

ORIGINAL ARTICLE

***Lactobacillus* strains stabilize intestinal microbiota in Japanese cedar pollinosis patients**

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ABSTRACT

A randomized double-blind, placebo-controlled trial was conducted to ascertain the intestinal microbiota-altering properties of LGG and *L. gasseri* TMC0356 (TMC0356) in Japanese cedar *Cryptomeria japonica* pollinosis patients. Fecal bacteria communities were examined before and after fermented milk administration using culture, FISH and T-RFLP methods. Test group subjects showed the presence of LGG and TMC0356 along with a significant increase in fecal lactobacilli ($P < 0.001$) after giving LGG and TMC0356 fermented milk. Culture and FISH analysis revealed no significant changes in other intestinal bacterial groups. Each subject exhibited a characteristic T-RFLP profile pattern that varied quantitatively and qualitatively with JCP shedding. Profile changes were observed in 53% of placebo group subjects and in 21% of test group subject's post-administration, indicating that LGG and TMC0356 suppressed intestinal microbiota changes in JCPsis patients. The results suggest that intestinal microbiota might be more sensitive to exposure to environmental allergens than expected from the results of general culture method studies. Stabilization of intestinal microbiota by selected probiotic strains such as LGG and TMC0356 could be beneficial to homeostasis of the intestinal microbiota and useful in the management of JCPsis.

Key words intestinal microbiota, *Lactobacillus*, pollen allergy, probiotic.

Rhinitis is an inflammation of nasal mucus caused by IgE-mediated hypersensitivity to environmental allergens (1–3). Allergic rhinitis, particularly seasonal allergic rhinitis, is caused by two major allergens (Cry j 1, Cry j 2) in Japanese cedar (*Cryptomeria japonica*) pollen (JCP), and has become a serious health problem in Japan (4, 5). Allergic rhinitis can significantly deteriorate the quality of life of both patients and their families, and can impact negatively on work productivity, school performance and social activities.

Anti-inflammatory drugs, such as histamine release antagonists and corticosteroids, demonstrate notable clinical

efficacy against allergic diseases; however, frequent recurrence of inflammation after drug treatments indicates the need for a therapy that will inhibit additional pathways of the allergic response (6, 7). Most anti-allergic pharmaceuticals are efficacious in the effector phase of the allergic response, and act mainly through inhibition of transcription regulatory molecules and enzymes (6–8). However, their pharmaceuticals are generally differentiated at stages and, in particular, are ineffective against specific IgE regulation in the immune system. Therefore, an innovative treatment aimed at host immunity is necessary in the management of JCPsis.

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List of Abbreviations: DAPI, 4',6-diamino-2-phenylindole; FISH, fluorescence *in situ* hybridization; IgE, immunoglobulin E; JCP, Japanese cedar pollen; JCPsis, Japanese cedar pollinosis; LGG, *Lactobacillus rhamnosus* GG; T-RFLP, terminal restriction fragment length polymorphism.

Fuller (9) suggested that a probiotic was a live microbial feed supplement that beneficially affected the host animal by improving its intestinal microbial balance. The definition of probiotic has been broadened with the expansion of knowledge on symbiotic interactions between microbes and host animals (10–12). A more current definition, published following Expert Consultation at a 2001 FAO/WHO meeting, is 'probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host'. At present, most probiotic organisms are lactic acid bacteria, especially lactobacilli and bifidobacteria, which possess adhesive properties and the ability to colonize, at least transiently, the human gastrointestinal tract. Evidence from recent clinical and animal studies has supported the idea that lactobacilli, especially some selected strains, can modify the immune responses of the host and protect against allergic disease (13–17). However, the underlying mechanisms by which lactobacilli alter allergic responses remain unclear.

In this 2006 double-blind, placebo-controlled study, fermented milk prepared with LGG and *L. gasseri* TMC0356 (TMC0356) was given orally for 10 weeks to subjects with JCPs during the JCP shedding season. The bacterial composition of fecal samples collected from study subjects was evaluated using culture and culture-independent methods, both before and after administration.

MATERIALS AND METHODS

Bacterial strains

LGG (ATCC 53103) was supplied by Valio (Helsinki, Finland). TMC0356 were stored in the Technical Research Laboratory of Takanashi Milk Products Co., Ltd (Yokohama, Japan). Lactobacilli were routinely cultured at 37 °C for 18 hr in MRS broth (Becton Dickinson, Sparks, MD, USA) and 10% skim milk until use.

Preparation of fermented milk

Pasteurized milk (8.0% solids non-fat [SNF], 2.0% milk fat) was inoculated with LGG and TMC0356 and incubated at 37 °C for 18 hr (test fermented milk). pH and acidity of the test fermented milk were 4.60 and 0.9%, respectively. The numbers of LGG and TMC0356 in the fermented milk were $>1.4 \times 10^8$ and $>1.0 \times 10^7$ cfu/mL, respectively. A yogurt placebo was made by adding lactic acid to the aforementioned pasteurized milk. There were no obvious differences between the test fermented milk and the placebo at packaging; the contents looked and tasted identical.

Subjects

Before JCP shedding season, 95 subjects with a clinical history of JCPs were recruited from Kanagawa Prefecture, Japan. Nasal cavity medical examination and blood chemistry determination were carried out at Sugano Clinic (Kawasaki, Japan). After examination, 44 patients (15 males and 29 females aged 20–57 years) were selected to participate in this study. All subjects had mild symptoms of JCPs, and were positive for IgE against JCP. None was allergic to milk or had received specific immunotherapy previously.

Giving fermented milk and collection of fecal samples

The study was carried out in accordance with the Declaration of Helsinki, and protocol was approved by the Ethics Committees of HUMA R&D Co., Ltd, and Sugano Clinic on Clinical Tests, as well as by the Ethics Committee of Shinagawa East One Medical Clinic, Tokyo. All subjects provided written informed consent for participation in the study.

The subjects were randomized and divided into two equal-sized groups: test and placebo ($n = 22$) and were given 100 g/day test fermented milk and placebo, respectively, for 10 weeks. Fecal samples were collected before and after administration once, respectively, and were stored at 4 °C under anaerobic conditions before culture and frozen at –80 °C before molecular analysis.

Fecal bacteria analysis using culture methods

Fecal bacterial analysis was conducted using the methods of Mitsuoka (18) with some modification (19, 20). Briefly, fecal samples were homogenized and a 10-fold serial dilution was made 6–10 hr after sampling. An appropriate dilution (0.1 mL) was streaked on agar plates. Anaerobic incubation was carried out by Anaeropack Kenki System (Mitsubishi-Gas Chemical Co., Ltd, Tokyo, Japan). The isolated bacteria were identified based on aerobic test, Gram stain, colony and cell morphologies and spore formation.

Eggerth Gagnon agar (EG agar; Nissui Seiyaku Co., Ltd, Tokyo, Japan), blood liver agar (BL agar; Nissui Seiyaku Co., Ltd), and trypticase soy blood agar (BBL, Cockeysville, MD, USA) were used as non-selective media. Selective media used were BS, NBGT, CW, ES and DHL (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) for detection of *Bifidobacterium*, Bacteroidaceae, *Clostridium*, *Eubacterium* and Enterobacteriaceae, respectively.

Isolates on LBS agar were selected as lactobacilli, recultured with MRS (BBL) and briefly identified according to

the methods of Kozaki *et al.* (21). For determination of LGG and TMC0356, large, white and creamy or transparent colonies, respectively, were enumerated. Final identification was based on carbohydrate fermentation profiles determined by the API 50CHL test strip (Bio Merieux S.A., Lyon, France) for LGG, and pulse field gel electrophoresis (PFGE) for TMC0356.

Fecal bacteria analysis using FISH

Fluorescence *in situ* hybridization analysis was conducted as described previously (22, 23). Briefly, samples were suspended in PBS and homogenized. Bacteria were fixed with paraformaldehyde and hybridized with a Cy3 indocarbocyanine-labeled oligonucleotide probe. Probes included Bifi164 (5'-CATCCGGCATTACCAC CC-3') for bifidobacteria, Bac303 (5'-CCAATGTGG GGGACCTT-3') for *Bacteroides*, Lab158 (5'-GGTAT TAGCA(T/C)GTGTTTCCA-3') for *Lactobacilli* and *Enterococcus*, and His150 (5'-TTATGCGGTATTAATCT (C/T)CCTTT-3') for the *Clostridium histolyticum* group. Total bacterial counts were determined after staining with DAPI. The bacteria were then washed with hybridization buffer, filtered through a 0.2 μ m polycarbonate filter, mounted on a slide, and counted visually using epifluorescence microscopy with Cy3- and DAPI-specific filters.

Fecal analysis using T-RFLP

Fecal samples were suspended in a solution containing 100 mM Tris-HCl (pH 9.0) and 40 mM ethylenediaminetetraacetic acid (EDTA) after washing three times with sterile distilled water, then beaten in the presence of glass beads using a mini-bead beater (BioSpec Products, Bartlesville, OK, USA). Thereafter, DNA was extracted from the bead-treated suspension using benzyl chloride as described by Zhu *et al.* (24), and purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK). The amplification of fecal 16SrDNA, restriction enzyme digestion and size-fractionation of T-RF data analysis were carried out as previously described (25, 26). Briefly, PCR was carried out using total fecal DNA and the primer of 5'HEX-labeled 516f (5' 532) and 1510r (5'-GGTTACCTTGTTACGACTT-3'; *E. coli* positions 1510 to 1492). The resulting 16SrDNA amplicons were treated with 2 U Bs/I (New England Biolabs, Ipswich, MA, USA) for 1 hr and digestive fractions were obtained using an automated sequence analyzer (ABI PRISM 310, Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) in GeneScan mode (injection time was 20 s and run-time was 40 min).

Statistical analysis

The results were expressed as mean values $\pm$ standard errors. Statistical differences comparing the values of the test and control groups were calculated by Student's *t*-test. The significance level was set at $P < 0.05$.

RESULTS

The bacterial composition of feces collected from 29 subjects (15 from the placebo group and 14 from the test group) both before and after administration was analyzed using the culture method (Table 1). There were no significant differences in total bacterial count, total anaerobes, total aerobes and other bacterial groups, both before and after administration within each group and between the two groups. However, the occurrence of bifidobacteria increased significantly ($P < 0.05$) from 18.54% to 33.88% in the test group after administration. Fecal lactobacilli in the stool samples of the test group were significantly increased ($P < 0.001$) after administration. Both LGG and TMC0356 were isolated from, and taxonomically identified in, all fecal samples collected from subjects given test fermented milk (data not shown).

Fecal bacterial compositions were also analyzed by FISH (Table 2). Total bacteria and fecal lactobacilli counts significantly increased in the fecal samples of test group subjects ($P < 0.05$), whereas *Bacteroides* significantly decreased in the placebo group subjects after administration. No differences were found in total bacteria, bifidobacteria, *Bacteroides*, *Lactobacillus*, *Enterococcus* and *Clostridium histolyticum* between test and placebo groups after administration.

Through T-RFLP analysis, the T-RFLP profile of each individual was characterized. The fecal T-RFLP profiling pattern varied both quantitatively and qualitatively during JCP shedding (Fig. 1). In the placebo group, 53% (8/15) showed apparent changes in their fecal T-RFLP profile pattern (i.e. 17 > 90% similarity in patterns before and after administration), whereas only 21% (3/14) of the test group subjects exhibited significant changes after administration.

DISCUSSION

Lactobacillus rhamnosus GG, a widely researched probiotic strain, can survive passage through the human gastrointestinal tract and characteristically adheres to intestinal epithelial cells and mucus (27, 28). Successful colonization in the intestine is believed to be a key mechanism for the health-promoting effects of this bacterium, including prevention of antibiotic-associated diarrhea, treatment and prevention of rotavirus diarrhea, treatment of relapsing

Table 1. Fecal bacteria composition in subjects before and after giving fermented milk prepared with LGG and TMC0356

Bacteria	Bacterial count (log10 cfu/g wet feces) (Rate of occurrence, No. of positive/No. of subjects)							
	Placebo group (n = 15)				Test group (n = 14)			
	Before		After		Before		After	
	Mean ± SD	Occurrence	Mean ± SD	Occurrence	Mean ± SD	Occurrence	Mean ± SD	Occurrence
Total cell count	10.50 ± 0.17		10.67 ± 0.35		10.74 ± 0.22		10.65 ± 0.19	
Anaerobes	10.50 ± 0.18		10.67 ± 0.35		10.74 ± 0.23		10.65 ± 0.19	
Aerobes	7.68 ± 1.43		7.63 ± 1.20		8.21 ± 0.62		8.34 ± 0.53	
Bacteroidaceae	10.08 ± 0.25	(15/15)	10.27 ± 0.39	(15/15)	10.29 ± 0.26	(14/14)	10.31 ± 0.34	(14/14)
<i>Bifidobacterium</i>	9.76 ± 0.49	(14/15)	9.90 ± 0.53	(14/15)	9.85 ± 0.48	(14/14)	10.05 ± 0.44	(14/14)
<i>Bifidobacterium</i> /Total cell count (%)	24.74 ± 16.64		27.70 ± 22.71		18.54 ± 14.99		33.88 ± 21.62 ^a	
<i>Eubacterium</i>	9.70 ± 0.40	(15/15)	9.66 ± 0.63	(15/15)	9.89 ± 0.34	(14/14)	9.64 ± 0.47	(14/14)
<i>Clostridium</i> (lecithinase positive)	3.81 ± 1.20	(7/15)	3.89 ± 1.26	(4/15)	4.18 ± 1.72	(6/14)	4.23 ± 1.36	(5/14)
<i>Clostridium</i> (lecithinase negative)	8.03 ± 1.05	(15/15)	8.24 ± 0.77	(15/15)	7.55 ± 2.00	(14/14)	8.14 ± 0.70	(14/14)
<i>Fusobacterium</i>	ND	(0/15)	8.78 ± 0.76	(4/15)	8.89 ± 0.70	(3/14)	8.88 ± 0.37	(5/14)
<i>Veillonella</i>	ND	(0/15)	9.66 ± 0.10	(3/15)	9.30 ± 0.00	(1/14)	9.10 ± 0.28	(2/14)
Peptococcaceae	9.31 ± 0.38	(8/15)	8.83 ± 0.50	(3/15)	9.03 ± 1.06	(6/14)	8.07 ± 1.21	(4/14)
<i>Lactobacillus</i>	4.54 ± 0.70	(12/15)	4.81 ± 1.40	(11/15)	5.43 ± 1.43	(12/14)	7.52 ± 1.11 ^b	(14/14)
<i>Streptococcus</i>	6.50 ± 1.79	(15/15)	6.57 ± 1.44	(15/15)	7.00 ± 1.06	(14/14)	7.21 ± 0.83	(14/14)
<i>Staphylococcus</i>	2.50 ± 0.17	(3/15)	3.33 ± 1.57 ^a	(13/15)	2.30 ± 0.00	(4/14)	4.35 ± 2.57	(6/14)
Enterobacteriaceae	6.70 ± 1.58	(15/15)	6.42 ± 1.41	(15/15)	6.86 ± 1.44	(14/14)	7.01 ± 1.11	(14/14)
<i>Bacillus</i>	6.97 ± 0.37	(9/15)	7.62 ± 0.86	(5/15)	7.50 ± 0.75	(11/14)	7.36 ± 0.85	(7/14)
Yeasts	3.58 ± 0.05	(4/15)	3.22 ± 0.67	(5/15)	3.42 ± 0.80	(4/14)	3.18 ± 0.93	(5/14)

ND, not detected (<10⁷/g).^aP < 0.05, to those before administration.^bP < 0.001, to those before administration.

Clostridium difficile diarrhea, prevention of acute diarrhea and enhancement of intestinal immunity (29, 30). LGG is reported to protect infants with a genetically high risk for allergic diseases from development of atopic disease (15, 16) and to alleviate atopic eczema-dermatitis syndrome by increasing interferon (IFN)- γ responses of peripheral lymphocytes in infants allergic to cow's milk and in in-

fants with IgE-associated atopic eczema-dermatitis syndrome (31, 32). However, LGG did not significantly alter birch-pollen allergy, an adult-type allergic disorder (33). In Japan, several selected lactobacilli and bifidobacteria have been evaluated for their ability to alleviate JCPsis in animal and human studies (14, 34–36). However, the clinical effects of these bacteria on JCPsis in humans have not

Table 2 Bacterial counts in fecal samples analyzed by FISH

Bacteria	Bacterial count (log10 cells/g wet feces)			
	Placebo group (n = 15)		Test group (n = 14)	
	Before Mean ± SD	After Mean ± SD	Before Mean ± SD	After Mean ± SD
Total cell counts	10.92 ± 0.33	11.01 ± 0.31	10.97 ± 0.38	11.17 ± 0.23*
Bifidobacteria	10.22 ± 0.59	10.21 ± 0.56	10.45 ± 0.35	10.53 ± 0.46
Bacteroides	9.48 ± 0.60	8.96 ± 0.39*	9.39 ± 0.75	9.03 ± 0.54
<i>Clostridium histolyticum</i>	8.49 ± 0.25	8.43 ± 0.39	8.31 ± 0.41	8.40 ± 0.37
Lactobacilli/Enterococci	8.85 ± 0.31	8.67 ± 0.40	8.80 ± 0.37	9.13 ± 0.30*

*P < 0.05 (to those before administration).

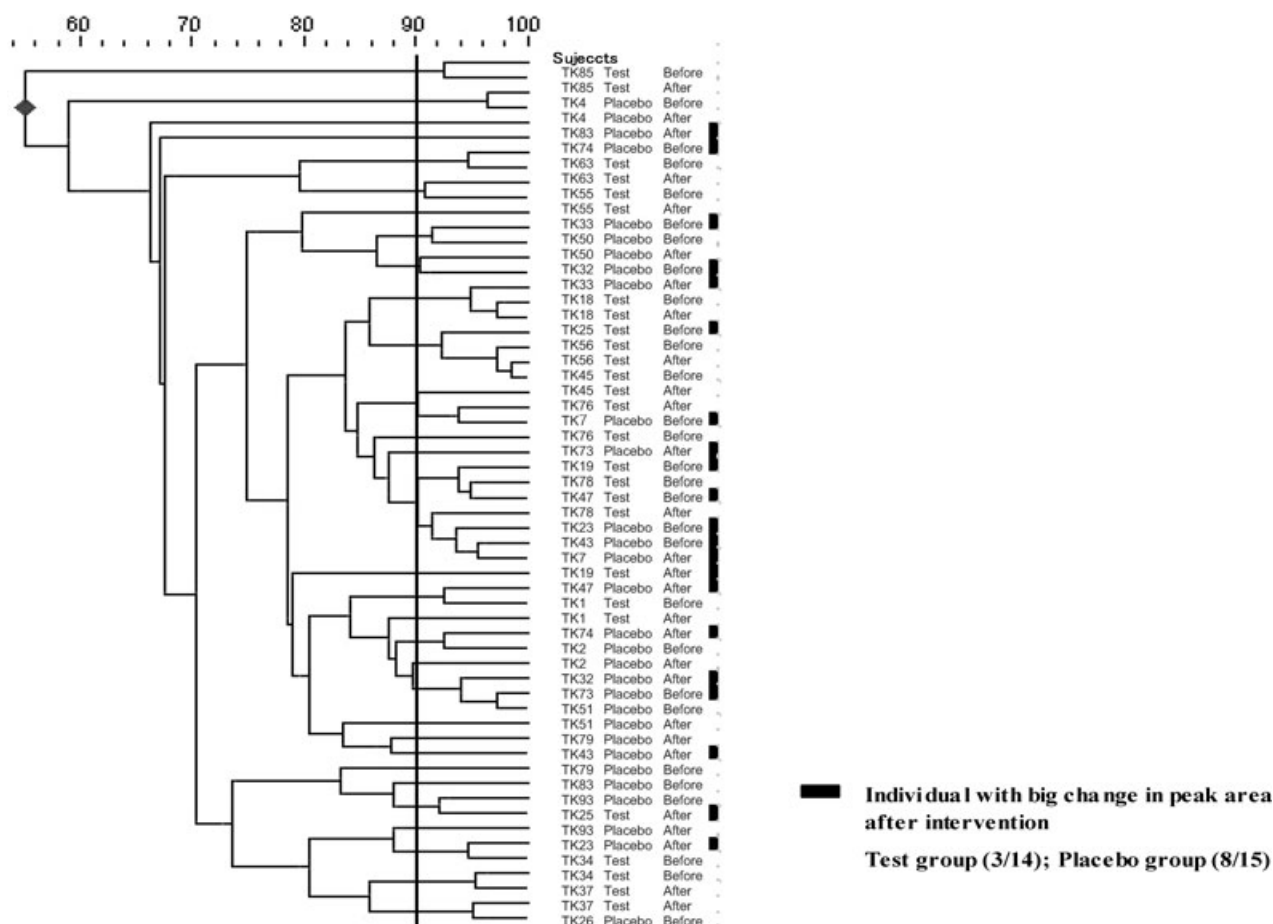


Fig. 1. Dendrogram of T-RFLP profiles of fecal samples collected from individuals allergic to Japanese cedar pollen.

been impressive, and are inconsistent with those expected from animal studies (14, 34). This suggests that adult allergic disorders, such as JCPsis, might be more difficult to manage because of genetic background heterogeneity affecting allergic sensitization, as well as other factors, such as lifestyle, behavior and environment, which may influence symptom development. Furthermore, adult intestinal microbiota, a target for probiotic therapies, are more complicated than those of infants, and might reduce the effects of probiotics on those aspects of the human immune system that are involved in allergies. It appears that probiotic regimens targeting JCP may need to be modified, and combinations of different probiotic strains have recently been suggested as a practical means of improving the potency of probiotic therapies in the treatment of allergic disorders (37, 38).

TMC0356 was originally isolated from the intestine of a healthy adult (19). This bacterium adheres to human enterocytes and does not enhance inflammatory responses (39); TMC0356 has been shown to in-

duce secretion of pro-inflammatory (interleukin [IL]-12) and anti-inflammatory (IL-10) cytokines from murine macrophages (40). Therefore, TMC0356 might be able to modulate specific immune responses by preventing or intensifying inflammation. TMC0356 has effectively inhibited antigen-augmented serum IgE in BALB/c mice immunized i.p. with ovalbumin, and altered serum IgE concentrations in subjects with high-serum IgE levels and perennial allergic rhinitis (41, 42). These studies suggest that TMC0356, in concert with LGG, may be effective against JCPsis because both can produce anti-allergic effects. For example, a combination of LGG and TMC0356 significantly has suppressed antigen-induced nasal vascular permeability and alleviated antigen-induced nasal blockage in rats and guinea pigs (17, 43).

In the current study, fermented milk prepared with LGG and TMC0356 was given orally to subjects suffering from JCPsis during the 2006 JCP shedding season. Consumption of the fermented milk significantly decreased the mean symptom score for nasal blockage after 9 weeks

($P < 0.05$), and mean symptoms-medication score after 9 and 10 weeks when compared with the placebo group [$P < 0.01$ and $P < 0.05$], respectively) (44). However, no significant changes in serum IgE and other biomarkers in the blood related to IgE immunity were observed in the present study, although LGG and TMC0356 altered Th2-type cytokine production by peripheral blood mononuclear cells (PBMC) isolated from subjects suffering from JCPsis. Therefore, the underlying mechanism remained unclear. To investigate the underlying mechanisms involved in the anti-allergic effects of LGG and TMC0356 on JCPsis, intestinal microbiota were examined. Our results indicated that both LGG and TMC0356 colonize in the intestine of allergic patients suffering from JCPsis, as has been observed in other human populations. We speculate that the anti-allergic effects of LGG and TMC0356 might have partially arisen from changes in the intestinal microbiota caused by the colonization of LGG and TMC0356. This is the first evidence that selected probiotic strains can colonize in the intestine of JCPsis patients. The results also indicated that no apparently aberrant microbiota were associated with JCPsis.

Bifidobacteria are a predominant group of normal microflora of the human intestine (45, 46). The presence of these bacteria in the human intestine has been considered an indicator of a healthy intestinal microflora (46). Allergic infants have been shown to have an aberrant composition of gut bifidobacteria (13, 47, 48). Also, fecal bifidobacteria were reported to be significantly lower in patients with atopic dermatitis. In particular, *Bifidobacterium* percentages were significantly lower in patients with severe skin symptoms than in those with mild symptoms (13). Recently, the presence of *B. pseudocatenulatum* in feces was associated with infant eczema and with exclusive formula-feeding, whereas the presence of *B. bifidum* was associated with breast-feeding (49). In a randomized, double-blind, placebo-controlled trial, *B. longum* BB536 relieved JCPsis symptoms, probably through the modulation of the Th2-skewed immune response and alteration of fecal microbiota (50). However, in the present study, no significant changes were detected in fecal bifidobacterial flora during JCP shedding season or after LGG and TMC0356 administration. Although the occurrence of bifidobacteria increased in our culture analysis, those changes were not validated by FISH analysis. Our results did not reveal a significant association between fecal *Bifidobacterium* and JCPsis. Thus, the anti-allergic effects of LGG and TMC0356 might not have arisen from association with intestinal bifidobacteria. Furthermore, *Escherichia coli*, *Staphylococcus aureus*, *Clostridium difficile* and yeast have been related to allergic diseases in previous studies (8, 22, 51–54). However, in our study, there was no significant alteration in abundance observed among these bacteria dur-

ing JCP pollen shedding or after probiotic intervention. Therefore, using culture methods, no predominant group of the intestinal bacteria detected in the present study could be characterized as having an etiologic role in JCPsis. This suggests that associations between intestinal microbiota and JCPsis should be explored using more rigorous methods (55).

The estimation of the number of bacteria species in intestinal microbiota by culture-independent approaches using a 16S rDNA clone library suggests that only 20–30% of the total intestinal bacteria can be cultured (56). Several 16S rRNA-based molecular tools have been developed and these are reportedly superior to culture-based methods with respect to detection limits and accuracy of microbial concentration determination (56, 57). T-RFLP is effective due to its higher throughput and reproducibility (25, 26), and has been used to track the intestinal bacterial communities associated with allergic diseases (58, 59). In the present study, placebo group subjects (53% (8/15), showed individual T-RFLP profiles, and their fecal T-RFLP profiling pattern varied qualitatively with JCP pollen shedding, whereas 21% (3/14) of the subjects in the test group exhibited the same changes after administration. The results from T-RFLP analysis suggest that bacterial composition changes, undetectable by traditional culture methods, might be profoundly involved in JCPsis. Thus, intestinal microbiota compositions and metabolic activities in JCPsis subjects may significantly change during JCP shedding. Further studies should attempt to identify those intestinal microbes that are sensitive to JCP-related environmental factors and to characterize their impacts on host immunity.

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