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ORIGINAL ARTICLE

Effects of Bifidobacterial Supplementation to Pregnant Women and Infants in the Prevention of Allergy Development in Infants and on Fecal Microbiota

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ABSTRACT

Background: Probiotic administration may be a useful method for preventing allergies in infants; however, there have been controversial results about the efficacy. We investigated the effects of bifidobacterial supplementation on the risk of developing allergic diseases in the Japanese population.

Methods: In an open trial, we gave *Bifidobacterium breve* M-16V and *Bifidobacterium longum* BB536 prenatally to 130 mothers beginning 1 month prior to delivery and postnatally to their infants for 6 months. Another 36 mother-infant pairs served as controls and did not receive the bifidobacterial supplementation. Development of allergic symptoms in the infants was assessed at 4, 10 and 18 months of age. Fecal samples were collected from the mothers and infants.

Results: The risk of developing eczema/atopic dermatitis (AD) during the first 18 months of life was significantly reduced in infants in the probiotic group (OR: 0.231 [95% CI: 0.084-0.628] and 0.304 [0.105-0.892] at 10 and 18 months of age, respectively). Pyrosequencing analyses indicated an altered composition of the fecal microbiota at 4 months for infants who developed eczema/AD at 4 and 10 months of age. The proportion of Proteobacteria was significantly lower ($P = 0.007$) in mothers at the time of delivery who received the supplementation when compared with the control group and was positively correlated ($r = 0.283$, $P = 0.024$) with that of infants at 4 months of age. No adverse effects were related to the use of probiotics.

Conclusions: These data suggest that the prenatal and postnatal supplementation of bifidobacteria is effective in primary preventing allergic diseases. Some limited changes in the composition of fecal microbiota by the bifidobacterial supplementation were observed.

KEY WORDS

allergy, *Bifidobacterium*, eczema, prevention, probiotics

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Conflict of interest: NY, NI, TO, FA and JZX are employees of Morinaga Milk Industry. The rest of the authors have no conflict of interest.

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INTRODUCTION

The prevalence of allergic diseases, such as eczema, allergic rhinitis, and asthma, has rapidly increased worldwide over the past several decades, particularly in industrialized countries. One explanation for the prevalence of allergies is the "hygiene hypothesis", which postulates that decreased exposure to immunostimulating pathogens in early childhood increases the prevalence of allergic diseases.[1] Several studies have suggested an association between the composition of the intestinal microbiota and the development of allergies.[2] Increasing numbers of clinical trials have focused on the use of *Lactobacillus* or *Bifidobacterium* strains (or a mixture of these) early in life for the prevention of atopic diseases; however, these trials have reported controversial results regarding the efficacy of such treatments.[3-6] A meta-analysis based on 14 studies demonstrated that probiotic use decreased the incidence of atopic dermatitis [relative risk = 0.79, 95% confidence interval (CI): 0.71-0.88], providing evidence in support of a moderate role of probiotics in the prevention of atopic dermatitis and IgE-associated atopic dermatitis in infants.[7] Similarly, a meta-analysis by Elazab *et al.*[8] has suggested that prenatal and/or early-life probiotic administration reduces the risk of atopic sensitization and decreases total IgE levels in children but might not reduce the risk of asthma or wheezing. They found that administration of *L. acidophilus* was associated with an increased risk of atopic sensitization (P = 0.002) when compared with other strains, indicating the importance of probiotic strain selection.[8] Heterogeneous factors involved in each clinical trial, such as the inclusion criteria for the study subjects, the timing of the probiotic intervention, the mode of administration, and the probiotic strains used, might contribute to the different outcomes observed between studies.

In the present study, we administered a combination of *Bifidobacterium* strains, *Bifidobacterium longum* BB536 and *B. breve* M-16V, prenatally to mothers during their last month (mo) of pregnancy and postnatally to their infants for 6 mo and assessed the effects of the bacteria on the prevention of atopic diseases in infants. An analysis of the fecal microbiota was performed to gain insight into the association of the microbiota with allergy development and probiotic ingestion. We employed a mixture of two *Bifidobacterium* strains, *B. longum* BB536 and *B. breve* M-16V, to attempt to achieve a wider spectrum of efficacy. These strains were originally isolated from healthy infants, and each strain has shown a high potential for anti-allergic effects in animals and clinical trials. *B. longum* BB536 alleviates the symptoms of certain allergic diseases, such as Japanese cedar pollinosis.[9-11] *B. breve* M-16V reduces allergic sensitization in ovalbumin-sensitized mice, alleviates IgE-mediated allergic symptoms, and prevents asthma-related symptoms in infants.[12-14] The study was performed as part of the Kinokunimoritsukuri project, which is supported by Wakayama Prefecture, Japan, an area abundant with Japanese cedar and known to have a high prevalence of allergies to pollen. Overall, the project aimed to investigate the prevalence of allergic diseases, including food allergy, atopic dermatitis (AD), allergic asthma, allergic rhinitis, allergic conjunctivitis, and Japanese cedar pollen allergy in Wakayama Prefecture and to develop a strategy for allergy care in the future.

METHODS

STUDY DESIGN AND PARTICIPANTS

This open-trial study was conducted in a single center (Hidaka General Hospital) in Wakayama, Japan. The trial flow is presented in Figure 1. Subject recruitment occurred between September 2008 and December 2010. Information about the study was distributed to pregnant women in the hospital. All families who were interested in participating contacted the research nurse during the recruitment period and were assessed for eligibility. Pregnant women with severe hepatic dysfunction, renal dysfunction, cardiovascular disorders, respiratory dysfunction, endocrine disorders, or metabolic dysfunction were excluded from the study. Other exclusion criteria included habitual ingestion of probiotic supplements. A total of 166 pregnant women were enrolled; 130 were assigned to the probiotic group, and 36 were assigned to the control group based on the willingness of the participants. The baseline characteristics of these participants in the probiotic and control groups are shown in Table 1. All of the participants provided written informed consent. All of the study protocols were approved and controlled by the Local Ethics Committee of Hidaka General Hospital and the Local Ethics Committee of the NPO (Nonprofit Organization) Japan Health Promotion Supporting Network, Wakayama, Japan.

SAMPLE INTAKE

Pregnant women in the probiotic group received daily two sachets of bifidobacterial powder (approximately 1 g per sachet, each containing approximately 5×10^9 colony-forming units of *B. longum* BB536 [ATCC BAA-999] and *B. breve* M-16V [LMG 23729]) beginning at approximately 4 wk before the date of expected delivery. The participants were advised to ingest the bifidobacterial powder by drinking it with milk or water. After delivery, the infants were given one sachet of the same bifidobacterial powder to be consumed daily in breast milk or water for breast-feeding infants or in formula or water for formula-feeding infants beginning approximately 1 wk after birth and continuing for 6 mo. The control group did not receive the probiotic supplementation. The moth-

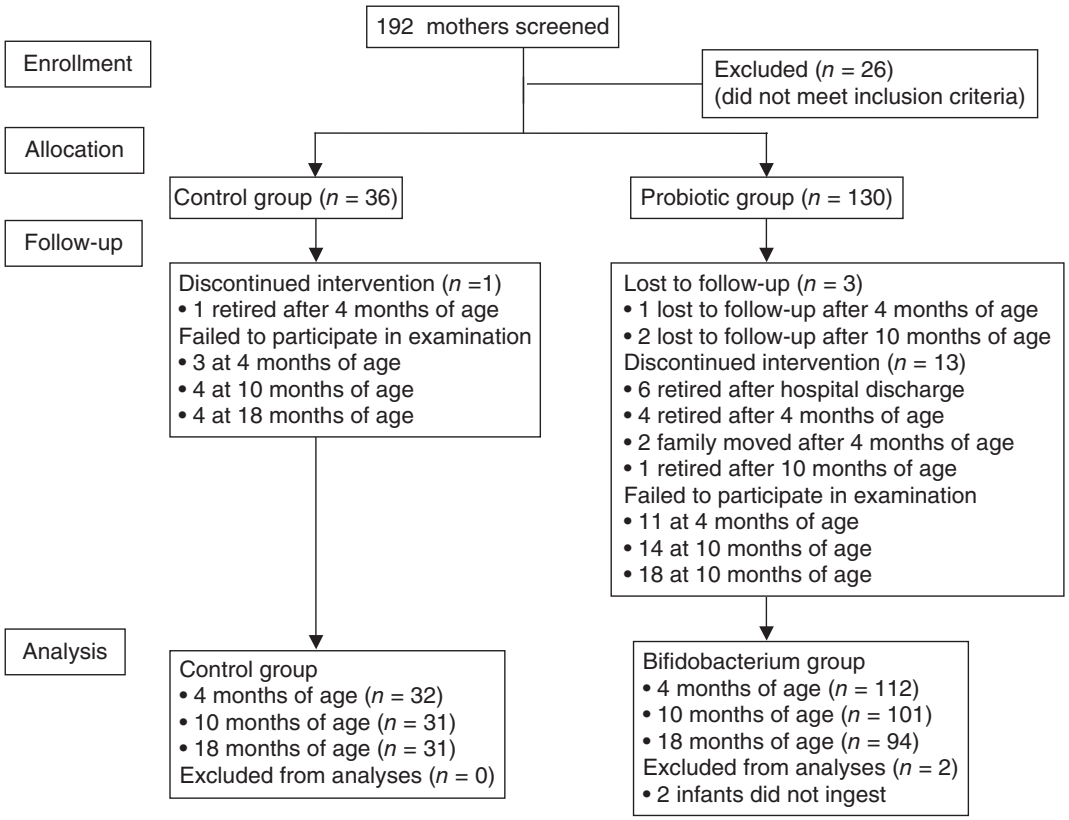

Fig. 1 Study flow.

Table 1 Background characteristics of mother-infant pairs

	Control group (*n* = 36)	Probiotic group (*n* = 122)	*P* †
Maternal			
Age (year), mean (range)	29.7 (21-38)	30.9 (22-41)	0.147
Food allergy (%)	8.8	15.3	0.337
Eczema/atopic dermatitis (%)	11.8	31.5	0.023
Allergic asthma (%)	0.0	9.8	0.057
Allergic rhinitis (%)	30.3	40.9	0.272
Allergic conjunctivitis (%)	6.1	16.4	0.134
With at least one allergy (%) ‡	36.1	55.7	0.038
Smoking (%)	9.1	5.4	0.443
Habitual ingestion of fermented milk during pregnancy (%)	90.9	89.0	0.753
Family			
Family history of allergy (%) §	68.6	81.1	0.111
Pets, ratio (%)	16.7	22.1	0.478
Smokers in family (%)	55.6	47.5	0.398
Infant			
Boy, ratio (%)	50.0	49.6	0.652
Older siblings, ratio (%)	69.4	61.7	0.533
Cesarean section, ratio (%)	5.5	16.4	0.137
Birth weight, ratio (% of <2500 g)	0.0	4.1	0.215
Primarily breast-fed, ratio (%)	56.1	47.9	0.699

† Groups were compared using Student's *t*-test for continuous variables and Fisher's exact test for categorical variables.

‡ Having at least one of the above allergic disorders.

§ Family history including grand fathers, grand mothers, fathers, mothers and siblings of infants.

ers and infants were advised to avoid ingestion of commercial probiotic supplements during the intervention. With the exception of the probiotic ingestion, all pregnant mothers and infants were advised not to change their normal lifestyles, such as food and exercise, during the intervention.

CLINICAL OBSERVATION

At the start of the intervention, each mother completed a questionnaire about her allergy history, smoking, and ingestion of fermented milk during pregnancy, as well as the allergy history of other family members of the newborn (*e.g.*, grandfathers, grandmothers, father, and older siblings), pet keeping, and smokers in the family. The infants were scheduled for follow-ups until the age of 36 mo. Clinical examination of the infants was performed in accordance with Japan's National Schedule for Health Checks of Infants and Children at 4, 10, 18, and 36 mo of age at health care centers in each municipality of Wakayama Prefecture. The physicians who performed the physical examination were informed about the study and were asked to determine whether the following allergic symptoms were present in the infants: eczema/AD, allergic asthma, allergic rhinitis, and allergic conjunctivitis. However, no detailed information was given to the physician about the intervention, including the assignment of probiotic supplementation, and no physician was directly involved in the intervention. The primary outcome measure of the trial was the incidence of eczema/AD in the infants. The occurrence of eczema/AD, allergic asthma, allergic rhinitis, and allergic conjunctivitis was examined in accordance with the Guidelines of the Japanese Dermatological Association on Eczema (2005), the Guidelines for the Treatment and Management of Childhood Asthma in Japan (2005), the Guidelines for Nasal Allergy Clinics in Japan (2005), and the Guidelines for the Clinical Management of Allergic Conjunctival Diseases (2005), respectively (All the guidelines are written in Japanese). Eczema/AD was diagnosed according to the presence of the following features: pruritus, typical morphology and distribution, and a chronic relapsing course.[15]

ANALYSIS OF FECAL MICROBIOTA BY NEXT-GENERATION SEQUENCING

Fecal samples were collected from the mothers before beginning sample intake and at postpartum and from the infants at the time points of each assessment using a sampling kit (Techno Suruga Laboratory, Shizuoka, Japan), which enabled the quick stabilization of DNA and sample processing at room temperature.

DNA was extracted from the fecal samples as described previously with some modifications.[16] 50 μL of 10% sodium dodecyl sulfate, 500 μL of phenol-chloroform, and 300 mg of glass beads (diameter, 0.1 mm) were added to 450 μL of fecal suspension. The mixture was vigorously vortexed for 30 s using a FastPrep™ FP 100A (Bio 101) at a power level of 5.0. After centrifugation at 14000× g for 5 min, 400 μL of supernatant was extracted with phenol-chloroform, and 250 μL of the supernatant was precipitated with isopropanol. Inhibitors were removed using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Switzerland). The purified DNA was suspended in 200 μL of Tris-EDTA buffer (pH 8.0).

The V6-V8 region of the bacterial 16S rRNA genes was amplified by PCR using the bacterial universal primer set, Q-968F-# (5'-CWSWSWWSHTWACGCGA RGAACCTTACC-3') and Q-1390R-# (5'-CWSWSWWS HTTGACGGGCGGTGWGTAC-3') (# indicates a series of 128 barcode sequence tags that are underlined in the sequence).[17] The DNA was amplified using the following program: preheating at 95℃ for 3 min, 20 cycles of denaturation at 95℃ for 15 s, annealing at 54℃ for 30 s, extension at 72℃ for 20 s, and a final terminal extension at 72℃ for 5 min. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The purified products were quantified using a NanoDrop ND-1000 microphotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, equal amounts (100 ng) of the amplicons from different samples were pooled and purified before pyrosequencing by ethanol precipitation. The amplicon DNA mixtures were clonally amplified by emulsion PCR (emPCR) using a GS FLX Titanium LV emPCR Kit (Lib-L) v2 according to the manufacturer's protocol (454 Life Sciences/Roche Diagnostics). Beads with amplified DNA were loaded onto a GS FLX Titanium PicoTiterPlate with dividers between the separate reaction chambers to accommodate two-mixture pools. The amplicon mixture was applied to the Genome Sequencer FLX454 Titanium System (454 Life Sciences/Roche Diagnostics).

The sequences that passed the quality filters were analyzed using the QIIME software package.[18] They were sorted into each sample batch with barcode sequences. The parameters used in this script were as follows: l (minimum sequence length) = 400; e (maximum number of errors in barcode) = 0; reverse primer mismatches = 2; and a (maximum number of ambiguous bases) = 3. Potential chimeric sequences were removed using UCHIME and assigned to operational taxonomic units (OTUs) using OTUpipe with a 97% threshold of pairwise identity.[19] The sequences were classified taxonomically using the Greengenes reference database and a confidence threshold of 60%.[20] For technical reasons, analysis of the fecal microbiota was performed using samples from 64 randomly selected mother-infant pairs (probiotic group, $n = 49$; control group, $n = 15$), which consisted of a total of 256 samples, including those collected from mothers before intervention and at postpartum and from infants at 4 and 10 mos of age. The baseline

Table 2 Background characteristics of mother-infant pairs included in microbiota analyses

	Control group (n = 15)	Probiotic group (n = 49)	P †
Maternal eczema or atopic dermatitis (%)	13.3	28.6	0.318
Family history of allergy (%) ‡	66.7	79.6	0.301
Boy, ratio (%)	53.3	53.1	0.781
Older siblings, ratio (%)	80.0	63.3	0.347
Cesarean section, ratio (%)	0.0	10.2	0.329
Mainly breast-fed, ratio (%)	60.0	63.3	0.939
Pets, ratio (%)	13.3	24.5	0.488
Occurence of infantile eczema/atopic dermatitis			
4 months of age	20.0	12.2	0.426
10 months of age	46.7	6.1	<0.001

† Groups were compared using Fisher's exact test for categorical variables.

‡ Family history including grand fathers, grand mothers, fathers, mothers and siblings of infants.

characteristics of these participants in the probiotic and control groups are shown in Table 2. Consequently, 735493 sequences were assigned to 256 samples in which there were 2,873 ± 2,021 (mean ± standard deviation) reads per sample.

STATISTICAL ANALYSIS

All statistical analyses were performed using SAS statistical software version 9.1.3 (SAS Institute, NC, USA). Continuous background data are expressed as means with standard errors (SD), and categorical data are expressed as percentages. Differences in background characteristics between the groups were compared using Student's *t*-test for continuous variables and Fisher's exact test for categorical variables. To assess the effects of the intervention on outcome, comparisons between groups were conducted using univariate logistic analyses and are expressed as *P* value and odds ratio (OR) with 95%CI. Microbiota data are expressed as the median and IQR (interquartile range) of the proportion of each bacterial category in the microbiota, and the intergroup difference at each time point was analyzed using the Mann-Whitney *U*-test. Correlation was analyzed using Spearman's correlation. $P < 0.05$ was considered statistically significant.

RESULTS

BASELINE CHARACTERISTICS OF PARTICIPANTS AND COMPLIANCE WITH SAMPLE INTAKE

The age ranges of the pregnant mothers were similar in both groups. No significant differences were found in the maternal histories for food allergy, allergic rhinitis, or allergic conjunctivitis of the pregnant mothers. However, the prevalence rates of eczema/AD (P = 0.023) and with at least one allergy (P = 0.038) were significantly higher in the probiotic group compared with the placebo group; the prevalence of allergic asthma tended to be higher in the probiotic group (P = 0.057) (Table 1). The prevalence of a family history of allergy was higher in the probiotic group, but this difference was not significant (P = 0.111) (Table 1). No significant differences were observed in the other baseline characteristics between the two groups. The pregnant women in the probiotic group received the intervention for 27.3 ± 7.5 d, and their compliance with probiotic ingestion was 92.0% ± 12.0%. The infants in the probiotic group received the intervention for 176.3 ± 30.0 d, and their compliance with probiotic ingestion was 88.0% ± 17.9%. No adverse effects related to probiotic supplementation were detected in the study subjects.

DEVELOPMENT OF ALLERGIC DISEASES IN INFANTS

Compared with the control group, the prevalence of eczema/AD was slightly lower at 4 mo of age and significantly lower at 10 and 18 mo of age in the probiotic group (Table 3). The prevalence of allergic asthma, allergic rhinitis, and allergic conjunctivitis was very low at all ages up to 18 mo for both groups, and there was no significant inter-group difference (Table 3).

SUBGROUP ANALYSIS OF THE PREVALENCE OF ECZEMA/ATOPIC DERMATITIS

When stratified for maternal allergy, the prevalence of eczema/AD at 10 mo of age in the probiotic group tended to be lower than that of the control group when comparing the subgroups with or without maternal allergy. At 18 mo of age, the prevalence was significantly lower than that of the control group (P = 0.005) only among the infants of mothers without allergy (Table 4). When stratified by family history of allergy, the prevalence of eczema/AD in the probiotic group was significantly decreased compared with the control group among infants with a family history at 10 mo of age (P = 0.025) and among infants without a family history at 4 (P = 0.049) and 18 (P = 0.002) mo of age (Table 4). Among the infants fed primarily formula milk, a reduced prevalence of eczema/AD was

Table 3 Occurrence of allergic diseases in infants

	Ratio (%)†		*P*‡	OR (95% CI)‡
	Control group	Probiotic group		
Eczema or atopic dermatitis				
4 months	4/32 (12.5)	12/112 (10.7)	0.755	0.840 (0.268-3.185)
10 months	10/31 (32.3)	10/101 (9.9)	0.007	0.231 (0.084-0.628)
18 months	8/31 (25.8)	9/94 (9.6)	0.033	0.304 (0.105-0.892)
Allergic asthma (%)				
4 months	0/32 (0)	1/111 (0.9)	ns	n/a
10 months	1/32 (3.2)	2/101 (2.0)	ns	n/a
18 months	1/31 (3.2)	2/94 (2.1)	ns	n/a
Allergic rhinitis (%)				
4 months	0/32 (0)	1/111 (0.9)	ns	n/a
10 months	0/31 (0)	0/101 (0)	ns	n/a
18 months	0/31 (0)	0/94 (0)	ns	n/a
Allergic conjunctivitis (%)				
4 months	0/32 (0)	0/111 (0)	ns	n/a
10 months	0/31 (0)	0/101 (0)	ns	n/a
18 months	0/31 (0)	0/94 (0)	ns	n/a

† Number with allergic diseases/total number (percentages).

‡ Groups were compared using univariate logistic analyses and expressed as *P* values and odds ratio (OR) with 95%C.

ns, not significant; n/a, not analyzed.

observed in the probiotic group when compared with the control group at 10 ($P = 0.0005$) and 18 ($P = 0.040$) mo of age (Table 4). No significant difference between the groups was observed at the other time points.

FECAL MICROBIOTA COMPOSITION

First, we compared the fecal microbiota composition of samples from infants with or without eczema/AD at 4 and 10 mo of age. At 4 mo of age, the proportion of Actinobacteria was significantly lower in the fecal microbiota of infants diagnosed with eczema/AD compared with those without symptoms, whereas Proteobacteria tended to be higher (Table 5). In addition to the above differences, a significantly higher proportion of Firmicutes was found in the fecal microbiota of 4-mo-old infants who developed eczema/AD compared with those without symptoms at 10 mo of age (Table 5). However, none of these differences were apparent in the fecal microbiota at 10 mo of age.

Next, we assessed the effect of the probiotic treatment on the fecal microbiota of pregnant mothers and infants. No significant difference was observed between the microbiota of mothers before beginning sample intake, whereas at postpartum, the proportion of Proteobacteria was significantly lower in the probiotic group than in the control group (Table 6). A significantly higher proportion of Bacteroidetes was observed in the microbiota of infants in the probiotic group than in that of the control group at 4 mo of age; however, no difference in the samples obtained at 10 mo of age was evident (Table 6). Significantly positive and negative correlations were found in the proportions of Proteobacteria and Firmicutes, respectively, between mothers at postpartum and infants at 4 mo of age (Table 7). In addition, significantly negative correlations were found between the proportions of Bacteroidetes in mothers and that of Actinobacteria in infants, the proportions of Firmicutes in mothers and that of Proteobacteria in infants, and the proportions of Proteobacteria in mothers and that of Bacteroidetes in infants, respectively. On the other hand, significantly positive correlations were found between the proportions of Bacteroidetes in mothers and that of Firmicutes in infants, and the proportions of Firmicutes in mothers and that of Actinobacteria in infants, respectively (Table 7).

DISCUSSION

In the present study, we found that prenatal and postnatal supplementation with a bifidobacterial combination significantly reduced the risk of developing eczema/AD when compared with a non-supplemented control group of infants. Because the prevalence of other allergic diseases such as allergic asthma, allergic rhinitis, and allergic conjunctivitis was very low at all ages up to 18 mo for both groups, the effect of probiotic supplementation could not be evaluated. This is the first study to demonstrate the primary prevention of atopic diseases by probiotic administration in the Japanese population.

Most studies seeking to prevent eczema/AD in infants have been performed by administering bacteria to pregnant women and then their infants. In the ma-

Table 4 Occurrence of eczema/atopic dermatitis in subgroups

	Ratio (%) †		*P* ‡	OR (95% CI) ‡
	Control group	Probiotic group		
Maternal allergy (at least one allergy)				
Yes				
4 months	1/13 (7.7)	6/70 (8.6)	1.000	1.125 (0.17-22.231)
10 months	3/11 (27.3)	5/67 (7.5)	0.080	0.215 (0.043-1.202)
18 months	1/12 (8.3)	6/56 (10.5)	1.000	1.294 (0.193-25.708)
No				
4 months	3/19 (15.8)	6/41 (14.6)	1.000	0.914 (0.212-4.758)
10 months	7/20 (35.0)	5/34 (14.7)	0.101	0.320 (0.081-1.184)
18 months	7/19 (36.8)	2/36 (5.6)	0.005	0.101 (0.014-0.483)
Family allergy history				
Yes				
4 months	0/22 (0)	10/91 (11.0)	0.205	n/a (1.22-na)
10 months	8/24 (33.3)	10/85 (11.8)	0.025	0.267 (0.090-0.795)
18 months	4/23 (17.4)	8/72 (11.1)	0.476	0.594 (0.167-2.419)
No				
4 months	4/9 (44.4)	2/21 (9.5)	0.049	0.132 (0.015-0.867)
10 months	2/6 (33.3)	0/14 (0)	0.079	n/a (n/a-n/a)
18 months	4/7 (57.1)	0/20 (0)	0.002	n/a (n/a-n/a)
Nutrition				
Mainly breast-fed				
4 months	3/24 (12.5)	11/75 (14.7)	1.000	1.203 (0.337-5.686)
10 months	3/18 (16.7)	7/56 (12.5)	0.698	0.714 (0.174-3.625)
18 months	5/23 (21.7)	7/60 (11.7)	0.299	0.475 (0.134-1.778)
Mainly formula-fed				
4 months	1/8 (12.5)	1/37 (2.7)	0.327	0.194 (0.007-5.300)
10 months	7/13 (53.8)	3/45 (6.7)	0.001	0.061 (0.011-0.280)
18 months	3/8 (37.5)	2/34 (5.9)	0.040	0.104 (0.011-0.770)

† Number with allergic diseases/total number (percentages).

‡ Groups were compared using univariate logistic analyses and expressed as *P* values and odds ratio (OR) with 95%C.

n/a, not analyzed.

jority of cases, a reduced risk of eczema was observed in the infants,[3-6] but a few studies found no evidence of reduced incidence.[21] Several studies have administered probiotics to mothers and their infants who had or did not have a high risk for allergy.[3,6,22,23] In addition, although recent works have suggested that maternal supplementation with probiotics during pregnancy and breastfeeding may reduce the risk of eczema in their infants,[23,24] prenatal intervention alone does not affect the risk of eczema in their infants.[25] To illustrate the association between background risk factors and the efficacy of probiotic ingestion in the prevention of allergy, we analyzed subgroups stratified by maternal allergy, family history of allergy, or style of nutrition. The results demonstrate that the preventive effects of probiotic supplementation on eczema/AD in infants at 10 and 18 mo of age were potentially maintained in the subgroup without maternal allergy and in the subgroup without a family history of allergy (Table 4), suggesting that the preventive effects of probiotic supplementation may be better in populations without a history of allergy. In the subgroup analysis that was stratified by nutrition, we observed significant differences at 10 and 18 mo of age among infants who were primarily formula-fed but not among those who were primarily breast-fed (Table 4), indicating that supplementation of infant formula with probiotics may be effective in the prevention of eczema/AD in infants. In the present trial, the mothers were not given probiotic supplementation during breastfeeding. Future studies are needed to evaluate the importance of prenatal and post-natal supplementation to mothers during breastfeeding or directly to infants for reducing the risk of eczema in infants.

Several studies have demonstrated differences in the gut microbiota between allergic and healthy infants with respect to the frequencies of genera, such as *Bifidobacterium*, *Bacteroides*, *Propionibacterium*, *Klebsiella*, *Acinetobacter*, *Escherichia*, *Staphylococcus*,

Table 5 Difference in the fecal microbiota composition between infants with and without eczema and atopic dermatitis at 4 and 10 months of age

Category at phylum level	Ages of infants assessed	Time of fecal sample collection	With disease (n = 9) Median (IQR, %)†	Without disease (n = 55) Median (IQR, %)†	P‡
Actinobacteria	4 months	4 months	27.98 (4.07-57.67)	55.97 (43.42-74.75)	0.048
		10 months	46.38 (19.01-58.82)	48.21 (41.93-58.02)	0.481
	10 months	4 months	29.55 (7.64-49.53)	57.31 (43.98-75.96)	0.012
		10 months	54.02 (47.62-58.63)	47.27 (35.66-57.72)	0.275
Bacteroidetes	4 months	4 months	0.03 (0-10.39)	4.95 (0.03-17.12)	0.332
		10 months	0.36 (0.04-12.69)	8.89 (0.18-15.07)	0.334
	10 months	4 months	0.2 (0-9.35)	4.07 (0.02-19.29)	0.253
		10 months	7.53 (0.22-14.69)	8.56 (0.08-14.62)	0.760
Firmicutes	4 months	4 months	28.87 (20.62-37.81)	16.16 (8.69-36.23)	0.091
		10 months	33.33 (32.86-46.1)	28.61 (22.96-40.46)	0.275
	10 months	4 months	27.8 (21.11-53.27)	16.2 (8.63-35.16)	0.042
		10 months	30.24 (23.99-33.33)	30.8 (23.12-44.27)	0.336
Proteobacteria	4 months	4 months	8.12 (2.7-31.37)	3.35 (1.57-6.2)	0.062
		10 months	2.62 (2.03-3.11)	2.82 (1.35-4.37)	0.505
	10 months	4 months	6.26 (4.33-28.49)	3.02 (1.48-6.1)	0.023
		10 months	1.66 (0.93-2.62)	2.91 (1.64-4.51)	0.112
Unclassified	4 months	4 months	2.27 (0.63-2.86)	2.05 (1.47-2.5)	0.915
		10 months	4.7 (2.29-5.49)	4.45 (3.39-5.12)	0.582
	10 months	4 months	2.15 (1.87-2.3)	2.05 (1.32-2.81)	1.000
		10 months	4.73 (3.89-5.04)	4.45 (3.14-5.27)	1.000

† Data are expressed as the median with IQR (interquartile range) of the proportion of each bacterial category in the microbiota. The proportions of Fusobacteria and Tenericutes were low and there were no inter-group difference (data not shown).

‡ Inter-group difference at each time point was analyzed using the Mann-Whitney U-test.

and *Clostridium*.[17,26-29] In the present study, we analyzed the composition of the fecal microbiota in mothers and infants. We observed distinct differences in the fecal microbiota between allergic infants and their non-allergic counterparts at 4 mo of age; however, these differences were not evident in the fecal microbiota at 10 mo of age. The major differences were found in the three predominant phyla: Actinobacteria, Firmicutes, and Proteobacteria. Allergic children have less bifidobacteria and more clostridia, and enterobacteria than nonallergic children.[2,30-33] He *et al*.[34] have reported that allergic infants have an adult-type *Bifidobacterium* flora and that healthy infants have a typical infant *Bifidobacterium* flora. Moreover, compared with healthy infants, allergic Japanese infants have more *B. catenulatum* group and *B. bifidum* at 1 and 6 mo of age, respectively.[35] The findings of these studies indicate the importance of the gut microbiota composition at an early stage of life in preventing the onset of eczema/AD. Although we have not analyzed differences at the genus level, those genera that were reported previously might be primary candidates for the three phyla that were observed in the present study. Whether the change in the microbiota is a trigger or a result of a particular disorder is often controversial; however, our data suggest that the microbiota present in the early stages of life may play an important function in regulating the development of AD in infants since the difference was only observed at early stage (4 mo) but not at later stage (10 mo).

When the effects of the supplementation of bifidobacteria on the composition of the fecal microbiota of the mothers and infants were assessed, we unexpectedly found only limited difference between the probiotic and control groups among either mothers or infants; a higher proportion of Bacteroidetes in the microbiota of infants at 4 mo of age and a lower proportion of Proteobacteria in the microbiota of mothers at postpartum in the probiotic group compared with the control group. Previous studies have also reported no major change in the microbiota composition upon probiotic administration.[36-38] McNulty *et al*.[39] demonstrated that ingestion of bifidobacteria and lactic acid bacteria resulted in no significant or only minimal changes in microbiota configuration, but significant changes in expression of microbiome-encoded enzymes involved in numerous metabolic pathways, from studies in human and mouse models. Further studies are needed to confirm whether the fecal microbiota differs at lower classification levels, such as at the species level, as well as to evaluate the possible transcriptional and metabolizing changes in the intestinal environments, in the present study.

Table 6 Comparison of fecal microbiota composition between the probiotic and control groups

Category at phylum level	Subjects	Time of fecal sample collection	Probiotic group (n = 49) Median (IQR, %)†,§	Control group (n = 15) Median (IQR, %)†,§	P‡
Actinobacteria	Mother	before study	8.87 (7.37-11.84)	6.88 (3.62-9.86)	0.200
		postpartum	11.23 (6.82-17.55)*	9.72 (5.3-17.07)**	0.560
	Infant	4 months	55.97 (43.16-68.59)	50 (28.78-73.63)	0.560
		10 months	47.97 (38.5-57.96)	53.65 (42.14-58.74)	0.770
Bacteroidetes	Mother	before study	11.81 (8.78-15.15)	11.71 (8.34-17.83)	0.510
		postpartum	10.89 (6.54-15.38)	9.09 (5.23-15.61)	0.540
	Infant	4 months	7.88 (0.03-21.1)	0.03 (0-8.31)	0.025
		10 months	8.23 (0.11-14.26)	11.16 (0.13-14.86)	0.770
Firmicutes	Mother	before study	72.88 (67.02-79.07)	71.38 (61.54-79.97)	0.570
		postpartum	70.18 (65.27-76.32)	71.36 (66.99-80.78)	0.410
	Infant	4 months	16.44 (8.81-33.3)	26.72 (11.97-45.55)	0.260
		10 months	31.67 (23.06-41.68)	28.28 (24.32-37.55)	0.570
Proteobacteria	Mother	before study	2.04 (0.89-3.63)	1.73 (0.88-3.47)	0.940
		postpartum	1.59 (0.46-2.63)*	2.77 (1.81-4.98)*	0.007
	Infant	4 months	3.08 (1.75-6.21)	5.57 (2.55-14.26)	0.230
		10 months	2.62 (1.49-4)	3.11 (1.9-5.02)	0.260
Unclassified	Mother	before study	1.42 (0.28-2.22)	2.01 (0.69-3.66)	0.083
		postpartum	0.75 (0.24-1.78)	0.21 (0-0.61)***	0.009
	Infant	4 months	2.05 (1.3-2.86)	2.07 (1.49-2.43)	0.830
		10 months	4.45 (3.1-5.49)	4.59 (4.16-4.98)	0.910

† Data are expressed as the median with IQR (interquartile range) of the proportion of each bacteria category in the microbiota. The proportions of *Fusobacteria* and *Tenericutes* were low and there were no inter-group or sequential difference (data not shown).

‡ Inter-group difference at each time point was analyzed using the Mann-Whitney U-test.

§ Intragroup difference in the microbiota of mothers before the study and at postpartum was analyzed using the Wilcoxon t test.

*$p < 0.1$; **$p < 0.05$; ***$p < 0.01$.

Table 7 Correlations between proportion of each major bacterial category in the microbiota of mothers at postpartum and infants at 4 months

Category at phylum level		Spearman's r coefficients				
		Fecal bacteria of infants at 4 months				
		Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria	Unclassified
Fecal bacteria of mothers at postpartum	Actinobacteria	-0.089	-0.166	0.159	0.200	0.010
	Bacteroidetes	-0.375**	0.052	0.252*	0.232	0.073
	Firmicutes	0.364**	0.012	-0.297*	-0.340**	-0.048
	Proteobacteria	-0.028	-0.278*	0.048	0.283*	-0.007
	Unclassified	0.021	0.029	-0.022	-0.015	0.165

*$P < 0.05$, **$P < 0.01$, Spearman's correlation.

Additionally, we observed significantly increased proportions of Actinobacteria in the microbiota of mothers at postpartum compared with their proportions before beginning the study (Table 6) in the probiotic and control groups. The proportion of Proteobacteria decreased in the probiotic group but increased in the control group of mothers at postpartum when compared with their proportions before beginning the study (Table 6). The reason for the changes in the microbiota in the pregnant mothers has not been established; however, previous studies showed that the gut microbiota changes dramatically during pregnancy, with an expansion of bacterial diversity and an overall increase in Proteobacteria and Actinobacteria.[40]

We observed significantly positive and negative correlations in the proportions of Proteobacteria and Firmicutes, respectively, between mothers at postpartum and infants at 4 mo of age. These results provide evidence of a possible horizontal influence of the composition of the microbiota of pregnant mothers on the development of their infants' microbiota. Con-

cerning the reason for negative correlation of Firmicutes, it may be an influence of possible interactions among intestinal bacteria in the infants' microbiota, since there were also some positive and negative correlations between the proportions in mothers and those in infants for inter-group bacteria. These interactions appear much complicated and need for further evaluation.

The mechanism for the efficiency of prenatal ingestion of probiotics by mothers in the prevention allergic diseases has not been clearly evaluated, however, it may not be an effect of solely bacterial transformation from mothers to infants during delivery. Rautava *et al*.[24] suggested that besides the possible modulating effect on the composition of maternal vaginal and intestinal microbiota, which provide an important colonizing inoculum to the newborn infant, the mechanisms of prenatal probiotic effects may also be more indirect. They have shown that maternal prenatal probiotic intervention significantly affects immune gene expression in the placenta and in the fetal gut in humans.[41]

This study had several limitations. At first, this study was an open trial. We were unable to perform a placebo-controlled trial due to ethical issues in enrolling participants in a placebo control in this hospital. However, the physicians who diagnosed the allergic symptoms were independent of the intervention and were uninformed about the details of the intervention. Thus, we think that diagnoses were made objectively. Next, the baseline characteristics in the probiotic group showed a higher prevalence of allergic history compared with the control group. This observation was not surprising because the allocation was based on the willingness of the participants who had been informed about the aims of the intervention and the possible effect of probiotic supplementation. A family history of allergy is a known risk factor for the development of allergies in infants.[42] Because it is not easy to administer probiotic samples to infants, it is likely that the participants with a history of allergy were more interested in probiotic supplementation in expectation of its potential benefits. Nevertheless, in spite of the higher incidence of allergy history in the probiotic group, the prevalence of eczema/AD was significantly lower in the probiotic group than in the control group at 10 and 18 mo of age, suggesting the potential power of probiotic administration to mothers and their infants for the prevention of eczema in infants.

In conclusion, our data suggest that the administration of a bifidobacterial combination to mothers (for 1 mo prior to delivery) and their infants (for 6 mo after birth) may reduce the risk of eczema development in the infants. These results indicate that probiotic supplementation to infant formula may be effective in the prevention of eczema/AD in infants. Our data also suggest that the composition of the microbiota during early infant life may be associated with the development of allergic diseases. Some limited changes in the composition of fecal microbiota of mothers and infants by the bifidobacterial supplementation were observed, further study is needed to understand the mechanism by which probiotics act in the prevention of allergic disease development. We are currently exploring the development of allergy in children up to 3 years of age.

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