Journal of Probiotics & Health
ISSN: 2329-8901

Maekawa. et al, J Prob Health 2016, 4:1
DOI: 10.4172/2329-8901.1000134

Research Article Open Access

Supplementation with *Lactobacillus pentosus* strain S-PT84 and Vitamin B Mixture Enhances Natural Killer Cell Activity in Healthy Humans

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Received date: Nov 27, 2015; **Accepted date:** Dec 23, 2015; **Published date:** Dec 28, 2015

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Abstract

Background: The maintenance of natural killer (NK) cell activity is critical for health, because NK cells target infected cells and cancer cells. In this study, we investigated the effect on human NK activity of supplementation with a combination of S-PT84 and vitamin B mixture (VBM: vitamin B1 (thiamin), vitamin B2 (riboflavin), and vitamin B6 (pyridoxine)).

Methods: We designed randomized, placebo-controlled, double-blind, parallel-group comparative studies. In the first study, we recruited healthy middle-aged (30- to 69-year-old) subjects who had low NK activity (Study 1). Subjects received a combination of S-PT84 (1.5×10^9 cells) and VBM or placebo supplement (derived from dextrin) for 4 weeks, with a 4-week follow-up phase. In the second study, healthy middle-aged (40- to 69-year-old) subjects received a combination of S-PT84 and VBM or placebo supplement for 12 weeks (Study 2). In both studies, we measured NK activity in peripheral blood mononuclear cells using the ^{51}Cr-release assay, and assessed safety by comprehensive analysis of blood hematology, serum biochemistry, urinalysis, and physical state.

Results: In Study 1, supplementation with 1.5×10^9 cells of S-PT84 and VBM enhanced NK activity during the dosing interval; NK activity subsequently returned to baseline during the wash-out period. In Study 2, supplementation with S-PT84 and VBM enhanced NK activity during the 12 weeks of ingestion. The increase of NK activity in the placebo group ($r=-0.193$, $p<0.01$) and S-PT84-VBM group ($r=-0.352$, $p<0.01$) was inversely correlated with NK activity at baseline, respectively. Moreover, the regression coefficient of S-PT84-VBM's regression line was smaller than that of placebo's significantly ($t=2.14$, $p=0.03$). No adverse effects were observed in either study.

Conclusions: These results suggest that daily supplementation with 1.5×10^9 cells of S-PT84 and VBM enhances NK activity in humans, even during long-term (12-week) administration. Therefore, our results indicate that S-PT84 and vitamin B supplementation may promote healthy state.

Keywords: *Lactobacillus pentosus* strain S-PT84; NK activity; PBMC; Human

Introduction

Natural killer (NK) cells are lymphocytes that have the ability to kill infected cells and cancer cells. Several lines of evidence show the importance of NK cells in defense of the human body. For instance, Biron *et al.* reported that patients who were genetically deficient in NK cells while harboring normal T cells and B cells suffer severe and frequent infection with herpes virus [1]. The mortality rate due to infectious disease is elevated in elderly patients with low NK activity compared to those with high NK activity [2]. These results suggest that NK cells are essential for preventing pathogenic infection and prolonging lifespan.

Lactic acid bacteria (LAB) can modulate mucosal and systemic immune responses and are widely used as health food ingredients. Notably, oral administration of *Lactobacillus casei* strain Shirota (LcS) yields increased NK activity *in vivo* [3], and fermented milk containing LcS enhances NK activity in humans by stimulation of interleukin-12 (IL-12) production [4-7]. Oral administration of the extracellular polysaccharides produced by *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 also induces interferon-γ (IFN-γ) production and augments NK activity in mice and in humans [8,9].

We have reported several physiological functions of *L. pentosus* strain S-PT84 *in vitro* and *in vivo*. Nonaka *et al.* reported that S-PT84 induces IL-12 production from macrophages *in vitro* and enhances NK activity in mice, and S-PT84 also exhibits anti-allergic effects by modulating the T helper (Th) type 1/type 2 balance toward a Th1-dominant state and by inducing regulatory T cells [10]. Izumo *et al.* reported that intranasal administration of S-PT84 augments NK activity in lung and increases IFN-α production in bronchoalveolar lavage fluids, protecting against influenza virus infection in mouse [11]. In addition, Koizumi *et al.* reported that S-PT84 stimulates IFN-γ and IL-12 production through interactions between Toll-like receptor (TLR)-2 and TLR-4 on dendritic cells and NK cells [12]. Therefore, S-PT84 has the potential to modulate immune responses and might be useful for health maintenance in humans.

In previous work, we performed preliminary investigations on the effect of S-PT84 supplementation on human NK activity. Daily intake of a supplement containing 1.5×10^9 cells of heat-killed S-PT84 for 4 weeks enhanced NK activity in humans (data not shown). Moreover, NK activity returned to the baseline level after a follow-up period, and no adverse effects were observed. Therefore, it was suggested that S-PT84 has the potential to enhance NK activity in humans and might be useful for health maintenance. Vitamins B1, B2, and B6 are well known to contribute to the maintenance of glucose metabolism, lipid metabolism, and protein turnover. According to the National Health and Nutrition Survey 2013 in Japan [13], an entire generation of the Japanese population may be deficient for the intake of vitamins B1, B2, and B6 as judged by the 2010 dietary reference intakes. Therefore, we considered that the combination of S-PT84 and a mixture of vitamins B1, B2, and B6 might serve as a valuable supplement for the maintenance of immune function and nutritional state.

In the present work, we designed randomized, placebo-controlled, double-blind, parallel-group studies and performed two clinical trials to address the following objectives. In Study 1, we recruited healthy middle-aged subjects who had relatively low levels of NK activity and investigated the effect on human NK activity of short-term (4-week) supplementation with heat-killed S-PT84 and vitamin B mixture (VBM: vitamin B1 (thiamin), vitamin B2 (riboflavin), and vitamin B6 (pyridoxine)). In Study 2, we assessed the effect of long-term (12-week) ingestion of heat-killed S-PT84 and VBM on NK activity in healthy middle-aged subjects. In order to investigate whether the combination of S-PT84 and VBM could enhance NK activity in the general population, we did not impose criteria for NK activity for entry into Study 2. Safety assessments were performed for both studies.

Material and Methods

Study design

Prospective, randomized, placebo-controlled, double-blind, parallel-group comparative studies were designed to assess the efficacy and safety of the experimental supplement. The study protocols were approved by the ethics committee on human experimentation of Suntory Holdings Ltd., and were conducted in accordance with the principles of the Declaration of Helsinki and "Ethical Guidelines for Epidemiological Research" (recognized by the Japanese Government in 2008). Before entry into a study, each prospective participant was given a full explanation of the objectives and methods of the study, and informed consent was obtained from each individual.

In Study 1, enrolled subjects ingested experimental supplements for 4 weeks, and then were monitored during a 4-week follow-up phase without the experimental supplement. The enrolled subjects underwent medical and physical examinations at baseline and at 1, 2, 4, and 8 weeks after the start of ingestion. Blood samples (from a peripheral vein) were collected at the time of each examination.

In Study 2, enrolled subjects ingested experimental supplements for 12 weeks. The enrolled subjects underwent medical and physical examinations at baseline and 12 weeks after the start of ingestion. Blood (from a peripheral vein) and urine samples were collected at the time of each examination.

In both studies, subjects were instructed not to change their daily life style (including dietary habits, physical activity, medicine, alcohol, smoking, and sleep) compared to that before the present study. Subjects self-reported their physical and mental state along with the frequency of supplement ingestion.

Subjects

For Study 1, we recruited subjects who had relatively low levels of NK activities (under 45%), because Takeda *et al.* reported a trial of the effect of LcS on human NK cell activity with relatively low levels (under 45%) of cytotoxicity [4]. Consequently, we were able to enroll subjects who had low NK activities (under 40%). Recruitment for Study 2 did not include criteria for NK activity. For both studies, individuals exhibiting any of the following criteria were excluded: a morbidity or history of a chronic disease or autoimmune disease or alimentary disease; a disease requiring treatment; pregnant or nursing; ingestion of a drug or functional food, for example the *Lactobacilli* supplement and the *Lactobacilli* fermented milk, expected to impact the study results; participation in an another study; participation in another clinical trial within the previous 4 weeks; or assessment by doctor's judgment of inappropriateness for participation in the study. In Study 2, we excluded subjects who reported no subjective symptoms of upper respiratory infection in the preceding 2 years. This additional criterion was added in order to exclude subjects who had elevated fundamental immune responses.

In Study 1, 44 healthy middle-aged (30- to 69-year old) subjects were enrolled. The subjects were separated into 2 groups, to be dosed with placebo (placebo group) or with the combination of S-PT84 and VBM (SVB group). Following initial assignment, we confirmed that subjects were evenly distributed for each of 3 parameters (age, sex, and NK activity) at the beginning of the trial; no significant differences were observed between the groups.

In Study 2, 417 healthy middle-aged (40 to 69-year old) subjects were enrolled. The subjects were separated into 2 groups, to be dosed with placebo (placebo group) or with the combination of S-PT84 and VBM (SVB group). Following initial assignment, we confirmed that subjects were evenly distributed for each of 3 parameters (age, sex, and NK activity) at the beginning of a trial; no significant differences were observed between the groups.

The experimenters were blinded to the group assignments during the studies. The identities of the groups were disclosed only upon the completion of data collection.

Experimental supplements

S-PT84 was isolated originally from Kyoto pickles "SHIBAZUKE" [10] and subsequently was cultivated in a medium containing glucose and yeast extract (AromildTM, SK yeast extract Hi-K) at 37°C for 24 hours. Cultured bacteria were collected by centrifugation, mixed with dextrin, and heat-killed at 90°C for 30 min. Heat-killed S-PT84 was lyophilized for use in the trials.

In Studies 1 and 2, we used S-PT84 and VBM supplements containing (per 3-tablet dose) 1.5×10^9 S-PT84, 25 mg vitamin B1, 12 mg vitamin B2, and 10 mg vitamin B6. For both studies, placebo supplement was made from dextrin. Experimental supplements were ingested with water once per day.

Efficacy assessment

NK activity was measured using peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood by the superposition method. PBMCs were washed with phosphate-buffered saline (PBS)

and then resuspended in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum. PBMCs were prepared at 2.0×10^6 cell/mL and used as effector cells. Target cells consisted of cells of K562, a human myeloid leukemia cell line. K562 cells were labeled with 200 μCi of ^{51}Cr for 60 min at 37°C and then were washed with PBS. K562 cells were prepared at 1.0×10^5 cells/mL. Effector cells were added to target cells at an effector-to-target (E/T) ratio of 20 and then incubated for 4 h at 37°C in 5% CO_2. NK activity was calculated according to the following formula:

NK Activity (%)=((Experimental Release)–(Spontaneous Release))/((Maximal Release)–(Spontaneous Release))×100

Maximal release of ^{51}Cr was measured by lysing cells using 1 N HCl solution.

Safety assessment

Safety assessments were performed for all subjects who ingested a supplement one or more times. Safety was assessed on the basis of the incidence and severity of adverse events reported throughout the treatment and follow-up periods, as well as changes in physical parameters and laboratory test variables including hematology, blood biochemistry, and urinalysis.

Statistical analysis

In Study 1, to test time and treatment effects for several time points and groups, two-way repeated-measures ANOVA was used to analyze differences in the effect of the intervention on outcome measures. Significant differences in NK cell activity between the groups at a given time point were determined using Student's t-test, and the time-dependent differences in NK cell activity compared to baseline level within a given group were determined using Bonferroni correction.

In Study 2, significant differences in NK cell activity between the groups at a given time were determined using Student's t-test, and the time-dependent differences in NK cell activity within a given group were determined using paired Student's t-test. The correlation between the increase of NK activity after the start of supplementation and baseline levels of NK activity was determined by Pearson's correlation coefficient test. Difference of correlation coefficients of the regression line obtained from placebo group and S-PT84-VBM group was determined by testing the t-value.

For all tests, p-values less than 0.05 were statistically-significant difference (two-sided statistical test). Statistical calculations were performed with SPSS statistical software (IBM SPSS Statistics, version 23).

Results

Baseline characteristics of study groups

In Study 1, a total of 87 participants were screened, and 44 subjects were randomly assigned to 2 groups, placebo group (n=23) and S-PT84-VBM group (n=21). All of the subjects completed the study. One subject in S-PT84-VBM group was removed from the study due to an influenza infection during the treatment period. The background characteristics of the Study-1 subjects for efficacy assessment are summarized in Table 1. No significant pre-study differences were observed between the groups in terms of age, sex, or NK activity.

	Placebo (n=23)	SVB (n=20)
Age (years)	47.7 ± 9.0	48.7 ± 12.2
Sex (male/female)	6/17	5/15
NK activity (%)	29.0 ± 10.9	29.2 ± 13.5

Table 1: Baseline characteristics of the subjects (Study 1).

All values are expressed as mean ± SD. There was no significant difference between the groups (Placebo; placebo group, SVB; S-PT84-VBM group) in baseline data. Natural killer (NK) cell activity was measured against K562 target cells in single point for each individual with an effector-to-target (E/T) ratio of 20 using peripheral blood mononuclear cells (PBMCs).

In Study 2, a total of 561 participants were screened, and 417 subjects were randomly assigned to 2 groups, placebo group (n=210) and S-PT84-VBM group (n=207). In the placebo group, 1 subject who received head bruise, lumbar bruise, and cervical sprain due to a traffic accident was withdrawn from the study at the doctor's discretion, and 1 subject withdrew informed consent during the course of the study. In the S-PT84-VBM group, 3 subjects withdrew informed consent during the course of the study. All of the remaining 412 subjects (n=208 in placebo group, n=204 in S-PT84-VBM group) completed the study. We subsequently excluded 12 subjects from the placebo group and 10 subjects from the S-PT84-VBM group because of deviations from the study protocol or the use of concomitant treatments that had the potential to interfere with the results. The background characteristics of the Study-2 subjects for efficacy assessment are summarized in Table 2. No significant pre-study differences were observed between the groups in terms of age, sex, or NK activity.

	Placebo (n=196)	SVB (n=194)
Age (years)	48.6 ± 6.4	49.3 ± 6.7
Sex (male/female)	81 / 115	76 / 118
NK activity (%)	24.1 ± 1.0	23.1 ± 0.9

Table 2: Baseline characteristics of the subjects (Study 2).

All values are expressed as mean ± SD. There was no significant difference between the groups in baseline data. NK activity was measured against K562 target cells in single point for each individual with an effector-to-target (E/T) ratio of 20 using peripheral blood mononuclear cells (PBMCs).

NK activity in peripheral blood mononuclear cells

Study 1: The combination effect of S-PT84 and VBM on NK activity

The increase of NK activity from baseline to 2 weeks in the S-PT84-VBM group was significantly larger than that in the placebo group (Table 3). NK activity was still nominally larger at 4 weeks, but the difference at the later time point fell short of statistical significance. After a follow-up (recovery) period, NK activity in both groups returned to baseline levels.

Study 2: The effect of S-PT84 and VBM on NK activity for a long period

The increase of NK activity from baseline to 12 weeks in the S-PT84-VBM group was significantly larger than that in the placebo group (Table 4). Negative correlation were found between baseline levels of NK activity and the increase of NK activity from baseline to 12 weeks in both placebo group (Figure 1A; r=-0.193, p<0.01) and S-PT84-VBM group (Figure 1B; r=-0.352, p<0.01). The regression lines using the increase of NK activity from baseline to 12 weeks as outcome variable (y) and baseline levels of NK activity as predictor variable (x) were y=-0.1381x+8.7407 (p<0.01) and y=-0.3505x +15.711 (p<0.01) for placebo group and S-PT84-VBM group, respectively. The regression coefficient of S-PT84-VBM group's regression line was smaller than that of placebo group's significantly (t=2.14, p=0.03).

	Placebo (n=23)	SVB (n=20)	Two-way ANOVA (Group × Time) P-value
NK activity (%)			
Baseline	28.3 ± 2.4	27.1 ± 2.9	0.526
1 week	29.3 ± 2.5	29.6 ± 3.5	
2 weeks	28.4 ± 2.7	31.5 ± 3.5	
4 weeks	30.3 ± 2.8	30.5 ± 3.6	
Post hoc	24.2 ± 2.2	24.5 ± 3.2	
NK activity (%)			
1 week	1.1 ± 1.6	2.5 ± 1.7	
2 weeks	0.1 ± 1.4	4.4 ± 1.7#	
4 weeks	2.0 ± 1.8	3.4 ± 1.4	
Post hoc	-4.1 ± 2.3	-2.6 ± 1.7	

Table 3: Change in NK activity during the study period (Study 1).

NK activity was measured 5 times against K562 target cells with an effector-to-target (E/T) ratio of 20 using peripheral blood mononuclear cells (PBMCs). ΔNK activity indicates the changes in values from baseline (before supplementation). Data are shown as mean ± SE. $^{\#}p$ <0.05 compared to placebo group using Student's t-test.

Safety

In Study 1, the safety analysis was performed with 44 subjects. During the study period, 18 adverse events (12 events in placebo group and 6 events in S-PT84-VBM group) were reported. Some events were considered possible consequences of supplement administration; these events included 2 events in the placebo group (constipation and bloating) and 2 events in the S-PT84-VBM group (loose stool and abdominal pain). However, these events were considered to fall within the range of normal physiological values. In Study 2, the safety analysis was performed with 417 subjects. During the study period, 205 adverse events (89 events in the placebo group and 116 events in the S-PT84-VBM group) were reported, and no side effects relating to the experimental supplement were observed. These findings indicate that supplementation with the combination of S-PT84 and VBM had no side effect, suggesting that this supplement is a safe food ingredient. Blood hematological and blood biochemical parameters were determined for the subjects who completed the study regimen (Supplementary Tables 1 and 2). Values were within normative ranges.

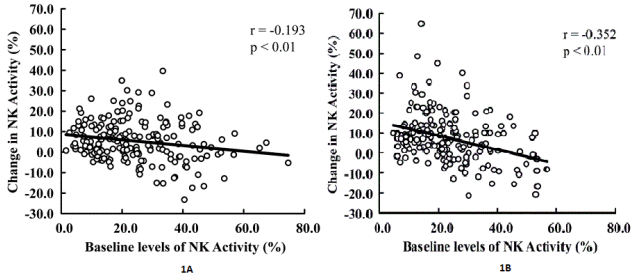

Figure 1: Relationship between the change in NK activity over 12 weeks of supplementation and baseline levels of NK activity.

Figure 1A and 1B show the result of placebo group and S-PT84-VBM group, respectively. The change of NK activity indicates the change in values from baseline (before supplementation) to 12 weeks (at the end of supplementation). The correlation was determined by Pearson's method. Statistical significance was set at p<0.05.

	Placebo (n=196)	SVB (n=194)
NK activity (%)		
Baseline	24.1 ± 1.0	23.1 ± 0.9
12 weeks	29.5 ± 1.1***	30.7 ± 1.0***
ΔNK activity (%)		
12 weeks	5.4 ± 0.7	7.7 ± 0.9#

Table 4: Change in NK activity during the study period (Study 2).

NK activity was measured 2 times against K562 target cells with an effector-to-target (E/T) ratio of 20 using peripheral blood mononuclear cells (PBMC). ΔNK activity indicates the change values from baseline (before supplementation). Data are shown as mean ± SE. $^{***}p$ <0.001 compared to the baseline (before supplementation) using paired Student's t-test. $^{\#}p$ <0.05 compared to placebo group using Student's t-test.

Discussion

We confirmed that daily ingestion of a supplement consisting of heat-killed S-PT84 and a vitamin B mixture (VBM, including vitamin B1 (thiamin), vitamin B2 (riboflavin), and vitamin B6 (pyridoxine)) enhanced NK activity, and that this enhancement persisted for a long-term (12-week) interval while dosing was continued.

In Study 1, we found that ingestion of 1.5×10^9 cells of heat-killed S-PT84 and VBM enhanced NK activity in PBMCs, and showed that NK activity returned to the baseline levels after a follow-up recovery period of 4 weeks in subjects who had relatively low levels of NK activities. Gill *et al.* similarly reported that ingestion of *L. rhamnosus* HN001 or *Bifidobacterium lactis* HN019 for 3 weeks enhanced NK activity in PBMCs in the elderly, with NK activity returning to baseline levels

following cessation of probiotic intake [14]. The same phenomenon was also observed in our study, suggesting that sustained intake of the S-PT84 and VBM supplement might be required to enhance NK activity.

Hirose *et al.* reported that 12 weeks ingestion of heat-killed *L. plantarum* strain L-137, which is genetically closely related to our *L. pentosus* strain, modulated Th1/Th2 balance toward a Th1-dominant state, however, NK activity was not determined [15]. Mañé J *et al.* reported that 12 weeks ingestion of a mixture of *L. plantarum* CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects, and increased the number of NK (CD56+CD16+) cells [16]. However, they did not investigate the enhancement of NK activity even though they confirmed the effect of *L. plantarum* strain on systemic immunity. To our knowledge, the effect of the long-term ingestion of *L. plantarum* or *L. pentosus* strain on human NK activity is not clear yet. Then, in Study 2, we investigated whether the long-term (12-week) ingestion of S-PT84 and VBM enhanced NK activity in patients with normal NK activity levels, a patient population expected to resemble the general population. After 12 weeks of dosing, the increases of NK activity in the S-PT84-VBM-treated group were significantly larger than those in the placebo group. Although NK activity in placebo group was significantly increased from baseline level, we thought that this phenomenon was affected by season variation due to perform the Study 2 from autumn to winter [17]. Hence, we confirmed that the supplementation with the combination of S-PT84 and VBM was efficacious in providing elevated levels of NK activity.

An earlier clinical trial demonstrated that LcS enhanced NK activity in healthy middle-aged volunteers who entered the study with relatively low levels of NK activity, such that the increase of NK activity was inversely correlated with the levels of NK activity before the initiation of dosing [4]. In Study 2, the increase of NK activity demonstrated an inverse correlation with baseline NK activity in general population (with baseline NK activities ranging from 1.6%~74.6%; Figure 1B). Furthermore, when Study-2 patients dosed with S-PT84 + VBM were classified based on their baseline NK activities, individuals with initial activities below 21.2% (a medium of baseline NK activity in all subjects; Study 2) exhibited significantly larger increases in NK activity than those with initial activities above the median (12.0 ± 1.1% vs. 3.1 ± 1.2%, respectively; $p<0.001$). Among individuals with baseline NK activities below the median, the increases in NK activities differed significantly when comparing the placebo group with the S-PT84-VBM group (6.8 ± 0.8% vs. 12.0 ± 1.1%, respectively; $p<0.001$). These results are consistent with those of Dong *et al.*, who reported that LcS consumption had a greater effect on NK activity enhancement in healthy volunteers who entered the study with lower NK activities [18]. In contrast, among the individuals with baseline NK activities above the 21.2% median, the increases of NK activity did not differ significantly between the placebo group (4.0 ± 1.1%) and the S-PT84-VBM group (3.1 ± 1.2%). When we summarize these results, we speculated that S-PT84 does not affect to enhance immune function above normal levels in healthy individuals, but rather modulates it back to normal levels in situations when immune function is impaired.

NK cells are activated by IL-12, specifically Th1 cytokine. IL-12 is produced by antigen-presenting cells such as macrophages, dendritic cells [19-21], and it was well-known that these antigen-presenting cells existed and modulated mucosal immunity in the intestine [22]. Koizumi *et al.* reported that S-PT84 stimulates IFN-γ and IL-12 production by dendritic cells via processes that depend on Toll-like receptor (TLR)-2 and TLR-4, resulting in enhanced NK activity in mouse spleen cells [12]. Nonaka *et al.* reported that S-PT84 increased IL-12 production by peritoneal macrophages, thereby enhancing NK activity in mice [10]. Therefore, we speculate that the mechanism of NK activity enhancement by S-PT84 might be mediated through intestinal immunity via activation of IL-12 production by dendritic cells or monocytes. To clarify the effect of S-PT84 on the increase of cytokine production, such as IL-12 and IFN-γ related to enhance the NK activity, further examinations are needed.

NK cell-mediated innate immunity is important for protection from various infections and/or cancer. Ogata *et al.* reported that lower NK activity in the elderly is associated with increased rates of infection (such as upper airway infection, pneumonia, and urinary tract infection) and reduced survival due to infection [2]. Imai *et al.* reported that medium and high cytotoxic activities of peripheral-blood lymphocytes are associated with reduced cancer risk [23]. Therefore, NK activity is critical to spend a healthy life. However, in modern society, we are exposed in our daily life to many stress factors that negatively influence NK activity. Previous reports indicate that Boscolo *et al.* reported that blood NK activity is impaired both by occupational stress and by job loss or insecurity [24]. Furthermore, NK cell activity is readily influenced by various mental stress or by other stressors, such as a cigarette smoking or hours of work [25,26]. In contrast, NK activity is enhanced by mirthful laughter (e.g., as with viewing of humorous videos) [27]. These findings strongly suggest that lifestyle and physical condition are closely related to NK activity, indicating that we should maintain well-conditions of daily life that lead to improved NK activity. From this point of view, supplementation with S-PT84 and the combination of vitamins B1, B2, and B6 has the potential for enhancing NK activity, and may be useful for maintaining quality of life.

Conflict of interest

T. Maekawa, M. Ida, Y. Furukawa, Y. Kitagawa, T. Izumo and H. Shibata are employees of Suntory Wellness Ltd., which markets health food products including S-PT84. T. Hayashi, K. Yasui and Y. Kowata declare no conflict of interest regarding these studies.

Author Contribution

T. Maekawa participated in planning the clinical protocol for Study 1, analyzed the data from both studies, and drafted the manuscript. M. Ida, Y. Furukawa and T. Izumo participated in planning the clinical protocol for both studies and analyzed the data from both studies. Y. Kitagawa and H. Shibata participated in planning the clinical protocol for both studies and helped in interpreting the findings. K. Yasui managed the subjects in Study 1. Y. Kowata managed the subjects in Study 2.

Acknowledgments

This research was not supported by any external grants. We thank all participants and Masahiro Ide for management and technical help with these studies.

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