Original Article

Effect of Probiotic *Bifidobacterium longum* BBS36 in relieving clinical symptoms and modulating plasma cytokine levels of japanese cedar pollinosis during the pollen season. A randomized double-blind, placebo-controlled trial

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Summary. Probiotic microorganisms have been shown to be effective in the treatment of allergic inflammation and food allergy, but their efficacy remains controversial. This study tested the effect of a yogurt supplemented with a probiotic strain *Bifidobacterium longum* BB536 in the treatment of Japanese cedar pollinosis (JCPsis).
Forty subjects with a clinical history of JCPsis were given yoghurt either containing BB536 (BB536 yoghurt) or without BB536 (placebo yoghurt) at 2 × 100 g per day for 14 weeks, in a randomized, double-blind, placebo-controlled trial. Subjective symptoms and self-care measures were recorded daily and blood samples were taken before and during the intervention (at weeks 4, 9, and 14) to measure the blood parameter levels related to JCPsis. Yoghurt supplemented with BB536 significantly alleviated eye symptoms compared with placebo yoghurt (odds ratio 0.31; 95% confidence interval 0.10–0.97; $p = 0.044$). Although no statistically significant differences were detected, nasal symptoms such as itching, rhinorrhea, and blockage, as well as throat symptoms tended to be relieved with the BB536 yoghurt. BB536 tended to suppress the decreasing blood levels of interferon-gamma (IFN-γ) and the increasing blood eosinophil rates; a significantly higher IFN-γ level was observed for the difference from baseline at week 4 . A decreased trend in the difference from baseline levels of JCP-specific IgE levels was also observed at week 4 in the BB536 group compared with the placebo group. In conclusion, these results suggest that intake of BB536-supplemented yoghurt may relieve JCPsis symptoms, probably through a modulating effect on Th balance.

Key words: *Bifidobacterium longum*, allergy, Japanese cedar pollinosis, cytokine, probiotic.

Resumen. Los microorganismos probióticos han demostrado surtir efecto en el tratamiento de la inflamación alérgica y alergia alimentaria, pero su eficacia sigue siendo controvertida. En este estudio se examinó el efecto de un yogur enriquecido con la cepa probiótica *Bifidobacterium longum* BB536 en el tratamiento de la polinosis por cedro japonés (PCJ).
Cuarenta pacientes con historia clínica de PCJ tomaron yogur con BB536 (yogur con BB536) o sin BB536 (yogur con placebo), 2 × 100 g al día durante 14 semanas, en un estudio aleatorizado, doble ciego y controlado con placebo. Las medidas de autocuidado y los síntomas subjetivos se registraron diariamente y se obtuvieron muestras de sangre antes y durante la intervención (a las semanas 4, 9 y 14) para determinar los niveles de los parámetros sanguíneos relacionados con la PCJ. El yogur enriquecido con BB536 alivió significativamente los síntomas oculares

en comparación con el yogur con placebo (cociente de posibilidades del 0,31; intervalo de confianza del 95% de 0,10–0,97; p = 0,044). Aunque no se detectaron diferencias estadísticamente significativas, los síntomas nasales, como prurito, rinorrea y obstrucción, y los síntomas de garganta tendieron a mitigarse con el yogur con BB536. BB536 mostró una tendencia a suprimir la disminución de los niveles sanguíneos del interferón gamma (IFN-g) y el aumento de la proporción de eosinófilos en sangre; se observó un nivel de IFN-g significativamente superior a la semana 4 respecto a la situación basal. También se detectó una menor tendencia en la diferencia respecto a los niveles basales de IgE específica de PCJ a la semana 4 en el grupo de BB536 en comparación con el grupo de placebo. En conclusión, estos resultados indican que la ingesta de yogur enriquecido con BB536 puede aliviar los síntomas de la PCJ, probablemente mediante un efecto modulador en el equilibrio de linfocitos T colaboradores.

Palabras clave: *Bifidobacterium longum*, alergia, polinosis por cedro japonés, citocina, probiótico.

Introduction

The prevalence of allergic diseases has rapidly increased worldwide over the past decades, especially in the industrialized countries [1]. One of the explanations for the prevalence of allergic disease is the "hygiene hypothesis", which postulates that the decrease in opportunity of exposure to immunostimulating pathogens in early childhood causes an increased prevalence of allergic diseases [2]. Association of the intestinal microbiota with the development of an ordinary immune system and the reduction of allergic risk has been proposed from many studies. Animal studies demonstrated that if sufficient microbial stimuli are not available to the developing immune system in infancy, a further maturation mechanism is inhibited, and persistent dysfunction of the Th2 response may result [3-4]. Studies on composition of the intestinal microflora between allergic and nonallergic 2-year-old children indicated that the prevalence of bifidobacteria was lower in allergic infants, whereas counts of *Staphylococcus aureus* and enterobacteria were higher [5]. In comparison with healthy infants, babies with allergies were found to be less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life [6]. These results indicate the importance of microflora in the development of allergic disease. In line with this hypothesis, Kalliomäki *et al*. [7] demonstrated an effect of *Lacotbacillus* GG (LGG) in prevention of early atopic disease in children at high risk by giving LGG prenatally to mothers with allergic relation and postnatally to children. Effect has also been shown in oral probiotic bacteriotherapy with lactic acid bacteria for the improvement of clinical symptoms of perennial allergic rhinitis in adults [8-9] and food allergy in small children [10,11]. Recently, LGG was reported to be effective in treatment of atopic eczema/dermatitis syndrome (AEDS) in IgE-sensitized infants but not in non-IgE-sensitized infants [12]. However, LGG was not shown to be effective in the treatment of birch-pollen allergy [13].

Substantial evidence exists that gram-positive lactic acid bacteria of the genera Lactobacillus and Bifidobacteria can survive transiently in the human intestinal tract when take by mouth, making contact with the gut mucosa, which can stimulate systemic, cell-mediated immunity that approximates Th1 type immune response [14]. *In vitro* and *in vivo* studies have suggested that probiotics can exert a wide array of immune effects, some of them potentially in contrast with characteristics of atopic constitution [15-16]. Dietary supplementation using particular defined strains was found to increase IFN activity in the blood of human volunteers [14].

Japanese cedar pollinosis (JCPsis) is an immunoglobulin E (IgE)-mediated type I allergy caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (JCP). It is one of the most common allergic diseases in Japan, with an increasing prevalence over the past decades [17]. Accumulation of JCP-specific IgE caused by exposure to JCP is known as a cue to the development of symptoms; however, JCP-specific IgE levels do not seem to be the only key factor for the onset and severity of pollinosis symptoms, since a certain population possessing JCP-IgE does not produce symptoms.

In the present study, a double-blind, placebo-controlled trial using yogurt with or without BB536 was carried out to evaluate if it is possible to relieve JCPsis symptoms with *Bifidobacterium longum* BB536, a probiotic strain originated from humans.

Materials and Methods

Participants

As a pilot study, forty adult volunteers (17 men and 23 women) with a clinical history of JCPsis recruited in the area around Togashi Clinic were enrolled in this intervention. Participants were recruited on the basis of a more than 2-year clinical history of JCPsis and the presence of serum JCP-specific IgE. No subject had carried out preventive JCPsis medications for the 2004 pollen season before the start of the intervention. Historical clinical severity of JCPsis for each participant was self-evaluated on a 4-point scale (1 = mild, endurable without prescribed drug intake; 2 = moderate, taking prescribed drug from time to time; 3 = severe, taking prescribed drug almost daily; and 4 = extremely severe, unendurable without daily prescribed drugs). Since use of prescribed pollinosis drug was set as a dropout criterion, participants with a self-reported score of 4 were excluded

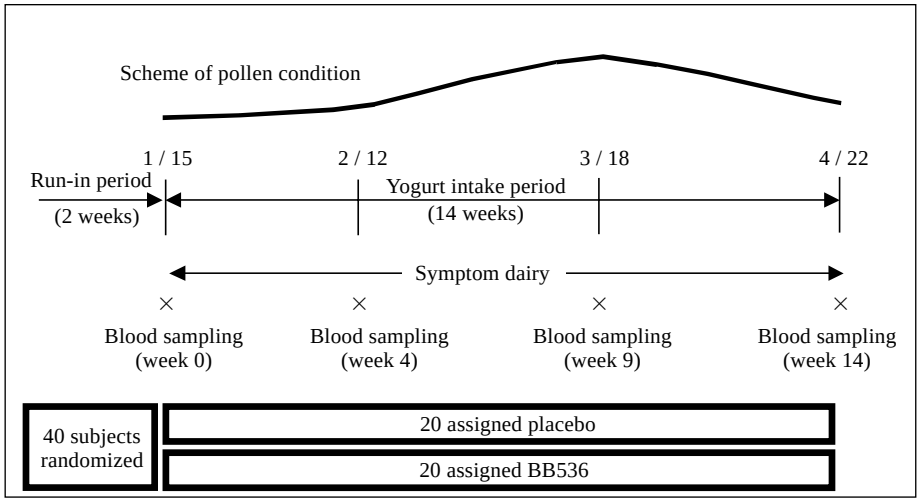

Figure 1. Study protocol and diagrammatic profile of pollen dispersion during the intervention.

from this study in order to avoid unnecessary dropouts. All participants provided informed consent. The study protocol was approved and controlled by the Local Ethics Committee of Morinaga Milk Industry Co., Ltd.

Yogurt intake

This was a randomized, double-blind, placebo-controlled trial. After a 2-week run-in period, participants were randomized according to computer-generated permuted-block randomization into two groups. During the run-in period, participants were instructed not to consume foods or supplements containing lactic acid bacteria or bifidobacteria. Each group was then randomly subjected to ingest yogurt either with or without supplementation with BB536, at 2 × 100 g per day.

In 2004, the onset of the pollen season of Japanese cedar, which is defined as the first count of at least two pollen grains/cm^2/day for successive days at sampling sites, was recorded in the middle of February in the study region of Kanagawa Prefecture. However, a small amount of pollen scattering was observed several weeks before the pollen season (preseason). The pollen season lasted to the end of April, with peak season from the end of February to the end of March. The total amount of pollen scattered in the 2004 season was about 10 times lower than the average. Yogurt intake started on 15 January 2004, which was approximately 4 weeks before the onset of the pollen season, and continued for 14 weeks, until 22 April 2004 (Fig. 1).

Preparation of yogurts

The BB536 yogurt was fermented with starters composed of *B. longum* strain BB536, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The placebo yogurt was fermented with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. BB536 and placebo yogurt packages and contents looked and tasted identical. Each batch of yogurt was consumed within 16 days after preparation to assure the freshness of the yogurt and the viability of the tested bifidobacteria. The yogurt was delivered to all subjects every 14 days, and they were instructed to store it in a refrigerator at less than 10 ºC to ensure viability. Each yogurt contained approximately 1.0 × 10^9 cfu of lactic acid bacteria. The BB536 yogurt contained 3.5 ± 2.4 × 10^8 and more than 2 × 10^7 cfu of living BB536 after preparation and at the end of the consumption period, respectively. Participants were instructed not to consume foods or supplements with possible antiallergic characteristics, or foods or supplements containing other lactic acid bacteria or bifidobacteria during the intervention.

Participant monitoring

During the study, participants were instructed to fill out a questionnaire on compliance, subjective symptoms, self-care measures, medication, health conditions, alcohol consumption, and exercise. Nasal blockage and nasal itching, eye symptoms (itching, redness, and weeping), and throat symptoms (redness and pain) were evaluated on a scale from 0 to 4 in accordance with the guidelines of the Nasal Allergy Clinic 2002, Japan, as follows: 0 = no sensation; 1 = mild; 2 = moderate; 3 = severe; and 4 = extremely severe. Counts of sneezing and nose blowing (rhinorrhea) per day were rated from 0 to 4 as follows: 0 = none; 1 = 1 to 5 counts; 2 = 6 to 10 counts; 3 = 11 to 20 counts; and 4 = more than 21 counts).

Participants were allowed to try self-care measures such as wearing a mask, eye drops, or local nasal sprays when they felt the need, and were asked to record use of these items daily. Mask wearing was recorded on a "yes or no" basis, and eye drops or local nasal sprays were reported by counts per day. Use of a prescribed pollinosis drug, which was set as a dropout criterion, was permitted when they felt the need, but further symptom evaluation and blood sampling were then terminated. The use of other medications, the presence of a fever or cold, alcohol consumption, and exercise were also recorded daily on a "yes or no" basis.

Physical examination and blood sampling

Physical examination and blood sampling were carried out before and during (at weeks 4, 9, and 14) the intervention. Blood samples were drawn from an antecubital vein after fasting overnight. The analysis of the collected blood samples was performed at Mitsubishi BCL Co. Ltd, Tokyo, Japan. Serum total IgE antibodies

and JPC-specific IgE antibodies were analyzed by the UniCap system (Diagnostic, Sweden). Plasma cytokine levels were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (human IFN-γELISA, Bendr Medsystems, Vienna, Austria; human IL-10 UltraSensitive, BioSource International, California, USA). Th2 type cytokines, such as IL-4 and IL-5, were not measured due to detection limits. Peripheral blood cell counting was carried out by autoanalyzer.

Statistical analysis

All statistical analyses were performed using SAS statistical software version 9.1.3 (SAS Institute, Cary, N.C, USA). The subjective symptom scores were recorded daily and expressed as weekly sums (scale 0-28 for each). The weekly summed scores were dichotomized either into no symptoms to mild symptoms (weekly score from 0 to 7) or moderate to severe symptoms (weekly score greater than 7). Because the subjective symptoms were collected repeatedly from the same subjects during the intervention period, the method of the generalized estimating equation for a multiple logistic regression was used to compute the odds ratios (ORs) in favor of the occurrence of the subjective symptoms. This approach accounts for the within-subject correlation nature of data resulting from repeated measures taken from the same subjects. In this analysis, subjective symptom scores between week 5 and 14 were analyzed, because this period corresponded to the pollen season. The treatment-by-time interaction was checked to assess whether the time effect influenced the relationship between the intervention and outcomes. ORs, after adjusting for historical clinical severity of JCPsis, and 95% confidence intervals (CIs) were calculated for each subjective symptom. The *t*-test and paired *t*-test were used to analyze inter-group and intra-group differences in blood data after logarithmic transformation, respectively. Limit values were used in analyses of blood parameters for data occurred under detection limits. A p value < 0.05 was considered statistically significant.

Table 1. Subject characteristics.

	Placebo group	BB536 group
Number of participants	20	20
Age (mean±SD), years	36.7±9.5	36.5±7.8
(range, years)	(24-55)	(23-61)
Body weight (mean±SD), kg	56.0±7.3	57.8±10.2
Gender		
Male	n=8	n=9
Female	n=12	n=11
Clinical history of JCPsis		
Years (mean±SD)	12.6±7.7	10.5±7.8
(range, years)	(2-30)	(2-30)
Severity (mean±SD)	2.3±0.7	2.2±0.6
Level 1 (mild)	n=2	n=2
Level 2 (moderate)	n=10	n=12
Level 3 (severe)	n=8	n=6

Results

Baseline characteristics of participants

Baseline characteristics were similar in the placebo and BB536 groups (Table 1). All participants completed the 14-week study. A high compliance rate (more than 95%) was obtained for all participants.

Clinical effects of *B. longum* BB536 on subjective symptoms

The primary outcome measures for assessment of efficacy of BB536 were the scores of subjective symptoms. Fig. 2 shows the mean scores of subjective symptoms in

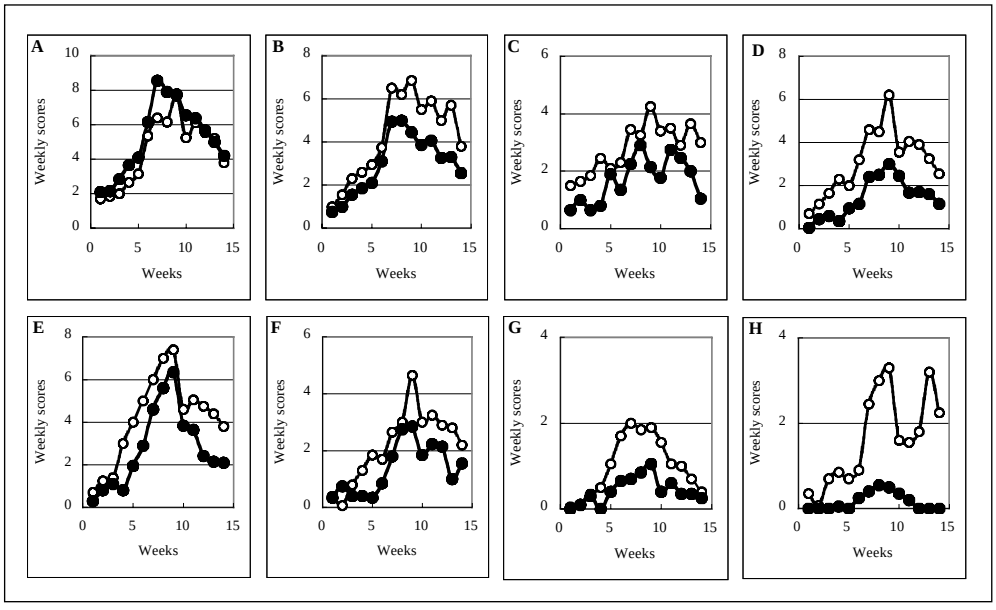

Figure 2. Means of weekly sum of subjective symptoms and self-care measures in the pollen season in the BB536 group (closed circles) and the placebo group (open circles).
Sneezing (A), rhinorrhea (B), nasal blockage (C), nasal itching (D), eye symptoms (E), throat symptoms (F), eye drops (G), and mask wearing (H).

Table 2. Numbers of subjects experiencing moderate to severe subjective symptoms in each group at each week during the pollen season.

		Number of subjects									
	Group	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14
Eye symptoms	Placebo	6	6	7	9	7	5	5	5	3	4
	BB536	1	1	3	7	4	2	2	1	1	1
Nasal itching	Placebo	2	3	3	3	6	3	3	3	4	2
	BB536	1	1	1	1	2	1	1	1	2	1
Rhinorrhea	Placebo	3	6	8	7	7	5	6	7	6	4
	BB536	0	1	2	6	5	2	5	4	4	2
Nasal blockage	Placebo	2	2	3	4	4	3	3	4	3	2
	BB536	2	0	0	1	1	0	3	2	1	0
Sneezing	Placebo	3	5	6	6	7	5	7	7	5	4
	BB536	4	6	10	9	8	5	7	3	6	4
Throat symptoms	Placebo	2	1	4	2	4	2	3	3	3	2
	BB536	0	0	1	1	2	0	2	1	2	2

each group. Along with the pollen prevalence, the subjective symptom scores increased remarkably from the middle of February and peaked in the middle of March. The subjective scores for nasal (rhinorrhea, blockage, itching), eye, and throat symptoms progressed at a lower level in the BB536 group during the pollen season compared with the placebo group. An exception was observed for sneezing, which progressed similarly in the two groups. When the weekly summed scores were dichotomized either into no symptoms to mild symptoms (weekly score from 0 to 7) or moderate to severe symptoms (weekly score greater than 7), numbers of subjects experiencing moderate to severe subjective symptoms during the pollen season were fewer in the BB536 group compared with the placebo group (Table 2). The ORs in favor of the occurrence of subjective symptoms between week 5 and 14 were computed by the method of the generalized estimating equations to identify the effect of BB536-containing yogurt on subjective symptoms as described in the statistical section. The odds of experiencing subjective eye symptoms for the BB536 group were about 68% lower than that of placebo group (OR 0.31, 95% CI 0.10-0.97) (Table 3). Other subjective symptoms, except for sneezing, appeared to be relieved with BB536 yogurt,

Table 3. Adjusted odds ratios and 95% confidence intervals for subjective symptoms in the pollen season in the BB536 group as compared with the placebo group.

	Odds ratios	95% CI	*P* value
Eye symptoms	0.31	0.10-0.97	0.044
Nasal itching	0.34	0.09-1.35	0.126
Rhinorrhea	0.51	0.17-1.56	0.238
Nasal blockage	0.45	0.12-1.72	0.244
Sneezing	1.28	0.48-3.38	0.624
Throat symptoms	0.52	0.12-2.19	0.373

Table 4. Changes in blood IgE, cytokine and eosinophil rate levels.

	Group	0 week	4 weeks	9 weeks	14 weeks
Total IgE (IU/ml)	Placebo	149.6 ± 206.9	158.8 ± 241.9	172.5 ± 266.3	190.2 ± 316.7
	BB536	90.3 ± 60.7	88.6 ± 67.0	88.1 ± 58.4	99.0 ± 63.1
JCP-specific IgE (UA/ml)	Placebo	15.2 ± 22.7	15.8 ± 24.9	15.8 ± 25.7	14.9 ± 24.5
	BB536	11.7 ± 5.1	10.9 ± 9.2	12.1 ± 9.8	12.4 ± 10.8
IFN-γ (pg/ml)	Placebo	12.6 ± 12.2	6.4 ± 9.3***	4.0 ± 3.6 ***	5.0 ± 3.0***
	BB536	11.6 ± 8.2	8.7 ± 10.0*	4.4 ± 3.2***	5.7 ± 3.3 **
IL-10 (pg/ml)	Placebo	2.1 ± 1.9	2.2 ± 0.9	3.2 ± 1.8***	2.1 ± 0.8
	BB536	2.9 ± 3.8	3.2 ± 3.3	4.3 ± 5.1***	3.4 ± 4.5
Eosinophil rate (%)	Placebo	3.1 ± 2.1	3.6 ± 2.6	4.0 ± 2.4*	3.8 ± 2.2*
	BB536	2.5 ± 1.7	2.1 ± 1.6	2.9 ± 1.9	2.6 ± 1.8

Means ± SD. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (significant intra-group difference from 0 week).

although none of the differences between groups were statistically significant: nasal itching (OR 0.34, 95% CI 0.09-1.35), rhinorrhea (OR 0.51, 0.17-1.56) and blockage (OR 0.51, 95% CI 0.17-1.56), and throat symptoms (OR 0.52, 0.12-2.19).

There was low use of local nasal sprays in both groups. No remarkable difference was observed in frequencies of use of other medications, a fever or cold, alcohol consumption, or exercise between the two groups (data not shown). However, there tended to be lower frequencies of self-care measures of mask wearing and eye drops in the BB536 group compared with the placebo group (Fig. 2-G, H).

Effect of BB536 on blood parameters

Before treatment, there were no significant inter-group differences in total IgE, JCP-specific IgE, IFN-γ, IL-10, or eosinophil rate levels (Table 4). No significant intra-group differences in the levels of total IgE and JCP-specific IgE from baseline were observed throughout the investigation period in either group. However, there was a decreasing trend (p = 0.081) in the difference from baseline of JCP-specific IgE at week 4 in the BB536 group (-0.78 ± 2.07) compared with that of placebo group (0.56 ± 2.61). In contrast, significant decreases from baseline in IFN-γ levels (placebo group, $p < 0.001$ at weeks 4, 9, and 14; BB536 group, p = 0.011 at week 4, $p < 0.001$ at week 9, and p = 0.005 at week 14). Significant increases ($p < 0.001$) from baseline in IL-10 levels at week 9 were observed in both the placebo and BB536 groups (Table 3). Furthermore, there was a significant increase from baseline in eosinophil rate at weeks 9 (p = 0.023) and 14 (p = 0.028) in the placebo group but not in the BB536 group. No remarkable changes were observed in the rates of basophil, neutrophil, monocyte, lymphocyte as well as other items from peripheral blood cell counting (data not shown).

Fig. 3 shows the difference from baseline of IFN-γ, IL-10 and eosinophil rate levels after sample intake in the two groups. Compared with the placebo group, a significantly higher (p = 0.035) level in the difference from baseline of IFN-γ was observed at week 4 in the BB536 group. Slight, though not significantly higher levels of IL-10 and lower levels of eosinophil rate were found in the BB536 group compared with the placebo group.

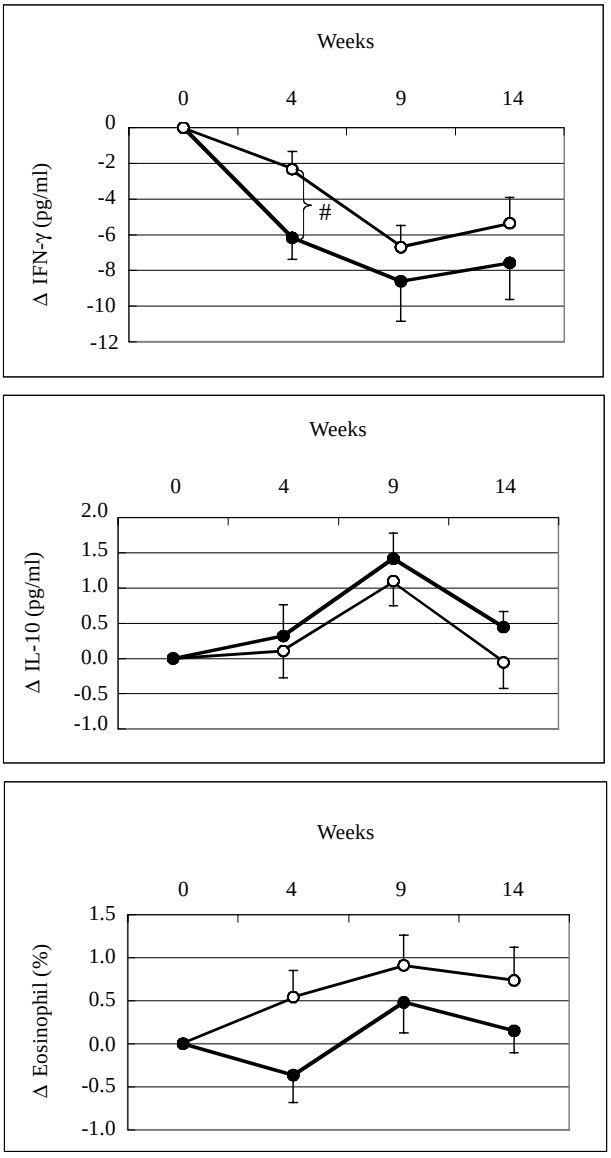

Figure 3. Differences from baseline of serum parameters in the pollen season in the BB536 group (closed circles) and the placebo group (open circles). Data are expressed as means ± SE. #, $p < 0.05$.

Discussion

Nasal and eye symptoms are known as the typical symptoms of JCPsis. In the present study, since there were no preliminary data on the probiotic effect in the treatment of JCPsis, as a pilot study, each symptom was evaluated separately. Subjective eye symptoms in subjects with JCPsis were significantly reduced over the pollen season (p = 0.044) in the BB536 group. Nasal and throat symptoms tended to be reduced as well, but did not reach statistical significance. The reason that BB536 did not affect sneezing scores is unclear. The higher frequency of mask wearing that occurred in the placebo group was speculated to have some influence on sneezing; however, statistical analysis did not show a sound correlation between mask wearing and sneezing (data not shown).

The efficacy of probiotics in preventing allergic development as well as in treating already established allergic diseases remains controversial and needs further study. Many reasons could account for the different outcomes in the clinical trials: the dose and intake period could vary, the type and severity of the symptoms involved may be different, and most importantly, the species and strains of the probiotics may differ. First of all, the

probiotic effects are assumed to be strain-specific, and extrapolation of results from studies using different strains or species should be done with caution. There may also be difference between the effect of treatment and prevention of allergic diseases. In addition, previous studies have not been able to give evidence in terms of blood parameters, which is considered to be important for evaluating the underlying mechanism(s). The present study implies that a decrease in Th1 cytokine secretion in the pollen season appears to be related to pollen allergy, and that BB536 appears to play a role in the suppression of this decrease, especially in the early stage of the pollen season. Concerning the Th1/Th2 balance, it has been proposed that marked skewing of the immune response to Th2 lineage can result in an allergic disorder [18, 19]. IFN-γ was described as a potential regulator of Th2 cytokine synthesis [19]. Although Th2 cytokines IL-4 and IL-5 were not measured in the present study, the results of IFN-γ suggested that intake of BB536 would modulate the Th balance, which would result in inhibiting specific IgE increases and eosinophil generation as well as relieving symptoms in the pollen season.

Bifidobacteria are the major components of the intestinal microflora in humans, and are frequently associated with health-promoting effects [20-24]. BB536 was used in the present intervention since many physiological effects such as stimulating immunity, reducing cancer risk, and preventing harmful bacterial infection, especially its pronounced promoting effect on the intestinal environment, have been reported for this strain [22, 25, 26]. Our *in vitro* experiments on OVA-primed splenocytes also indicated that BB536 stimulate Th1 cytokine IFN-γ secretion and inhibit Th2 cytokine and IgE generation (data not published). BB536-supplemented yogurt has been demonstrated to have a pronounced promoting effect on intestinal environments after 2 weeks of intake at a dose of 100 g per day [25]. Therefore, we started this trial several weeks before the onset of the pollen season to obtain a promoting effect on the intestinal environments. Furthermore, analyses of the fecal samples obtained from 23 of the 40 participants indicated that BB536 appears to have a positive impact on the formation of an anti-allergic microflora (data not published). Thus, both local effects from interactions between cell components on the intestinal surface and systemic effects from an impact on the microflora, may contribute to the immunomodulatory effects of BB536.

In the present study, the evaluation of the efficacy of BB536 was performed by comparison of BB536-supplemented yogurt with a placebo yogurt. However, some anti-allergic effects of the placebo yogurt cannot be excluded. *In vitro* experiments have demonstrated the potential for immuno-modulation by some strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [27]. Nevertheless, our results demonstrate a superior effect of the BB536 yogurt compared with placebo yogurt in the treatment of JCPsis. We are also interested to learn if there is a synergistic effect of each strain.

Acknowledgements

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