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Original article

Effects of yogurt containing *Lactobacillus plantarum* HOKKAIDO on immune function and stress markers

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ABSTRACT

Lactobacillus plantarum HOKKAIDO (HOKKAIDO strain) was isolated from well-pickled vegetables in Hokkaido, Japan. We report a randomized, double-blind, placebo-controlled study evaluating the effects of *L. plantarum* HOKKAIDO on immune function and stress markers in 171 adult subjects. Subjects were divided into three groups: the *L. plantarum* HOKKAIDO yogurt group, the placebo-1 group who ingested yogurt without the HOKKAIDO strain, and the placebo-2 group who ingested a yogurt-like dessert without the HOKKAIDO strain. Hematological tests and body composition measurements were performed before and after 4 and 8 weeks of blinded ingestion. Although no significant differences in natural killer cell activity were observed, it was found that neutrophil ratio significantly decreased and lymphocytes tended to increase in the HOKKAIDO strain yogurt group compared with the yogurt-like dessert group. In addition, the neutrophil-to-lymphocyte ratio, a stress marker, tended to improve in the HOKKAIDO strain yogurt group compared with the yogurt-like dessert group. These results suggest that the ingestion of HOKKAIDO strain yogurt tends to improve immune activity and decrease stress markers.

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1. Introduction

Several types of microorganisms reside in the intestine and comprise an individual's "microbial flora."[1] Probiotic microorganisms serve various purposes, such as increasing beneficial bacteria-growth, promoting digestion and absorption, and suppressing infectious diseases.[2]

The human immune system performs activities of "innate immunity," otherwise known as the nonspecific immune system that functions as the body's first line of defense, and "adaptive immunity," referred to as the acquired immune system or the specific immune system. Natural killer (NK) cells are innate lymphoid cells that play a role in natural immunity against tumors and infected cells.[3] NK cell numbers decrease because of various reasons, such as aging,[4] stress,[5] and smoking cigarettes.[6,7] Thus, it is worthwhile to investigate whether functional foods or the bioactive components of certain foods can improve NK cell activity. Recently, probiotic organisms, such as *Lactobacillus*, have been shown to have several functional properties, including stimulation of the immune system.[8] The mechanism of NK cell activation by *Lactobacillus* is thought to involve increased production of several cytokines following macrophage phagocytosis of *Lactobacillus*.[9]

Abbreviations: BMI, body mass index; BW, body weight; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HPA, hypothalamic-pituitary-adrenal; LDL-C, low-density lipoprotein cholesterol; NK, natural killer; NLR, neutrophil-to-lymphocyte ratio; TC, total cholesterol; TG, triglyceride.

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The gut–brain-microbiota axis is increasingly recognized as an important regulator of intestinal physiology.[10] Exposure to psychological stress causes activation of the hypothalamic–pituitary–adrenal (HPA) axis that influences intestinal barrier function. Microbial flora affect postnatal development of the HPA stress response in mice,[11] and germ-free mice exhibit more anxiety than their conventional counterparts. These findings suggest that the modulation of intestinal microbiota, through probiotics or symbiotics, could alter human behavior and reduce stress. However, there are limited number of clinical trials that have investigated effects of probiotics on both the immune system and stress-induced disorders.

There are currently three *Lactobacillus* biotope classifications: vegetable *Lactobacillus*, animal *Lactobacillus*, and intestinal *Lactobacillus*. Vegetable *Lactobacillus* exists in fermented foods that are often eaten in Japanese cuisine. It was recently reported that vegetable *Lactobacillus* isolated from vegetarians' intestines[12] activates immune functions and prevents infection and cancer.[13] In addition, vegetable *Lactobacillus* contains plant-derived antibacterial agents, including tannins, and can persist in high-salt and low-pH environments.

Lactobacillus plantarum HOKKAIDO (HOKKAIDO strain) was isolated from well-pickled vegetables in Hokkaido, Japan, by the Hokkaido Food Processing Research Center. This strain is resistant to salt, alcohol, and pH, protects individuals who consume it from bacterial infection, regulates intestinal functions, and improves isoflavone absorption.[14] Regarding immune regulation, it was reported that coincubation of the HOKKAIDO strain with human dendritic cell lines increased Interleukin (IL)-12 mRNA expression.[15] In addition, the HOKKAIDO strain induced the production of IL-8, a neutrophil chemotactic factor, in human monocytes (HTP-1) and OSC70 epithelial cells. These reports demonstrate that the HOKKAIDO strain activates NK cells and likely improves immune function.

These findings warranted a clinical trial to determine whether yogurt containing the HOKKAIDO strain improves immune function and decreases stress markers. However, very few clinical studies have been conducted to specifically assess responses to the HOKKAIDO strain. Here we evaluated whether ingestion of HOKKAIDO strain yogurt improves immune activity and decreases stress markers in Japanese adults.

2. Methods

2.1. Test meal preparation and ingestion method

The composition of the HOKKAIDO strain yogurt investigated in this study is presented in Table 1. The production and packing of test meals were performed by the Hokkaido Milk Product Co. Ltd.

Table 1
Composition of HOKKAIDO strain yogurt compared with placebo yogurt and yogurt-like dessert per 90 g.

Component	HOKKAIDO strain yogurt	Placebo yogurt	Yogurt-like dessert
Calories (kcal)	77	77	82
Water (g)	73.0	73.0	71.8
Proteins (g)	3.1	3.1	2.5
Lipids (g)	2.6	2.6	2.4
Carbohydrates (g)	10.3	10.3	12.7
Ash (g)	0.8	0.8	0.6
Sodium (mg)	45	45	40
HOKKAIDO strain	$\geq 5.0 \times 10^9$ CFU	–	–

Subjects were instructed to ingest daily 90 g of yogurt containing *L. plantarum* HOKKAIDO ($\geq 5.0 \times 10^9$ CFU, Hokkaido strain yogurt) or yogurt fermented with *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus* (Chr. Hansen, Hoersholm, Denmark) without Hokkaido strain (placebo-1) or a yogurt-like dessert without the Hokkaido strain (placebo-2). The HOKKAIDO strain was originally isolated from well-pickled vegetables in Hokkaido, Japan, by the Hokkaido Research Organization.[14]

2.2. Subjects

In this study, 171 subjects (28 males and 143 females, age range 31–69 years) with NK cell activity below 50% were enrolled. Subjects with a recent history of gastrointestinal disorders, pregnancy, significant disease, surgery, severe allergic reaction to food, or current use of any medication were excluded. Mean subject age, body weight (BW), height, body mass index (BMI), body fat percentage, and NK cell activity for each group are reported in Table 2.

The clinical intervention was conducted as a double-blind, placebo-controlled trial. At randomization, the 171 eligible subjects were randomly assigned to one of the three groups (Hokkaido strain yogurt group, placebo-1 group, and placebo-2 group) with adjustment for age, sex, and NK cell activity. The time schedule for this clinical study is shown in Fig. 1.

We performed hematological examinations and body composition (BW, BMI, and body fat percentage) measurements at baseline (week 0) and post-intervention (weeks 4 and 8) for the three groups. The hematological examinations were performed by Sapporo Clinical Laboratory, Inc. (Sapporo, Japan). Each subject's body compositionwas measured with an In-Body device (Biospace Co., Tokyo, Japan).

All subjects provided written informed consent before undergoing any study-related tests, and the study protocol was approved by the Ethics Committee of Hokkaido Information University. The study protocol conformed to the Helsinki Declaration and was registered at the UMIN Clinical Trial Registration System (certificate number UMIN000014138).

2.3. Statistical analysis

The mean and standard deviation of subject characteristics were calculated for each group. Changes in subject values were analyzed using Student's *t*-tests comparing means between the test group and placebo-1 group or between the test group and placebo-2 group. Statistical analyses were performed using SPSS Statistic 19 (IBM, Armonk, NY, USA). *P*-values <0.05 were considered significant.

Table 2
Characteristics of the subjects in the placebo and HOKKAIDO strain yogurt intake groups.

Characteristic	HOKKAIDO strain yogurt	Placebo yogurt	Yogurt-like dessert	*P* value
Subjects, *n*	57	55	59	–
Males, *n* (%)	9 (15.79%)	8 (14.55%)	11 (18.64%)	0.831
Age, years	49.58 ± 8.62	51.15 ± 9.90	51.34 ± 10.94	0.275
Height, cm	159.89 ± 5.96	158.39 ± 6.77	158.11 ± 6.35	0.698
Body weight, kg	54.82 ± 8.96	54.04 ± 7.99	53.48 ± 8.62	0.954
BMI, kg/m^2	21.38 ± 2.83	21.51 ± 2.66	21.36 ± 2.97	0.667
Body fat percentage, %	27.04 ± 6.47	27.40 ± 7.02	26.30 ± 6.55	0.580
NK cell activity, %	32.46 ± 13.39	28.85 ± 11.91	28.85 ± 13.50	0.580

Values shown are mean ± standard deviation. Analysis by analysis of variance was performed for age, height, body weight, BMI, body fat percentage, and natural killer cell activity and by chi-square test for gender. BMI, body mass index; *n* = number of subjects.

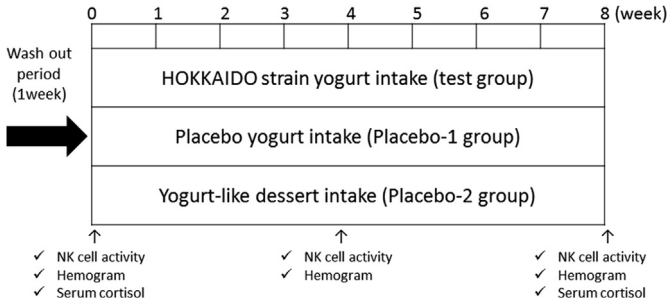

Fig. 1. Clinical study time schedule in weeks. Hematological measurements were conducted at baseline (week 0), week 4, and week 8.

3. Results

3.1. Effects of HOKKAIDO strain yogurt on NK cell activity

There were no significant differences among the three study groups in age, height, BW, BMI, body fat percentage, or NK cell activity at baseline (Table 2). To determine the effect of HOKKAIDO strain yogurt on immune activity, we evaluated NK cell activity (Fig. 2a). There was no significant difference between the test group and placebo-1 group or between the test group and placebo-2 group. Next, to clarify the effects of age on response, we divided the 171 subjects into subgroups with respect to median age: younger subjects (<50 years; $n = 86$) and older subjects (≥50 years; $n = 85$). For the older subjects, there was no significant difference among the three study groups (Fig. 2c). In the younger subjects, however, the NK cell activity of the test group increased significantly compared with that of the placebo-1 group at 8 weeks (placebo-1: −3.04% ± 13.87%; HOKKAIDO strain yogurt: 3.40% ± 9.58%, reported as the change in the percentage of NK cell activity from baseline to 8 weeks, $P = 0.043$) (Fig. 2b).

3.2. Effects of HOKKAIDO strain yogurt on hemogram

Furthermore, we examined the effect of HOKKAIDO strain yogurt on subject hemograms. Although no significant between-group differences were observed in serum monocyte, eosinophil, and basophil ratio (Fig. 3c–e), neutrophils were significantly decreased in the HOKKAIDO strain yogurt group compared with the placebo-2 group (Fig. 3a) (placebo-2: 1.98% ± 9.07%; HOKKAIDO strain yogurt: −0.93% ± 5.21%, reported as the change in the percentage of neutrophil from baseline to 8 weeks, $P = 0.038$). In addition, lymphocytes were slightly increased at 8 weeks following the ingestion of HOKKAIDO strain yogurt (Fig. 3b) (placebo-2: −1.47% ± 8.00%; HOKKAIDO strain yogurt: 0.63% ± 4.47%, reported as the change in the percentage of lymphocyte from baseline to 8 weeks, $P = 0.087$).

3.3. Effects of HOKKAIDO strain yogurt on stress markers

Neutrophil-to-lymphocyte ratio (NLR) and serum cortisol are commonly used as stress-induced biomarkers. The HOKKAIDO strain yogurt intake group showed a slight decrease in NLR compared with the placebo-2 intake group at 8 weeks (Fig. 4a) (placebo-2: 0.05 ± 0.84; HOKKAIDO strain yogurt: −0.09 ± 0.52, reported as the change in the rate of NLR from baseline to 8 weeks, $P = 0.093$). In addition, serum cortisol was suppressed by intake of the HOKKAIDO strain yogurt compared with the placebo-2 group, but only marginally (Fig. 4b) (placebo-2: 0.29 ± 2.21 μg/dl; HOKKAIDO strain yogurt: −0.25 ± 2.6 μg/dl, reported as the change in serum cortisol level from baseline to 8 weeks, $P = 0.236$).

3.4. Levels of biomarkers of lipid metabolism, glucose metabolism, and body composition after the ingestion of HOKKAIDO strain yogurt

We examined the levels of several biomarkers of blood metabolism, lipid and glucose metabolism, and body composition. In the Hokkaido strain yogurt group, minimal changes were observed in the parameters of lipid metabolism (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride) and glucose metabolism (fasting plasma glucose and hemoglobin A1c), as well as body composition (BW, BMI, and body fat percentage) as shown in Table 3. These results suggest that the ingestion of HOKKAIDO strain yogurt has no or minimal unfavorable effects on these parameters even at the dose of 90 g/day.

4. Discussion

The results of our randomized, double-blind, placebo-controlled, parallel-group clinical study indicate the potential effects of the HOKKAIDO strain yogurt on stress and immune activity. Although no significant differences in NK cell activity were observed, the younger subjects (<50 years) in the HOKKAIDO strain yogurt group showed increased NK cell activity compared with those in the placebo yogurt group. In addition, the trends of decreased neutrophil and NLR were observed in the HOKKAIDO strain yogurt group when compared with yogurt-like dessert

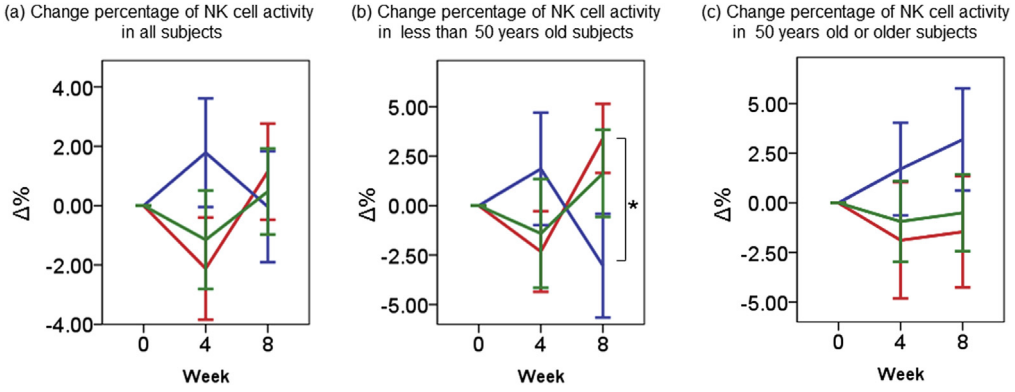

Fig. 2. Changes in the percentage of NK cell activity from pre- and post-HOKKAIDO strain yogurt intake. Values are the mean ± standard error. *$P < 0.05$, Student's t-test. (a) NK cell activity in all subjects. (b) NK cell activity in less than 50 years old subjects. (c) NK cell activity in 50 years old or older subjects. Red line: HOKKAIDO strain yogurt; blue line: placebo-1; green line: placebo-2. NK, natural killer.

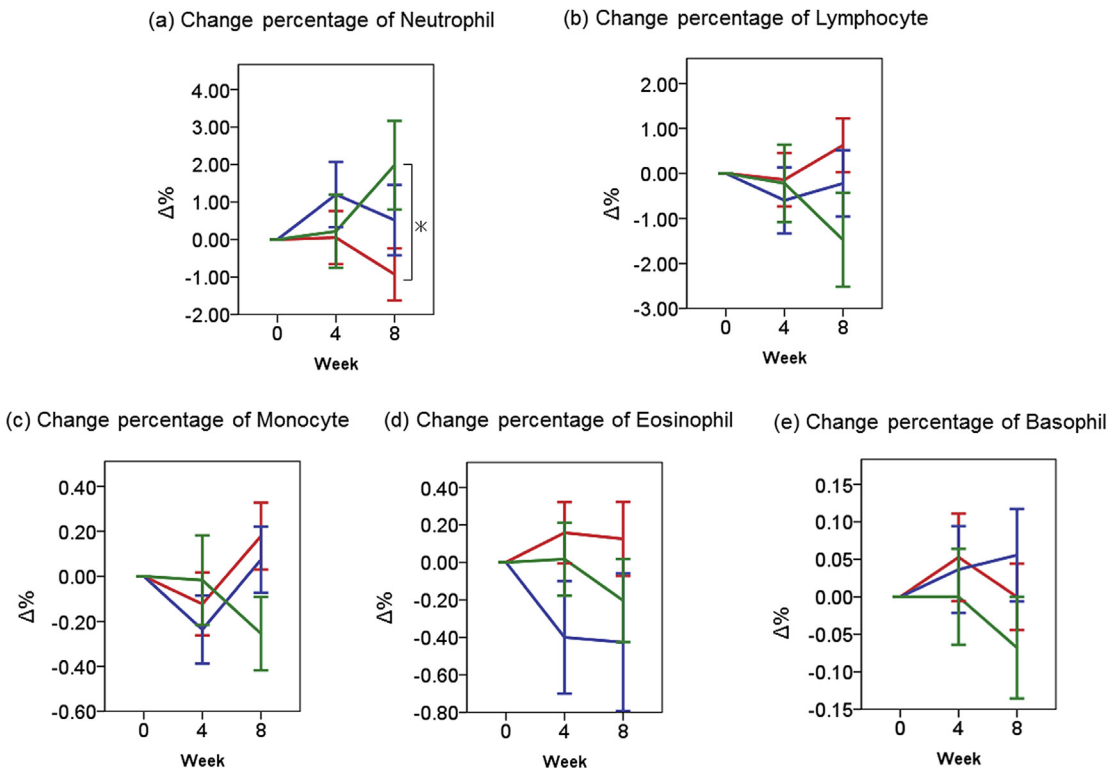

Fig. 3. Changes in the percentage of hemogram from before to after HOKKAIDO strain yogurt intake. Values are the mean ± standard error. *$P < 0.05$, Student's *t*-test. (a) Neutrophil. (b) Lymphocyte. (c) Monocyte. (d) Eosinophil. (e) Basophil. Red line: HOKKAIDO strain yogurt; blue line: placebo-1; green line: placebo-2.

(placebo-2) group. Therefore, the potential effects of HOKKAIDO strain yogurt include improved immune function and stress reduction.

We observed no statistically significant differences in the percentage change in NK cell activity among the three study groups when evaluated in all age subjects. However, the percentage change in NK cell activity increased in the younger subjects (<50 years) of the HOKKAIDO strain yogurt group compared with the placebo-1 group at study week 8. There have been various reports on the influence of age on NK cell activity. Vital et al. reported that NK cell activity is decreased with aging.[16] Conversely, Krishnaraj et al. suggested that NK cell activity increases with aging.[17] Moreover, DelaRosa et al. suggested that overall NK cell activity was unchanged with age because individual cell activity decreased, but cell numbers increased.[18] In our clinical study, initial NK cell values were widely varied in subjects over 50 years old compared with subjects less than 50 years old. This fact might influence the lack of change in NK cell activity with diet change in the older cohort. This possibility requires further investigation to determine how HOKKAIDO strain yogurt ingestion increased NK cell activity in the younger subjects. Moreover, the initial percentage of NK cell activity at baseline was correlated strongly with the percentage change in NK cell activity at week 8 (Pearson correlation coefficient: 0.379, $P = 0.01$), but no differences were observed between

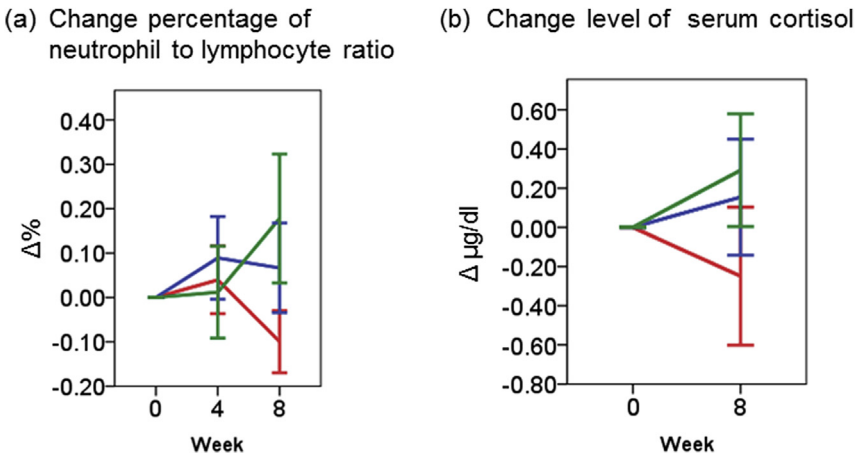

Fig. 4. Changes in the percentage and levels of neutrophil-to-lymphocyte ratio and serum cortisol from before to after HOKKAIDO strain yogurt intake. Values are the mean ± standard error. (a) *$P < 0.05$, Student's *t*-test. Neutrophil-to-lymphocyte ratio. (b) Serum cortisol. Red line: HOKKAIDO strain yogurt; blue line: placebo-1; green line: placebo-2.

Table 3
Biochemical data.

		Week 0	Week 4	Week 8
TC (mg/dl)	Test yogurt	212.18 ± 38.47	209.98 ± 38.10	212.68 ± 42.34
	Placebo-1	203.55 ± 37.05	205.49 ± 36.63	201.63 ± 34.56
	Placebo-2	211.90 ± 35.38	213.64 ± 33.87	211.61 ± 32.10
LDL-C (mg/dl)	Test yogurt	129.35 ± 32.66	127.82 ± 31.53	131.88 ± 36.40
	Placebo-1	121.02 ± 32.66	122.98 ± 33.27	120.91 ± 32.19
	Placebo-2	130.97 ± 36.64	132.02 ± 36.44	132.05 ± 33.71
HDL-C (mg/dl)	Test yogurt	77.04 ± 17.85	75.16 ± 17.21	74.66 ± 17.75
	Placebo-1	76.65 ± 15.46	75.84 ± 16.14	75.44 ± 14.81
	Placebo-2	75.08 ± 18.92	74.58 ± 18.07	74.12 ± 18.53
TG (mg/dl)	Test yogurt	78.39 ± 46.03	81.25 ± 41.53	83.84 ± 45.51
	Placebo-1	72.73 ± 33.75	75.11 ± 29.21	78.56 ± 36.27
	Placebo-2	81.64 ± 40.98	90.86 ± 61.36	90.83 ± 46.65
FPG (mg/dl)	Test yogurt	87.23 ± 7.65	87.42 ± 7.00	86.27 ± 6.00
	Placebo-1	84.40 ± 5.18	85.27 ± 5.63	83.94 ± 6.37
	Placebo-2	88.17 ± 9.85	88.05 ± 9.56	87.15 ± 8.30
HbA1c (%)	Test yogurt	5.26 ± 0.33	5.21 ± 0.32	5.27 ± 0.31
	Placebo-1	5.27 ± 0.27	5.19 ± 0.27	5.27 ± 0.29
	Placebo-2	5.31 ± 0.34	5.27 ± 0.36	5.33 ± 0.40
BW (kg)	Test yogurt	55.06 ± 9.06	54.97 ± 9.24	54.88 ± 9.37
	Placebo-1	54.01 ± 8.18	53.95 ± 8.33	53.86 ± 8.45
	Placebo-2	53.41 ± 8.57	53.56 ± 8.87	53.59 ± 8.85
BMI (kg/m^2)	Test yogurt	21.46 ± 2.87	21.42 ± 2.93	21.34 ± 2.99
	Placebo-1	21.52 ± 2.87	21.49 ± 2.91	21.34 ± 2.96
	Placebo-2	21.33 ± 2.95	21.38 ± 3.01	21.39 ± 3.00
Body fat (%)	Test yogurt	27.26 ± 6.63	27.49 ± 6.21	27.31 ± 6.16
	Placebo-1	27.55 ± 7.08	27.44 ± 7.00	27.63 ± 7.29
	Placebo-2	26.29 ± 6.50	26.73 ± 6.46	26.97 ± 6.46

BMI, body mass index; BW, body weight; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride. Values are mean ± standard deviation.

the overall groups. These results imply that the increase of NK cell activity in the HOKKAIDO strain yogurt group could be the result of a low initial percentage of NK cell activity in the Hokkaido strain yogurt group compared with the placebo-1 group. Further investigation is required to evaluate the effects of HOKKAIDO strain yogurt on NK cell activity.

In our clinical study, neutrophils were significantly decreased in the HOKKAIDO strain yogurt group compared with the placebo-2 group. In addition, lymphocytes were slightly increased at week 8 following ingestion of the HOKKAIDO strain yogurt. Moreover, the HOKKAIDO strain yogurt group showed a slightly decreased NLR compared with the placebo-2 group at week 8. NLR has been associated with inflammation and stress.[19] Serum cortisol increases the neutrophil half-life and induces neutrophil release from myeloid or splenic neutrophil pools to the blood. Therefore, cortisol increases serum neutrophil counts.[20] Moreover, the states of sympathetic dominance increase NLR, which is the reason why adrenalin facilitates an increase in neutrophil proliferation through binding to the adrenalin receptors expressed by neutrophils.[21] In addition, it was reported that patients with Alzheimer's disease had high NLRs compared with healthy subjects, and that NLR was correlated with beta amyloid deposition.[22] These findings suggest that the HOKKAIDO strain yogurt exerts both anti-stress and anti-inflammatory effects. In a stressful environment, humans activate the HPA axis and the sympathetic nervous system to maintain homeostasis. It was recently reported that intestinal flora are involved in HPA.[23] For example, increasing adrenocorticotropic hormone by exposure to an acute stressor was higher in germ-free mice than control mice in vivo.[11] Moreover, ingestion of probiotics significantly improved depressed mood and the degree of conscious stress in a human trial.[20] In our clinical study, serum cortisol was more suppressed in the HOKKAIDO group than the placebo-2 group, although this suppression was not significant. Cortisol has been used in many studies as an indicator of the HPA axis stress response, which is activated by exposure to an acute stressor. These findings may suggest that the mechanism of *L. plantarum* HOKKAIDO in HOKKAIDO strain yogurt reduction in stress is through HPA via regulation of intestinal flora, resulting in a decrease in stress markers, NLR, and serum cortisol. In addition, it was reported that mental stress and NK cell activity are closely related. NK cell activity in students declined on the day of examinations compared with 1 month before examinations, suggesting that mental stress decreases NK cell activity.[24] In our clinical study, NLR improved more in <50-year-old subjects ($P = 0.079$, data not shown), which indicated that improvement of NK cell activity and NLR was correlated with HOKKAIDO strain ingestion. This possibility requires further investigation to clarify the effects of the Hokkaido strain yogurt on stress relief in subjects who are subject to daily stressors.

5. Conclusion

In our clinical study, the younger subjects (<50 years) in the HOKKAIDO strain yogurt group showed increased NK cell activity compared with those in the placebo yogurt group. Neutrophils were decreased in subjects who ingested HOKKAIDO strain yogurt compared with those who ingested the yogurt-like dessert. In addition, NLR, which is associated with stress, trended lower in the HOKKAIDO strain yogurt group compared with the yogurt-like dessert group. In conclusion, HOKKAIDO strain yogurt may potentially stimulate immune functions and reduce stress markers. This possibility requires further investigation.

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Conflicts of interest

The authors state that they have no conflicts of interest to declare.

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