expression in PBMC. qRT-PCR was performed using total RNA from PBMC. Relative MxA gene expression was normalized by

β -actin gene expression. Data are presented as changes from the baseline (W0). Data are presented as mean \pm standard error for the LG2055 group (closed circle) and placebo group (open circle), ##: $p < 0.01$, between-group comparisons by t test. *: $p < 0.05$, ***: $p < 0.001$, within-group comparisons between W0 and each subsequent time point (W7 and W16) using Bonferroni multiple comparisons test.

Influenza Virus-infected Subjects. Over the entire study period (W0 to W16), three subjects in each group were diagnosed as having influenza by physicians. No statistically significant difference between the two groups was detected.

DISCUSSION:

There are many reports suggesting that administration of LAB stimulates innate immunity, e.g., NK cell activity [27, 28, 29] and type I IFN production [30, 31], as well as adaptive immunity, e.g., specific antibody production against a vaccine [32, 33] in humans. However, there are few reports to show LAB strains stimulating both innate and adaptive immunity simultaneously. Oral administration of LG2055 induces the expression of antiviral genes by type I IFN signaling in the mouse lung [22] and IgA production in the mouse small intestine [23], indicating that LG2055 elicits both innate and adaptive immunity. In this clinical study, oral administration of DY containing LG2055 in healthy adult volunteers increased vaccine-specific antibody production against A/H1N1 and B, total IgG levels in plasma, NK cell activity, and gene expression of an antiviral factor in PBMC. These findings demonstrate, to our knowledge for the first time, that intake of probiotics can enhance both innate and adaptive immunity.

Vaccination is the most effective way to prevent influenza virus infections, reducing the risks of death and hospitalization associated with complications. Influenza vaccines induce antigen-specific antibody and cytotoxic T cells to specifically target the virus. Some strains of lactobacilli and bifidobacteria have been reported to enhance vaccine responses [32, 33]. However, not all probiotic strains have this ability. Some lactobacillus strains were reported to have no effects on immune responses to influenza vaccination in healthy adult [34] and elderly [12] volunteers. Specific probiotic strains have unique properties, and it is not possible to extrapolate the effect of one probiotic strain to others [35]. In our results, HI antibody titers against A/H1N1 and B were significantly higher in LG2055 group compared with placebo group. On the other hand, there were no differences in HI titers against A/H3N2 between the placebo

group and the LG2055 group. The effectiveness of influenza vaccination varies from year to year [36]. The results of the current study, based on the 2014–2015 vaccine, revealed high HI titers against A/H3N2, with a seroprotection rate >90% at W16 in the placebo group, whereas seroprotection rates against A/H1N1 and B were 70% and 45% at W16, respectively. Therefore, LG2055 is likely to be more effective when the influenza vaccination efficacy is moderate or low. Immune function declines with aging, and antibody responses to vaccines also decrease in the elderly [37]. We speculate that LG2055 would be more effective with lower influenza vaccination efficacy. Therefore, studies on the effectiveness of LG2055 with an intervention trial in the elderly may provide additional data to support our speculation.

Total IgG in plasma is an important biomarker of immune function in healthy subjects. Hypogammaglobulinemia is associated with an increased risk of opportunistic infections, particularly in transplant recipients [38] and with congenital immunodeficiencies [39]. On the other hand, high IgG levels in plasma are associated with autoimmune disease. In our results, the IgG levels in plasma were higher in the LG2055 group than in the placebo group. Notably, the effect of LG2055 intake on plasma IgG was greater in subjects whose IgG levels were relatively low (lower than median value), compared with that in subjects whose IgG levels were relatively high (higher than median value). These results suggest that LG2055 intake is more effective in increasing plasma IgG levels in subjects with low plasma IgG, and that LG2055 should cause no concern about the possibility of increasing an already high plasma IgG level, which is an indicator for autoimmune disease.

In mucosal tissue, IgA is the most abundant immunoglobulin isotype, with up to 3 g of sIgA secreted into the human intestinal lumen per day [40, 41]. IgA plays important roles in host defense against mucosally transmitted pathogens, preventing commensal bacteria from binding to epithelial cells, and neutralizing toxins to maintain homeostasis at mucosal surfaces [42]. We previously demonstrated that administration of LG2055 to mice stimulated IgA production in mucosal tissues, such as the intestine [23] and breast milk [43]. In this study, LG2055 intake not only increased plasma IgA but also stimulated sIgA in saliva. These findings suggest that LG2055 stimulates humoral immunity, as indicated by the production of IgG in plasma and IgA in mucosal tissues.

Mechanisms of antibody production enhancement by LAB are not fully understood. LABs are reported to stimulate cytokine production via Toll-like receptors (TLR) on macrophages and dendritic cells [44]. We previously revealed that LG2055 stimulates cytokines production from

dendritic cells and IgA production from B cells, and that TLR2 signal is critical for this stimulation [23]. We speculate that LG2055 helps augment nonspecific and vaccine-specific antibody production by functioning as an adjuvant.

NK cells are involved in innate immune responses and play a major role in recognizing and killing both virus-infected cells and tumor cells. Our previous study demonstrated that intake of fermented milk containing LG2055 and *Bifidobacterium longum* SBT2928 for 12 weeks elevated NK cell activity in healthy volunteers [24]. In this study, the change in NK cell activity from the baseline (W0) to W7 in the LG2055 group was significantly greater than that in the placebo group. These findings revealed that intake of fermented milk which only contained LG2055 and did not contain *Bifidobacterium longum* SBT2928 can potentiate NK cell activity. Significantly, increases in NK cell activity following LG2055 intake were greatest in the low NK cell activity layer at the baseline. A previous study showed that daily intake of *Lactobacillus casei* Shirota (LcS) increased NK cell activity, and the effects of LcS were remarkable in subjects with low baseline NK cell activity [45]. Furthermore, Real M. et al. reported that LcS intake prevented smoking-induced NK activity reduction [46], and Makino S. et al. reported that dietary intake of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 increased NK cell activity in subjects with low baseline NK cell activity [28]. Increases in NK cell activity following administration of probiotics that contain LG2055 are likely to be more effective in subjects with low NK cell activity. An epidemiological study suggests that high NK cell activity is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk [47]. Patients with a congenital lack of NK cell activity tend to have repeated polymicrobial infections [48], and elderly subjects with low NK cell activity experience short survival times following infection [49]. These reports indicate that maintaining high basal NK cell activity is critical for host defense against cancer and infection. We expect that increases in NK cell activity following LG2055 intake in people with low NK cell activity can reduce risks of infectious disease and cancer.

IFN-induced human myxovirus resistance protein A (MxA) exhibits broad antiviral activity, including against influenza A virus [50]. We previously reported that oral administration of LG2055 to mice induced gene expression of Mx1, a human MxA ortholog, in lung tissues, and protected against influenza A virus infection. In this study, MxA gene expression levels in PBMC were higher in the LG2055 group than in the placebo group, in agreement with our previous study in mice. To our knowledge, this is the first demonstration of an increase MxA gene

expression in human PBMC by administrations of probiotics. MxA expression is tightly regulated by type I and type III IFN, but no detectable IFN- α and β in plasma by ELISA in this study (data not shown). MxA expression is widely used as a surrogate marker for IFN activity in various experimental and clinical settings [51]. We assume that LG2055 increases MxA gene expression through induction of type I or III IFN production, because LG2055 has the ability to induce IFN- β production from macrophage-derived RAW 264.7 cell in vitro [22]. Induction of type I or III IFN by LG2055 may be transient or undetectable. MxA inhibits influenza virus infection by blocking an early step of the viral replication cycle [52]. MxA is considered an important component of the early innate immune defense in humans. Therefore, daily intake of LG2055 would be beneficial for the inhibition of viral replication via induction of MxA gene expression.

Intake of some LAB have been reported to be effective in reducing the prevalence of influenza virus infection or common colds in humans [27, 53, 54, 55]. In this study, the incidence of influenza virus infection during the trial was not significantly different between the placebo and LG2055 groups, because of a very low incidence in both groups. Therefore, it would be worthwhile to assess the effect of LG2055 intake on influenza incidence in a large-scale RCT investigation.

We used two test DYs in this study. One was a control DY, which contained a conventional yogurt starter (*Streptococcus thermophilus*) without LG2055 cells. The other was an active DY, to which LG2055 cells were added to the control DY. The control DY itself could be considered as potential probiotics, but we found little effects in the placebo group (the control DY) concerning the total IgG and IgA concentrations in plasma or NK cell activity or MxA gene expression by administration of control DY compared to those at the baseline (W0). Moreover, the control and active DYs were essentially identical in nutrient content. As a result, we believe that observations in this study are attributed to specific features of LG2055.

In this study, all subjects received a vaccine against influenza virus; therefore, we should take into consideration that these results were obtained from vaccinated subjects administered LG2055. Since we have demonstrated the efficacy of LG2055 on some immune parameters in unvaccinated mice and humans [22, 23, 24], LG2055 may also potentiate the host immune response without vaccination. In this context, we need further clinical trials to evaluate the efficacy of LG2055 on host immune responses without vaccination. To clarify the mechanism of immunomodulatory effect of LG2055. Additionally, we plan to investigate the role of regulatory

T cells and other potential factors, e.g., discrepancy of male/female immune responses.

CONCLUSION:

The present study shows that the intake of DY containing LG2055 increases HI titers against influenza A/H1N1 and B, as well as the seroprotection rate against B after vaccination, as compared with HI titers in healthy volunteer administered a placebo. Furthermore, we revealed that total IgG production in plasma, sIgA production in saliva, NK cell activity, and MxA gene expression in PBMC were enhanced by intake of LG2055. These data suggest that LG2055 activates both adaptive and innate immune responses in humans. We speculate that LG2055 potentiates host defense systems and protects against human pathogenic viruses and bacteria. Further studies are required to understand the precise mechanisms and effects with clinical endpoints.

Competing Interests:

The authors have no financial conflict of interest.

Authors' Contributions:

All the authors contributed to this study

Abbreviations:

IFN, interferon; NK, natural killer; LAB, lactic acid bacteria; PBMC, peripheral blood mononuclear cells; sIgA, secretory immunoglobulin

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