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Fluctuation of Fecal Microbiota in Individuals With Japanese Cedar Pollinosis During the Pollen Season and Influence of Probiotic Intake

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Abstract

Background: We have previously reported the results of a randomized, double-blind, placebo-controlled trial that found the intake of yogurt supplemented with a probiotic strain, *Bifidobacterium longum* BB536, alleviates symptoms and affects blood parameters in individuals with Japanese cedar pollinosis (JCPsis) during the pollen season.
Objective: In the present study, fecal microbiota were investigated to examine whether any changes occur during the pollen season and whether any influence is exerted by probiotic intake.
Methods: Yogurt either with BB536 (BB536 yogurt) or without BB536 (placebo yogurt) was administered for 14 weeks at 2 × 100 g per day to 40 subjects (17 men, 23 women) with a clinical history of JCPsis. Fecal samples were obtained from 23 subjects (placebo group, n = 13; BB536 group, n = 10) before and during the intervention (weeks 4, 9 and 13) and fecal microbiota were analyzed using terminal-restriction fragment length polymorphism and real-time polymerase chain reaction (PCR) methods.
Results: From the fluctuation patterns of terminal-restriction fragments, the *Bacteroides fragilis* group and bifidobacteria were among the species that changed most with pollen dispersion. Real-time PCR analyses indicated that the cell numbers of the *B fragilis* group increased significantly along with pollen dispersion in both BB536 and placebo groups. Cell numbers of bifidobacteria were significantly higher in the BB536 group compared with the placebo group ($P < .05$ at weeks 4 and 9). The ratio of cell numbers of the *B fragilis* group to bifidobacteria increased significantly during the pollen season in the placebo group ($P < .01$ at weeks 9 and 14), but not in the BB536 group. An in vitro study using peripheral blood mononuclear cells from JCPsis subjects indicated that strains of the *B fragilis* group induced significantly more helper T cell (T_H) type2 cytokines (interleukin [IL]-6) but fewer T_H1 cytokines (IL-12 and interferon) compared with those of bifidobacteria.
Conclusions: These results suggest a relationship between fluctuation in intestinal microbiota and pollinosis allergy. Furthermore, intake of BB536 yogurt appears to exert positive influences on the formation of anti-allergic microbiota.

Key words: *Bifidobacterium longum*. Allergy. Japanese cedar pollinosis. Cytokine. Probiotic.

Resumen

Antecedentes: Anteriormente habíamos presentado los resultados de un estudio aleatorizado, a doble ciego y controlado con placebo, que determinó que el consumo de yogur suplementado con una cepa probiótica, *Bifidobacterium longum* BB536, alivia los síntomas y afecta los parámetros sanguíneos de las personas con polinosis por cedro japonés (PCJ) durante la estación de polinización.
Objetivo: En el presente estudio, se investigó la microbiota fecal para examinar si se producían cambios durante la estación de polinización y si la ingesta de probióticos ejercía alguna influencia.

Métodos: Durante 14 semanas, se administró yogur con BB536 (yogur BB536) o sin BB536 (yogur placebo) en cantidades de 2 × 100 g al día a 40 sujetos (17 hombres y 23 mujeres) con un historial clínico de PCJ. Se obtuvieron muestras fecales de 23 sujetos (grupo placebo, n = 13; grupo BB536, n = 10) antes y después de la intervención (semanas 4, 9 y 13) y se analizó la microbiota fecal utilizando los métodos de polimorfismo de longitud de fragmentos de restricción terminal y reacción en cadena de la polimerasa (PCR) en tiempo real.
Resultados: Por los patrones de fluctuación de los fragmentos de restricción terminal, las bifidobacterias y el grupo *Bacteroides fragilis* fueron las especies que más cambiaron con la dispersión del polen. Los análisis de la PCR en tiempo real indicaron que los recuentos celulares del grupo *B fragilis* aumentaron significativamente junto con la dispersión de polen tanto en el grupo BB536 como en el placebo. Los recuentos celulares de bifidobacterias fueron significativamente superiores en el grupo BB536 que en el grupo placebo ($P < 0,05$ en las semanas 4 y 9). La proporción de recuentos celulares del grupo B fragilis frente a las bifidobacterias aumentó significativamente en la estación de polinización en el grupo placebo ($P < 0,01$ en las semanas 9 y 14), pero no en el grupo BB536. Un estudio in vitro realizado utilizando células mononucleares de sangre periférica de sujetos con PCJ indicó que las cepas del grupo B fragilis indujeron un número mucho mayor de citocinas tipo Th2 (interleucina (IL)-6), pero menos citocinas Th1 (IL-12 e interferón) comparado con las bifidobacterias.
Conclusiones: Estos resultados sugieren la existencia de una relación entre la microbiota intestinal y la alergia al polen. Lo que es más, la ingesta de yogur BB536 parece ejercer una influencia positiva en la formación de microbiota antialérgica.

Palabras clave: Bifidobacterium longum. Alergia. Polinosis por cedro japonés. Citocina. Probiótico.

Introduction

The hygiene hypothesis, which postulates that the decrease in opportunity of exposure to immunostimulating pathogens in the early childhood causes increased prevalence of allergic diseases, has been proposed to explain the increase in asthma and atopic disease in industrialized countries [1,2]. Animal studies have demonstrated that if sufficient microbial stimuli are not available to the developing immune system during infancy, further maturation becomes inhibited, potentially resulting in persistent dysfunction of helper T cell (T_H) type 2 responses [3,4]. Studies on the composition of intestinal microflora between allergic and non-allergic 2-year-old children have indicated that the prevalence of bifidobacteria is lower in allergic infants, whereas counts of *Staphylococcus aureus* and enterobacteria are higher [5]. In comparison with healthy infants, babies with allergies are less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life [6]. In line with this hypothesis, oral probiotic bacteriotherapy with lactic acid bacteria reduces the risk of atopic eczema, improves the clinical symptoms of perennial allergic rhinitis [7-9] and alleviates the intestinal inflammation associated with food allergies [10, 11].

Japanese cedar pollinosis (JCPsis) is an immunoglobulin (Ig) E-mediated type I allergy caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen. JCPsis represents one of the most widespread allergic diseases in Japan. It constitutes a public health issue, affecting over 16% of the Japanese population, and its prevalence has increased over the past decades [12].

A human trial investigated the effect of yogurt supplemented with *Bifidobacterium longum* BB536, a probiotic strain originally discovered in humans, on the treatment of JCPsis, and found that subjective symptoms were alleviated while increased blood eosinophil rates and decreased interferon (IFN)-γ levels were suppressed [13]. The present study assessed the fecal microbiota of individuals with JCPsis to determine whether any changes occur during the pollen season and whether probiotic intake exerts any influence.

Methods

Clinical Study

Subjects comprised 40 individuals (17 men, 23 women) with a clinical history of JCPsis. They were enrolled in a randomized, double-blind, placebo-controlled trial, as described previously [13]. Briefly, after 2-week run-in period, participants were randomly assigned to groups that ingested 2 × 100 g of yogurt daily either with BB536 (BB536 group, $3.5 \pm 2.4 \times 10^8$ colony forming units [cfu] of living BB536 mL^{-1}) or without BB536 (placebo group) from 15 January until 22 April 2004 (total, 14 weeks comprising 4 weeks before and 10 weeks during pollen season). Both BB536 and placebo yogurt contained approximately 1.0×10^9 cfu of lactic acid bacteria. During the run-in period, participants were instructed not to consume foods or supplements containing lactic acid bacteria or bifidobacteria. Subjective symptoms were recorded daily and blood samples were taken before and during (at weeks 4, 9, and 14) the intervention to measure levels of blood parameters related to JCPsis. The 2004 Japanese cedar pollen season began in mid-February and lasted until the end of April, with a peak from the middle to the end of March in the study region of Kanagawa Prefecture. Participants were instructed not to consume foods or supplements with possible anti-allergic characteristics, or foods or supplements containing other lactic acid bacteria or bifidobacteria during the intervention.

Fecal Sampling for Microbiota Study

Among the 40 individuals, 23 subjects (13 subjects from the placebo group; 10 subjects from the BB536 group) provided written informed consent to participate in the microbiota study (Table 1). Each of these subjects provided fecal specimens before and during the intervention (weeks 4, 9 and 14). Participants were instructed to collect specimens in sterile plastic bags, cool the bag immediately to <10 °C and to deliver the sample within 24 hours. Upon receipt, fecal specimens were stored at –80 °C.

Table 1. Patient Characteristics*

	Placebo Group	BB536 Group
No. of participants	13	10
Age, y†	37.69 (26-61)	36.3 (23-55)
Body weight, kg†	56.1 (41.4-72)	59.28 (47-69.6)
Gender, male/female	5/8	5/5
Clinical history of JCPsis		
Years†	14.58 (2-30)	10.0 (2-30)
Severity		
Level 1 (mild)	2	2
Level 2 (moderate)	5	5
Level 3 (severe)	6	3

* JCPsis indicates Japanese cedar pollinosis

† Data are presented as means, with ranges given in parentheses

DNA Extraction From Fecal Samples

Fecal samples (20 mg) were washed 3 times in 1.0 mL of PBS and centrifuged at 14 000*g*. Fecal pellets were resuspended in 450 μL of extraction buffer (100 mM Tris-HCl, 40 mM EDTA, pH 9.0) and 50 μL of 10% SDS. Glass beads (300 mg; diameter, 0.1 mm) and 500 μL of buffer-saturated phenol were added to the suspension and the mixture was vigorously vortex mixed for 30 seconds using a FastPrep kit (Bio 101, Vista, California, USA) at a power level of 5.0. After centrifugation at 14 000*g* for 5 minutes, 400 μL of supernatant was extracted with phenol-chloroform and 250 μL of the supernatant was precipitated with isopropanol. Pellets were suspended in 200 μL of Tris-EDTA buffer (pH 8.0) and DNA purified using a High Pure Polymerase Chain Reaction (PCR) Template Preparation kit (Roche, Basel, Switzerland) was suspended in 200 μL of TE buffer.

Terminal-Restriction Fragment Length Polymorphism Analysis

Terminal-restriction fragment (T-RF) length polymorphism (T-RFLP) analysis was performed as described previously [14]. Universal primers comprising 529F (ACGTGCCAGCAGCCGCGG) labeled with 6-FAM (6-carboxyfluorescein; Applied Biosystems, Foster City, California, USA) and 1492R (GGTTACCTTGTTACGACTT) were used for PCR amplification [15]. The reaction mixture (50 μL) contained 1 μL of DNA, 1.25 U of TaKaRa Ex TaqTM, 10 × TaKaRa Ex TaqTM buffer, 4 μL of dNTP mixture (2.5 mM each) (TaKaRa Shuzo,Tokyo, Japan) and 10 pmol of primers. DNA was amplified in a Thermocycler T Gradient (Biometra, Goettingen, Germany) according to the following program: preheating at 95 °C for 3 min; followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 90 seconds; a final terminal extension at 72 °C for 10 minutes. Amplified DNA was verified by 1.5% agarose gel electrophoresis. Fluorescently labeled PCR products (50 μL) were purified using MultiScreen FB filter plates (Millipore, Bedford, Massachusetts, USA).

Mung bean nuclease digestion removed pseudo-T-RFs as described by Egert & Friedrich with some modification [16]. Briefly, approximately 1000 ng of PCR products was digested for 30 minutes at 30 °C with 1 U of mung bean nuclease (TaKaRa Shuzo) and 5 μL of 10 × mung bean nuclease buffer in a total volume of 50 μL. The reaction was stopped by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and DNA was precipitated with ethanol.

The DNA was subsequently digested with 20 U of Rsa I (Nippon Gene, Toyama, Japan) plus Bfa I, or Bsl I (New England BioLabs,Ioswich, Massachusetts, USA), in a total volume of 10 μL at 37 °C or 55 °C for 3 hours as previously described [17]. The product (1 μL) was mixed with 8 μL of deionized formamide and 1 μL of 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, Foster City, California, USA). Samples were denatured at 95 °C for 2 minutes, then placed immediately on ice. Lengths of T-RFs were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in GeneScan mode. Fragment sizes were estimated by the local Southern method using GeneScan 3.1 software (Applied Biosystems).

Assignment of each T-RF was based on the RDP database (http://wdcm.nig.ac.jp/RDP/html /analyses.html). To confirm correspondence with the database, several strains of bifidobacteria and the *Bacteroides fragilis* group were analyzed according to T-RFLP. To verify the accuracy of this method, T-RF peak heights and cell numbers of bifidobacteria in samples were measured by T-RFLP and real-time PCR, respectively. Peak heights closely correlated with cell numbers ($\rho = 0.814$, $P < .001$, $n = 92$).

To estimate the fluctuation of each T-RF during intervention, ratios of relative peak areas at weeks 4, 9 and 14 relative to week 0 were calculated for each T-RF. After logarithmic transformation, absolute values at weeks 4, 9 and 14 were summed for each subject as a change index.

Real-Time PCR for Quantitative Determination of Cell Numbers

Cell numbers were estimated by real-time PCR using group-specific primers as described by Matsuki et al [18]. According to this method which DNA extracts from each of specific strains were used as PCR controls for each of representative species, cell numbers of predominant bacteria in feces determined by real-time PCR were found to be in general agreement with values measured by FISH or culture methods [18]. Primer sets used were g-Bfra-F (ATAGCCTTTCGAAAGRAAGAT) and g-Bfra-R (CCAGTATCAACTGCAATTTA) for the *B fragilis* group; and g-Bifid-F (CTCCTGGAAACGGGTGG) and g-Bifid-R (GGTGTTCTTCCCGATATCTACA) for

bifidobacteria cells, respectively [18]. Real-time PCR was conducted using a Smart Cycler II system (Cepheid, Sunnyvale, California, USA) and SYBR Premix Ex Taq (TaKaRa Shuzo). Annealing temperatures were 50°C for *B fragilis* and 55°C for bifidobacteria. The amplification program consisted of 1 cycle of 94°C for 10 seconds, followed by 40 cycles of 94°C for 5 seconds, annealing temperature for 20 seconds, and 72°C for 30 seconds. Fluorescent products were detected at the last step of each cycle. Melting curves were obtained by heating from 60°C to 95°C in 0.2°C per 1 second increments, with continuous fluorescence collection. DNA extracted from *B longum* JCM 1217T and *Bacteroides vulgatus* JCM 5826T were used as real-time PCR controls [18].

Cytokine Induction in Peripheral Blood Mononuclear Cells

A total of 31 strains of Bifidobacteria and 21 strains of the *B fragilis* group were used in the cytokine induction study (Table 2). All clinical isolates were from fecal samples of JCPsis volunteers participating in the present intervention. All bacterial strains were first cultured on GAM broth (Nissui Pharmaceutical, Tokyo, Japan), then collected by centrifugation and washed with PBS and distilled water twice. Cells were heat-killed (121°C, 15minutes) and lyophilized at –40°C. Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood of 3 JCPsis subjects by density gradient centrifugation (1077 g mL^{-1}, Histopaque-1077; Sigma, St Louis, Missouri, USA). Induction and assay of cytokine were conducted as previously described with some modifications [19]. Briefly, cells were resuspended in RPMI 1640 culture medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% (vol/vol) heat-inactivated (55°C, 30 minutes) fetal bovine serum and penicillin-streptomycin (1%). Cells (1×10^6 mL^{-1}) were cultured in complete medium with 100 μg of each bacterium mL^{-1} in 48-well plates (NUNC A/S, Roskilde, Denmark) at 37°C for 24 hours with 5% CO_2. Levels of cytokines (interleukin [IL]-6, IL-10, IL-12p40 and interferon [IFN]-γ) in culture supernatants were measured by capture enzyme-linked immunosorbent assay (ELISA), and determined in each case against a standard curve generated by employing known amounts of recombinant cytokine, using ELISA development kits (R&D Systems, Minneapolis, Minnesota, USA).

Statistical Analysis

All statistical analyses were performed using SPSS statistical software version 11.0. The Mann-Whitney U test was used to analyze inter-group differences between groups. Intra-group differences were analyzed using the Wilcoxon signed rank test with Bonferroni correction. Values of $P<.05$ were considered statistically significant.

Results

T-RFLP Analyses of Fecal Microbiota

Over 200 T-RFs were distinguished in T-RFLP profiles after digestion with *Rsa* I plus *Bfa* I and *Bsl* I. Table 3 shows base pairs, relative peak areas and change indices of the predominant T-RFs. Since T-RFLP is a semi-quantitative tool and subject numbers were relatively small, relative peak areas and change indices were averaged for total participants. Total area of these predominant peaks accounted for ≥80% of

Table 2. Strains Used for Cytokine Induction in Human Peripheral Blood Mononuclear Cells.

Species	Strains
Bifidobacterium longum	JCM 1217^T, ATCC15708 and 9 clinical isolates*
Bifidobacterium adolescentis	JCM1275^T, ATCC15706 and 5 clinical isolates
Bifidobacterium catenulatum	JCM 1194^T and 2 clinical isolates
Bifidobacterium pseudocatenulatum	JCM 1200^T, JCM 7041 and 2 clinical isolates
Bifidobacterium infantis	ATCC 15697
Bifidobacterium breve	ATCC 15700^T
Bifidobacterium angulatum	ATCC 27535^T, ATCC 27669
Bifidobacterium dentium	JCM 7135, ATCC 15424
Bacteroides fragilis	JCM 11019^T and 2 clinical isolates
Bacteroides ovatus	JCM 5824^T and 2 clinical isolates
Bacteroides distasonis	JCM 5825^T
Bacteroides vulgatus	JCM 5826^T and 3 clinical isolates
Bacteroides thetaiotaomicron	JCM 5827^T
Bacteroides uniformis	JCM 5828^T and 3 clinical isolates
Bacteroides stercoris	JCM 9496^T
Bacteroides merdae	JCM 9497^T
Bacteroides caccae	JCM 9498^T and 2 clinical isolates

* All clinical isolates were isolated from fecal samples of Japanese cedar pollinosis volunteers participating in the present study.

Table 3. Predominant Terminal-Restriction Fragments and Corresponding Organisms

Digestion Type	by	% Peak Area*				Change Index†	Corresponding Organisms (Major Large Intestinal Bacterial Genera)
		Week 0	Week 4	Week 9	Week 14		
Rsa + *Bfa* I	114	3.27±4.19	3.87±6.19	3.63±5.39	3.10±4.47	0.99‡	Eubacteria, *Prevotella* species
	129	9.80±10.04	11.64±11.69	11.68±12.32	8.52±8.11	1.12‡	Bifidobacteria
	138	7.10±4.26	7.82±3.55	4.50±3.36	6.35±3.32	0.98‡	Clostridia
	144	2.18±2.07	1.94±2.48	1.65±1.50	1.92±2.50	0.99	Clostridia, eubacteria, *Bacteroide* species, *Prevotella* species
	145	1.59±2.83	2.46±5.18	3.67±5.40	4.06±6.80	1.66	*Bacteroides, Prevotella* species
	167	4.71±4.57	4056±3.73	4.22±3.86	3.73±4.11	0.58	Clostridia, eubacteria, peptostreptococci, *Atopobium* species
	168	28.98±12.39	28.40±11.32	24.70±9.28	25.79±7.56	0.46	Clostridia, ruminococci, eubacteria, *Atopobium* species
	311	9.28±4.83	10.80±7.03	12.02±4.82	11.26±4.21	0.70	Clostridia, peptostreptococci, eubacteria
	312	4.19±2.87	3.07±3.07	6.26±3.36	5.00±3.48	0.66	Streptococci, lactobacilli
	376	1.12±2.19	1.29±2.44	3.37±4.64	3.69±4.25	1.55	*Bacteroides*, *Prevotella* species
	379	2.23±2.59	0.75±1.40	2.81±2.35	2.31±1.51	0.82	Enterobacteria, *Escherichia coli*, enterococci
	380	6.33±4.03	4.39±2.73	5.54±3.87	4.44±3.30	0.86	Enterococci, lactobacilli
Bsl I	130	19.47±19.24	23.13±21.93	19.43±19.58	17.95±15.38	1.00	Bifidobacteria
	470	2.65±4.22	5.17±8.63	7.39±10.81	9.06±11.37	1.40	*Bacteroides, Prevotella* species
	490	6.24±4.48	5.89±5.34	6.03±4.36	4.97±4.01	0.73	Clostridia, ruminoccocci, eubacteria
	755	3.71±1.96	3.07±3.29	3.34±2.38	2.51±2.23	0.56	Clostridia
	921	8.10±6.66	8.23±7.44	8.26±8.67	7.81±4.72	0.77	Enterococci
	937	21.18±8.50	18.00±11.16	19.14±11.28	18.21±8.33	0.78	Ruminococci
	959	4.58±2.74	3.05±2.09	2.71±1.19	3.41±2.17	0.92	Clostridia, ruminococci
	993	17.35±7.48	16.17±10.10	13.77±8.66	17.80±11.80	0.79	Clostridia, ruminococci, eubacteria, enterococci

* Percentage of each peak area relative to total terminal-restriction fragments (T-RF) areas of each participant (mean±SD) of all participants, n = 23.
† Change index for each T-RF during intervention. Ratios of peak area at weeks 4, 9 and 14 in relative to week 0 were calculated for each T-RF and change index was calculated by summing absolute logarithmic values of ratio at weeks 4, 9 and 14 for each participant (means of all participants, n = 23).
‡ Each T-RF was ascribed to the corresponding organism using the Ribosomal Database Project database.

Table 4. Estimated Fecal Microbiota Cell Counts for the *Bacteroides fragilis* and Bitidobacteria Groups and Ratios Betwen Counts During the Intervention Period

Bacteroides fragilis Group*				
Group	Week 0	Week 4	Week 9	Week 14
Placebo	9.69±0.63	9.85±0.57	10.34±0.32‡	10.52±0.40‖
BB536	9.33±0.79	9.57±0.68	10.21±0.34‡	10.32±0.41‡
Bifidobacteria Group*				
Group	Week 0	Week 4	Week 9	Week 14
Placebo	9.61±0.95	9.41±0.53	9.30±1.13	9.36±1.17
BB536	9.98±0.37	10.02±0.50§	10.04±0.56§	10.16±0.61
Bacteroides fragilis Group / Bifidobacteria Ratio†				
Group	Week 0	Week 4	Week 9	Week 14
Placebo	0.84 (0.45-6.12)	4.61 (0.60-5.42)	8.75‖ (1.96-27.8)	13.89‖ (3.32-38.6)
BB536	0.57 (0.02-1.34)	0.34§ (0.10-1.83)	1.34§ (0.67-2.94)	1.01§ (0.73-3.26)

* Cell numbers (mean±SD, log 10) per gram of wet-weight feces. All data represent triplicate assays.
† Medians (interquartile ranges).
‡ $P<.05$
§ $P<.05$, significant inter-group difference
‖ $P<.01$, significant intra-group difference from baseline (week 0)

the total. Predominant T-RFs were assigned to *Clostridium-Ruminococcus, Eubacterium, Peptostreptococcus, Atopobium, Bacteroides, Prevotella* and *Bifidobacterium* species. Judging from relative peak areas and change indices, T-RFs corresponding to the *B fragilis* group (T-RFs 145 and 376 digested with Rsa I plus Bfa I; and T-RF 470 digested with Bsl I) and bifidobacteria (T-RF 129 digested with *Rsa* I plus *Bfa* I; and T-RF 130 digested with *Bsl* I) fluctuated the most during the intervention.

Quantification of Cell Numbers of Bacteroides fragilis *Group and Bifidobacteria by Real-Time PCR*

Table 4 shows cell numbers estimated by real-time PCR of the *B fragilis* group and bifidobacteria at weeks 0, 4, 9 and 14. Cell numbers of the *B fragilis* group increased significantly from week 0 in both groups (placebo, $P<.05$, and $P<.01$ at weeks 9 and 14, respectively; BB536, $P<.05$ at weeks 9 and

Table 5. Profile of Cytokines Secreted by Human Peripheral Blood Mononuclear Cells in Response to Stimulation by Strains of the *Bacteroides fragilis* Group and Bifidobacteria

Group	Concentration of Cytokines (ng mL^{-1})*			
	IL6	IL-10	IL-12p40	IFN-γ
Bifidobacteria (31 strains)	10.60±8.91†	2.22±1.43	0.37±0.35‡	0.18±0.25§
Bacteroides fragilis Group (21 strains)	20.83±12.09	2.22±1.42	0.17±0.21	0.04±0.06

* Mean±SD of each treatment from duplicate wells. Human peripheral blood mononuclear cells were isolated from peripheral blood of 3 Japanese cedar pollinosis volunteers. Cells were cultured in complete medium at a concentration of 1×10^6 cells mL^{-1} with a bacterial concentration of 100 μg mL^{-1} in a 5% CO_2-humidified incubator at 37° C for 24 hours.
† $P<0.001$; ‡ $P<0.01$; § $P<0.05$, significant difference compared with the *Bacteroides fragilis* group.
IL indicates interleukin; IFN, interferon.

14), but with no significant inter-group differences. On the other hand, cell numbers of bifidobacteria were significantly higher in the BB536 group compared with the placebo group ($P < .05$ at weeks 4 and 9, $P = .068$ at week 14). Furthermore, cell ratios of *B fragilis* group to bifidobacteria increased significantly from week 0 to weeks 9 and 14 in the placebo group ($P < .01$). However, no marked changes were found in the BB536 group. Significant inter-group differences were observed at weeks 4, 9 and 14.

Induction of Major Immunoregulatory Cytokines

Since the *B fragilis* group and bifidobacteria fluctuated the most during the pollen season, the immunoregulatory ability of these bacteria was investigated by measuring cytokine secretion patterns in PBMCs from JCPsis subjects stimulated using heat-killed bacteria (Table 5). Strains of *B fragilis* group induced significantly large amounts of IL-6 ($P < .001$) in PBMCs compared with bifidobacteria. In contrast, bifidobacteria induced significantly more of the T_H1 cytokines IFN-γ and IL-12 ($P < .05$, $P < .01$, respectively). No difference was found in IL-10 levels induced by these 2 groups. No marked differences were found in cytokine levels induced by different species of bifidobacteria or different species of the *B fragilis* group.

Discussion

The association between allergy and microbiota has recently received considerable attention. However, to the best of our knowledge, the microbiota has usually been characterized in comparative studies of allergic and non-allergic individuals and few studies have focused on microbiota changes accompanying allergic sensitization. Furthermore, although modification of the microbiota has been postulated as part of the mechanisms through which bacteriotherapy alleviates allergic symptoms, the effect of probiotic administration on the microbiota has not been sufficiently investigated.

We determined bacterial species significantly fluctuated during the pollen season using T-RFLP, which is a rapid and powerful method of comparing spatial and temporal changes in bacterial communities [14,15]. We found that bifidobacteria and the *B fragilis* group were among the species that fluctuated most extensively.

Since T-RFLP provided only semi-quantitative results, cell numbers of the *B fragilis* group and bifidobacteria in fecal microbiota were further investigated using real-time PCR. The method developed by Matsuki et al [18] is a quantitative PCR detection method for investigation of the composition of human intestinal flora and has been shown to be higher sensitivity, easy sample handling, simple procedures, and give results in general agreement with values measured by FISH or culture methods. We found that cell numbers of the *B fragilis* group increased significantly from week 0 in both groups, but with no significant inter-group differences. Cell numbers of bifidobacteria were significantly higher in the BB536 group compared with the placebo group during the pollen season. Furthermore, marked increases were found in ratios of the *B fragilis* group to bifidobacteria in the placebo group, but not in the BB536 group, with a significant inter-group difference identified.

Bifidobacterium species are one of the major components of gut microbiota in humans, and are frequently associated with health-promoting effects [20]. Studies on the gut microflora between allergic and non-allergic individuals have indicated distinct compositions, including different *Bifidobacterium* colonization [21]. Studies have also shown the immunostimulating and antitumor effects of bifidobacteria on oral administration [22-24]. Many physiological effects such as stimulating immunity, reducing cancer risk, and preventing harmful bacterial infection, particularly a pronounced promoting effect on the intestinal environment, have been reported for BB536, and this strain has been commercially applied to various aspects of the food industry in many countries [24-26]. Our in vitro and in vivo studies have also implied the potential of BB536 for immune modulation (results not published). Yogurt supplemented with BB536 promotes a healthy intestinal environment by increasing counts and relative ratios of bifidobacteria, in addition to defecation frequency after consumption for 2 weeks [25].

Conversely, Kirjavainen et al [27] found a direct correlation between serum total IgE titer and bacteroides counts in the gut microbiota of high-risk subjects with atopic disorders, and bifidobacterial supplementation prevented increases in bacteroides and *Escherichia coli* during weaning. Fukuda et al [28] found that IgG titers against *B vulgatus* were significantly higher among school children with any 2 of the allergic symptoms of asthma, rhinitis, eczema or food allergy than among non-allergic groups. These findings suggest a beneficial role of Bifidobacteria and a deteriorating effect of the *B fragilis* group on allergic conditions. To support this hypothesis, we measured the induction of major immunoregulatory cytokines. In vitro study indicated that strains of the *B fragilis* group induced significantly more IL-6 that is one of the T_H2-type cytokines, but significantly less T_H1-type cytokines (IFN-γ and IL-12) compared with those of *Bifidobacterium* species in PBMC derived from JCPsis volunteers. Cytokine induction in PBMC from four healthy subjects was also assayed with each of 10 strains of bifidobacteria and *B fragilis* group; the pattern was similar to JCPsis subjects (data not shown). The present results indicate different capacities in the induction of cytokines by the *B fragilis* group and bifidobacteria in PBMCs, and thus potentially suggests the immunoregulatory ability of these bacteria in the intestinal tract of JCPsis subjects, particularly given the different fluctuation patterns during the pollen season.

An alternative explanation is that allergic sensitization promotes the growth of bacteroides by causing modifications in the gut microbiota. Matsuki et al [18] reported that the number of the *B fragilis* group cells in feces from 6 healthy individuals remained comparatively stable over 8 months. This finding supports the notion that bacteria in the present study might be influenced by allergic sensitization during the pollen season, although the influence of long-term yogurt consumption requires further investigation.

In conclusion, the present results indicate a noticeable

fluctuation in fecal microbiota during the pollen season. Furthermore, supplementation with BB536 yogurt modulates the microbiota in a manner that could contribute to the alleviation of allergic symptoms. However, since placebo yogurt containing ordinary lactic acid bacteria was used in the present study, some effects from placebo yogurt on microbiota cannot be excluded. Total amount of pollen scattered in the 2004 season was about 10-fold lower than average, so the influences of pollen sensitization and the effects of probiotic administration on fecal microbiota should be further evaluated in a season of normal or heavy pollen dispersion. Further studies are underway to evaluate seasonal changes and the effects of BB536 intake on microbiota in JCPsis subjects in comparison with non-JCPsis individuals.

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