Influence of *Bifidobacterium longum* BB536 intake on faecal microbiota in individuals with Japanese cedar pollinosis during the pollen season

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It has been reported that intake of yogurt or powder supplemented with the *Bifidobacterium longum* BB536 probiotic strain alleviated subjective symptoms and affected blood markers of allergy in individuals with Japanese cedar pollinosis (JCPsis) during the pollen seasons of 2004 and 2005, based on randomized, double-blind, placebo-controlled trials. Furthermore, the 2004 study found that intestinal bacteria such as the *Bacteroides fragilis* group significantly fluctuated during the pollen season in JCPsis individuals and intake of BB536 yogurt tended to suppress these fluctuations. The present study investigated faecal microbiota to examine whether any changes occurred during the pollen season and whether any influence was exerted by intake of BB536 powder in the 2005 pollen season, which happened to be a heavy season, to confirm the 2004 findings and to evaluate the relationship of microbiota with symptom development. In a randomized, double-blind, placebo-controlled trial, 44 JCPsis subjects received BB536 or a placebo for 13 weeks during the pollen season. Another 14 Japanese cedar pollen (JCP)-specific IgE negative healthy subjects received placebo for the same period. Faecal samples were collected before (week 0), during (weeks 4, 8 and 13) and after (week 17) intervention, and out of JCP season (week 28). Faecal microbiota were analysed using terminal-RFLP (T-RFLP) and real-time PCR methods. Principal component analysis based on T-RFLP indicated distinct patterns of microbiota between healthy subjects and JCPsis subjects in the placebo group, but an intermediate pattern in the BB536 group at week 13, the last stage of the pollen season. The coordinate of principal component 1 at week 13 correlated with composite scores of JCPsis symptoms recorded during the pollen season. *Faecalibacterium prausnitzii* and the *Bacteroides fragilis* group were identified as the main contributors to microbiotal fluctuations. Real-time PCR indicated that BB536 intake suppressed increases in the *Bacteroides fragilis* group compared with the placebo group ($P < 0.05$). These results suggest that faecal microbiota in JCPsis subjects, but not healthy subjects, fluctuate at the end of the pollen season and that BB536 intake plays a role in maintaining normal microbiota.

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INTRODUCTION

Japanese cedar pollinosis (JCPsis), an IgE-mediated type I allergy caused by exposure to (*Cryptomeria japonica*) Japanese cedar pollen (JCP), represents a public health issue affecting over 16% of the Japanese population (Enomoto, 2004).

Abbreviations: JCP, Japanese cedar pollen; JCPsis, Japanese cedar pollinosis; PAD-HCM, phylogenetic assignment database for T-RFLP analysis of human colonic microbiota; PC, principal component; PCA, principal component analysis; T-RFLP, terminal-RFLP; T-RF, terminal restriction fragment.

The prevalence of allergic diseases has increased rapidly worldwide over the few past decades, particularly in industrialized countries (Holt *et al.*, 1997). One explanation for this prevalence is the 'hygiene hypothesis', which postulates that decreased opportunities for exposure to immunostimulating pathogens in early childhood cause increased prevalence of allergic diseases (Warner, 1999). Associations of the intestinal microbiota with the development of an ordinary immune system and reductions in allergic risk have been proposed from many studies (Sudo *et al.*, 1997; Bjorksten *et al.*, 2001; Oyama *et al.*, 2001). Noverr & Huffnagle (2005) recently proposed the 'microflora hypothesis', which suggests that perturbations in gastrointestinal microbiota due to antibiotic use and dietary differences in 'industrialized' countries have disrupted the normal microbiota-mediated mechanisms of immunological tolerance in the mucosa, leading to increased incidence of allergic airway disease.

In the 2004 JCP season, we performed a human trial to investigate the effects of yogurt supplemented with *Bifidobacterium longum* BB536, a probiotic strain that was originally discovered in humans, in the treatment of JCPsis during the pollen season. We found that subjective symptoms were alleviated, whilst increased blood eosinophil rates and decreased gamma interferon levels were suppressed by BB536 intake (Xiao *et al.*, 2006a). In addition, cell numbers of the *Bacteroides fragilis* group significantly fluctuated during the pollen season (Odamaki *et al.*, 2007).

However, as placebo yogurt containing ordinary lactic acid bacteria was used in that study, some effect from the placebo on microbiota could not be excluded. In addition, the 2004 season was a mild season for JCP dispersal, and changes in microbiota and in the efficacy of BB536 in a normal or heavy pollen season needed to be seen. Furthermore, the fluctuation in microbiota among JCPsis subjects during the JCP season needed to be compared with healthy non-JCPsis subjects. We therefore performed a trial using BB536 lyophilized powder in the JCP season of 2005, which happened to be a heavy season. The total number of JCP grains dispersed in 2005 season was $>10\,000$

grains cm^{-2} around the study area, which was about 30 times the level in 2004. BB536 intake was found to be associated with significant decreases in subjective symptoms and modulation of blood immunological parameters (Xiao *et al.*, 2006b).

We report herein the changes to faecal microbiota in JCPsis subjects in comparison with healthy subjects during the 2005 JCP season, and the effects of BB536 intake on faecal microbiota in JCPsis.

METHODS

Clinical study. Samples for this study came from a clinical study reported by Xiao *et al.* (2006b) evaluating the effects of BB536 on clinical symptoms of JCPsis and blood parameters related to pollen allergy. A total of 44 adult volunteers (26 men, 18 women) was recruited on the basis of a >2 year clinical history of JCPsis and the presence of serum JCP-specific IgE (Table 1). Historical clinical severity of JCPsis for each participant was self-evaluated on a four-point scale (1, mild, endurable without prescribed medication; 2, moderate, taking prescribed medication from time to time; 3, severe, taking prescribed medication almost daily; and 4, extremely severe, unendurable without daily prescribed medication). Subjects were randomized for ingestion of BB536 powder (approx. 5×10^{10} c.f.u.) or placebo powder twice daily, in a randomized, double-blind design for 13 weeks during the pollen season of 2005. To study changes in faecal microbiota during the pollen season, 14 healthy volunteers (JCP-specific IgE-negative and with no prior history of spring allergic rhinitis) were administered placebo powder during the same intervention stage in an identical manner to JCPsis subjects.

All participants provided written informed consent. All study protocols were approved and controlled by the local ethics committee of the non-profit organization Japan Health Promotion Supporting Network, Wakayama, Japan, and the local ethics committee of Morinaga Milk Industry (Tokyo, Japan).

Clinical symptoms. During the study period, participants were instructed to record subjective symptoms daily in accordance with the guidelines of the Nasal Allergy Clinic 2002, Japan. Subjective symptom scores were recorded daily and expressed as weekly totals (scale of 0–28). Weekly total scores for sneezing, rhinorrhea, nasal blockage, nasal itching, eye symptoms and throat symptoms were totalled as composite scores (scale of 0–252) for the pollen season (weeks 5–13) (Xiao *et al.*, 2006b).

Table 1. Subject characteristics

Characteristic	Group		
	Placebo	BB536	Healthy
No. of participants	22	22	14
Mean age in years (range)	36.00 (22–48)	36.50 (26–57)	33.36 (22–50)
Gender (male/female)	13/9	13/9	11/3
Clinical history of JCPsis			
Mean no. of years (range)	11.90 (3–20)	11.20 (2–30)	–
Severity			
Level 1 (mild)	5	8	–
Level 2 (moderate)	9	9	–
Level 3 (severe)	8	5	–

Faecal samples. Each subject provided faecal specimens before (week 0), during (weeks 4, 8 and 13) and after (week 17) intervention, and out of JCP season (week 28). Participants were instructed to collect specimens in a plastic tube, cool the bag immediately to <10 °C and deliver the sample within 12 h. Collected specimens were stored at –80 °C until analysis.

DNA extraction from faecal samples. DNA extraction from faecal samples was performed as described previously (Odamaki *et al.*, 2007). Faecal samples (20 mg) were washed twice in 1.0 ml PBS and centrifuged at 14 000 g. Faecal pellets were resuspended in 450 µl extraction buffer (100 mM Tris/HCl, 40 mM EDTA, pH 9.0) and 50 µl 10% SDS. Glass beads (300 mg, 0.1 mm diameter) and 500 µl buffer-saturated phenol were added to the suspension and the mixture was vortexed vigorously for 30 s using a FastPrep FP 100A (Bio 101) at a power level of 5. After centrifugation at 14 000 g for 5 min, 400 µl supernatant was extracted with phenol/chloroform and 250 µl supernatant was precipitated with propan-2-ol. Inhibitors were removed using a High Pure PCR template preparation kit (Roche). Purified DNA was suspended in 200 µl Tris/EDTA buffer (pH 8.0).

Terminal-RFLP (T-RFLP) analysis. T-RFLP analysis was performed as described previously (Odamaki *et al.*, 2007) with some modifications. Briefly, two pairs of universal primers comprising 1492R and either 27F or 529F labelled with 6-carboxyfluorescein (Applied Biosystems) (Table 2) were used for PCR amplification (Sakamoto *et al.*, 2003). DNA was amplified according to the following program: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1.5 min; and a final terminal extension at 72 °C for 10 min. Fluorescently labelled PCR products (50 µl) were purified using MultiScreen FB filter plates (Millipore). PCR products amplified by 27F and 1492R primers were subsequently digested with 20 U *HhaI*, *MspI*, *AluI* or *HaeIII* (TaKaRa Shuzo). DNA amplified by 529F and 1492R primers was digested with 20 U *RsaI* (Nippon Gene) plus *XspI* (TaKaRa Shuzo), in a total volume of 10 µl at 37 °C for 3 h (Nagashima *et al.*, 2003). A 1 µl aliquot of the product was mixed with 8 µl deionized formamide and 1 µl DNA fragment length standards. The standard size marker was a 1:1 mixture of GS 500 ROX and GS 1000 ROX size standards (Applied Biosystems). The length of terminal restriction fragments (T-RFs) was determined using an ABI PRISM 3100 genetic analyser (Applied Biosystems) in the GENESCAN mode.

T-RFLPs obtained after digestion with *HhaI*, *MspI*, *AluI*, *HaeIII* or *RsaI* plus *XspI* were combined and analysed using BIONUMERICS

version 2.5 software (Applied Maths). T-RFs with a peak area of less than 2% of the total area were excluded from the analysis. Principal component analysis (PCA) was performed using the Pearson coefficient and Ward's algorithm.

Real-time PCR for quantitative determination of cell and rRNA gene copy number.

Real-time PCR was performed using an ABI PRISM 7500 fast real-time PCR system (Applied Biosystems), SYBR Premix Ex *Taq* (TaKaRa Shuzo) and ROX reference dye II (TaKaRa Shuzo) as an internal standard. Table 2 shows the primer sets used. The amplification program consisted of one cycle of 94 °C for 10 s, followed by 40 cycles of 94 °C for 5 s, the appropriate annealing temperature for 30 s and 72 °C for 30 s. Fluorescent products were detected at the last step of each cycle. Melting curves were obtained by heating from 60 to 95 °C in 0.2 °C s⁻¹ increments, with continuous fluorescence collection. Plasmid DNA prepared as described by Bartosch *et al.* (2004) was used as the standard for determination of rRNA gene copy number.

Statistical analysis. All statistical analyses were performed using SPSS version 14.0 statistical software. Inter- and intragroup differences in faecal bacterial and 16S rRNA gene copy number were analysed using the Mann–Whitney *U* test and Wilcoxon signed rank test, respectively. Spearman's correlation coefficient was used to determine the relationships of the coordinate of principal component (PC) 1 axis with composite scores of JCPsis symptoms. Values of *P* < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Diversity of faecal microbiota based on T-RFLP datasets

T-RFLPs obtained from each individual at weeks 0, 4, 8, 13, 17 and 28 were analysed using PCA (Fig. 1). No precise grouping was found for samples collected before the season (week 0) or during the early and peak season (weeks 4 and 8, respectively). However, an exception was found at week 13, the last stage of the pollen season. At week 13, although no distinct aggregation was found in the BB536 group, placebo subjects were found to aggregate on one side, whilst healthy subjects were on the other side (Fig. 1). A similar trend was somewhat apparent in week 17

Table 2. Primer sets used in this study

Type of analysis/target organism	Primer name	Sequence (5'→3')	Product size (bp)	Annealing temp (°C)	Reference
T-RFLP analysis					
All eubacteria	27F	AGAGTTTGATCCTGGCTCAG	1485	50	Hayashi <i>et al.</i> (2004)
	529F	ACGTGCCAGCAGCCGCGG	981	50	
	1492R	GGTTACCTTGTTACGACTT			
Real-time PCR analysis					
<i>Clostridium coccoides</i> group	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	50	Matsuki <i>et al.</i> (2004)
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			
<i>Faecalibacterium prausnitzii</i>	Fprau223F	GATGGCCTCGCGTCCGATTAG	199	58	Bartosch <i>et al.</i> (2004)
	Fprau420R	CCGAAGACCTTCTTCTCTCC			
<i>Bacteroides fragilis</i> group	g-Bfra-F	ATAGCCTTTTCGAAAAGRAAGAT	495	50	Matsuki <i>et al.</i> (2004)
	g-Bfra-R	CCAGTATCAACTGCAATTTTA

Fig. 1. PCA based on T-RFLP datasets derived from combined *AluI*, *HaeIII*, *HhaI*, *MspI* and *RsaI*+*XspI* digestion of faecal samples in JCPsis and healthy subjects. PC1 and PC2 indicate the percentage variation covered by each PC. ●, BB536; ○, placebo; ×, healthy subject.

(post-season), but not in week 28 (out of season). Analysis including low-contributing PCs up to a total contribution rate of >60% was also performed, but no further distinct groupings were detected. As some JCPsis subjects prematurely terminated the intervention (Xiao *et al.*, 2006b), the influence of decreased subject number was checked by analysing data from subjects who completed the whole intervention and yielded similar results to those shown in Fig. 1 (data not shown).

These results indicated that there were no marked differences in the bacterial components of microbiota between JCPsis and healthy subjects before the pollen season. However, the pollen season appeared to have caused a difference in the microbiota between healthy and JCPsis subjects. The reason for the absence of such a change in weeks 4 and 8, the early and peak stages of pollen dispersal, respectively, remains unclear. One explanation is that the fluctuation of microbiota occurred gradually, leading to distinct results that were only apparent by the late stage of pollen dispersal. Another explanation is that such changes in microbiota resulted from stress due to symptoms. Similarly, we found a dramatic increase in serum total and JCP-specific IgE levels in JCPsis subjects, but not in healthy subjects, at week 13, but not in weeks 4 and 8 (Xiao *et al.*, 2006b). Although PCA indicated

different patterns of microbiota between placebo and healthy groups, the BB536 group was found to straddle these groups. This may suggest diverse responses to probiotic treatment among JCPsis subjects. Supporting this proposal, we found a significant correlation between composite scores for JCPsis symptoms recorded during the pollen season (weeks 5–13) and the coordinate of the PC1 axis in week 13 ($\rho = -0.366$, $P = 0.011$), suggesting that subjects located on the left side of the PCA plot experienced severer symptoms during the pollen season. In addition, among subjects in the BB536 group, composite symptom scores tended to be higher for those located on the left side ($n = 10$) compared with those on the right side ($n = 10$) [median (interquartile range): 47.0 (44.8–68.8) vs 32.6 (20.9–37.5); $P = 0.064$]. These results suggested that BB536 intake directly or indirectly impacts on microbiota. Stressors such as fasting conditions, overcrowding and maternal deprivation have been reported to influence the intestinal microbiota (Morishita & Ogata, 1970; Suzuki *et al.*, 1983; Bailey & Coe, 1999). Fluctuations in intestinal microbiota have been described during psychological stress in humans (Holdeman *et al.*, 1976; Takatsuka *et al.*, 2000). Under such stress conditions, changes may occur in gastric juice secretion and peristalsis. These and other changes in the intestinal environment may modify the balance of intestinal microbiota.

Prediction of composite T-RFs

As the correlation study indicated that subjects on the left side of the PCA plot experienced severer symptoms, T-RF contributions to the left-side plot in PCA in week 13 were analysed to investigate bacterial involvement in the fluctuation. There were distinct individual differences in the T-RFs before the pollen season, which made it to be difficult to pick up those T-RFs involved in the fluctuation. To eliminate individual differences in faecal microbiota at the start of this study, we excluded T-RFs associated with the placement of plots in week 0. As shown in Table 3, PCA was performed with five T-RFLP patterns including the 27F + 1492R primer set with *HhaI*, *MspI*, *AluI* and *HaeIII*, and the 529F + 1492R primer set with *RsaI* plus *XspI*, using the phylogenetic assignment database for T-RFLP analysis of human colonic microbiota (PAD-HCM; Matsumoto *et al.*, 2005) and the GenBank nucleotide sequence database. Theoretically, there should be five types of peak fitted to the same bacterium; however, we could not find any candidates with such peaks, probably due to the incompleteness of the PAD-HCM database. Thus, the selection criterion was set for contributions with more than three types of peak fitted to the same bacterium. Based on this criterion, a total of 14 peaks was picked up (Fig. 2), which were thus chosen for detailed analysis. These peaks were designated as bacteria in the

Fig. 2. T-RFs that contribute to the left-side plot of the PCA plot of week 13. Representative T-RFs that contributed to the heavy symptom area on the PCA plot of week 13 are indicated. ■, *Clostridium* subcluster XIVa; ×, *Clostridium* cluster IV; ◇, *Bacteroides fragilis* group (listed in Table 3). Alphanumeric designations represent restriction enzymes and T-RF lengths (bp). A, *AluI*; H, *HhaI*; Ha, *HaeIII*; M, *MspI*; R, *RsaI* + *XspI*.

database that matched the set (Table 3). Major components were the *Clostridium* subcluster XIVa, *Faecalibacterium prausnitzii* and the *Bacteroides fragilis* group.

Table 3. T-RFs contributing to the left-side plot on the PCA plot of week 13

T-RFs were selected using the criterion of those with more than three types of peak fitted to the same bacterium using PAD-HCM and GenBank.

T-RFs digested with each restriction enzyme*					Genus	Species	Group†	GenBank accession no.
<i>HhaI</i>	<i>MspI</i>	<i>HaeIII</i>	<i>AluI</i>	<i>RsaI</i> + <i>XspI</i>				
188	205	271	252	–	Uncultured <i>Ruminococcus</i>	sp.	XIVa	AB064751
188	205	271	252	–	<i>Ruminococcus</i>	sp.	XIVa	AB064896
36	287	263	471	–	Uncultured human intestinal bacterium		UC	AB099785
188	220	271	252	–	Uncultured firmicute		XIVa	AB064771
188	220	271	252	–	Uncultured firmicute		XIVa	AB064778
188	220	271	252	–	Uncultured bacterium		XIVa	AB181447
188	220	271	252	168	Uncultured bacterium		XIVa	AF371546
188	220	271	252	314	<i>Ruminococcus (Peptostreptococcus)</i>	<i>productus</i>	XIVa	D14144
37	287	307	473	–	Uncultured human intestinal bacterium		UC	AB080852
188	220	271	259	–	Uncultured firmicute		XIVa	AB064718
188	220	271	438	–	Uncultured firmicute		XIVa	AB064774
37	281	257	72	–	Uncultured bacterium		IV	AB099731
37	181	224	72	–	<i>Fusobacterium</i>	sp.	IV	AB064908
36	181	224	72	314	<i>Faecalibacterium</i>	<i>prausnitzii</i>	IV	AJ413954
37	281	257	72	168	<i>Eubacterium</i>	<i>siraeum</i>	IV	L34625
561	205	203	160	166	<i>Clostridium</i>	<i>polysaccharolyticum</i>	XIVa	X77839
36–38	283–285	224–6	72–74	314	Butyrate-producing bacterium		IV	AJ270469
102	97	263	32	376	<i>Bacteroides</i>	<i>uniformis</i>	Bact	L16486
102	97	263	32	376	<i>Bacteroides</i>	<i>eggerthii</i>	Bact	L16485
101	96	263	32	376	<i>Bacteroides</i>	<i>vulgatus</i>	Bact	M58762

–, No data in GenBank.

*T-RFs contributing to the left-side plot on the PCA plot of week 13 are shown in bold.

†Bact, *Bacteroides fragilis* group; IV, *Clostridium* cluster IV; XIVa, *Clostridium* subcluster XIVa; UC, unclassified *Clostridium*.

Associations of bifidobacteria with allergic disorders such as atopic eczema have been reported (He *et al.*, 2001; Tejada-Simon *et al.*, 1999). In the present study, bifidobacteria were not identified as a major contributor to changed microbiota in week 13. One possibility is that the primer sets used here limited the detection of bifidobacteria (Hayashi *et al.*, 2004).

Quantification of 16S rRNA gene copies in faecal microbiota before and after the JCP season

The 16S rRNA gene copy numbers of the three changed groups were investigated using real-time PCR. Copy numbers of the 16S rRNA gene for *F. prausnitzii* increased significantly by week 13 compared with week 0 in both JCPsis groups, but not in the healthy group (placebo group, $P=0.034$; BB536 group, $P=0.001$; Table 4). Significant intergroup differences were also observed between placebo and healthy groups at week 13 ($P=0.022$).

In the *Bacteroides fragilis* group, significant increases in 16S rRNA gene copy number from week 0 were found at week 13 for both JCPsis groups but not in the healthy group (placebo group, $P=0.003$; BB536 group, $P=0.002$). Significant intergroup differences were also seen for both placebo and BB536 groups at week 13 compared with the healthy group (placebo group, $P=0.001$; BB536 group, $P=0.011$). Significantly higher copy numbers were found in the placebo group compared with the BB536 group at week 13 ($P<0.05$). We also found a fluctuation in microbiota among the JCPsis subjects in the 2004 study (Odamaki *et al.*, 2007). The most fluctuating group of bacteria was found to be the *Bacteroides fragilis* group, with the cell number increasing along with pollen dispersal, especially at the end of the pollen season (Odamaki *et al.*, 2007). These results obtained in the 2004 and 2005 studies suggested that the fluctuation in microbiota during the pollen season resulted from pollen sensitization and/or

symptom development. Furthermore, the present results show that administration of BB536 lyophilized powder suppressed the increase in the *Bacteroides fragilis* group, as observed in the 2004 study (Odamaki *et al.*, 2007). In order to confirm this at a species level, a total of 288 real-time PCR products (each of 96 clones from three samples) of the *Bacteroides fragilis* group was analysed. Although *Bacteroides uniformis*, *Bacteroides ovatus*, *Bacteroides intestinalis*, *Bacteroides plebeius* and *Bacteroides coprocola*, as well as uncultured *Bacteroidales*, were the main components, there was no significant difference in the bacterial composition between weeks 0 and 13. We suggest that this result shows that the increased 16S rRNA gene copy number of these groups indicates an increase in their cell number.

No significant differences were noted between the BB536 and placebo groups or between JCPsis and healthy subjects in the *Clostridium coccoides* group, mainly of *Clostridium* subcluster XIVa. As the *C. coccoides* group is known to be the most dominant group in faecal microbiota and displays many species or phenotypes (Hayashi *et al.*, 2006), differences at some species or phenotype level may thus exist for this group.

Analysis of the three bacterial groups mentioned above was also performed at other stages (weeks 4, 8, 17 and 28); however, no differences from baseline values were found (data not shown).

As previously reported, BB536 intake was found to relieve JCPsis symptoms efficiently, probably through the modulation of a Th2-skewed immune response (Xiao *et al.*, 2006b). This report revealed changes to faecal microbiota in individuals with JCPsis during the pollen season and showed that such changes were unique to JCPsis subjects. We also showed that intake of the BB536 probiotic strain may have suppressed fluctuations in the microbiota of some subjects, possibly as a result of relieved subjective

Table 4. Real-time PCR quantification of 16S rRNA gene copies in faecal microbiota before and after the pollen season

Gene copies of the 16S rRNA gene (mean $\pm$ SD) were determined as $\log_{10}$ cells (g wet weight faeces) $^{-1}$ compared with a plasmid DNA standard. All data represent triplicate assays.

Group	Week	Placebo	BB536	Healthy
<i>Clostridium coccoides</i> group	0	12.23 $\pm$ 0.54	12.39 $\pm$ 0.41	12.38 $\pm$ 0.46
	13	12.56 $\pm$ 0.29	12.49 $\pm$ 0.60	12.58 $\pm$ 0.51
<i>Faecalibacterium prausnitzii</i>	0	10.22 $\pm$ 0.81	9.94 $\pm$ 1.11	10.04 $\pm$ 0.94
	13	11.04 $\pm$ 0.54*†	10.54 $\pm$ 1.37‡	10.22 $\pm$ 0.96
<i>Bacteroides fragilis</i> group	0	10.19 $\pm$ 0.73	10.10 $\pm$ 0.51	10.05 $\pm$ 0.53
	13	11.14 $\pm$ 0.38‡§	10.65 $\pm$ 0.57†‡	10.27 $\pm$ 0.46

* $P<0.05$, significant intragroup difference from baseline (week 0).

† $P<0.05$, significant intergroup difference compared with the healthy group at each time point.

‡ $P<0.01$, significant intragroup difference from baseline (week 0).

§ $P<0.01$, significant intergroup difference compared with the healthy group at each time point.

|| $P<0.05$, significant intergroup difference between the placebo and BB536 groups at each time point.

symptoms. Fluctuations in the *Bacteroides fragilis* group were suppressed in the BB536 group compared with the placebo group. We reported previously that strains of the *Bacteroides fragilis* group induced significantly more interleukin-6, a Th2-type cytokine, but significantly less Th1-type cytokines (gamma interferon and interleukin-12) compared with *Bifidobacterium* spp. in human peripheral blood mononuclear cells (Odamaki *et al.*, 2007). Kirjavainen *et al.* (2002) found a direct correlation between serum total IgE titre and *Bacteroides* counts in gut microbiota of high-risk subjects with atopic disorders, and bifidobacterial supplementation prevented increases in *Bacteroides* and *Escherichia coli* during weaning. Fukuda *et al.* (2004) found that IgG titres against *Bacteroides vulgatus* were significantly higher among school children with any two of the allergic symptoms of asthma, rhinitis, eczema or food allergy than among non-allergic groups. These lines of evidence imply a deteriorating effect of the *Bacteroides fragilis* group on allergic disorder. Given such a perspective, fluctuations in microbiota might also exacerbate subjective symptom development. Exacerbating circulation may thus occur between subjective symptom development and microbiota fluctuations among JCPsis subjects during the pollen season.

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