

RESEARCH ARTICLE

Signatures in the gut microbiota of Japanese infants who developed food allergies in early childhood

Masaru Tanaka¹, Yuki Korenori¹, Masakazu Washio^{2,3}, Takako Kobayashi¹, Rie Momoda¹, Chikako Kiyohara⁴, Aki Kuroda³, Yuka Saito³, Kenji Sonomoto^{1,5} and Jiro Nakayama^{1,*}

¹Laboratory of Microbial Technology, Division of Applied Molecular Microbiology and Biomass Chemistry, Faculty of Agriculture, Graduate school, Kyushu University, Fukuoka 812-8581, Japan, ²Department of Community Health and Clinical Epidemiology, St. Mary's College, Kurume 830-8558, Japan, ³St. Mary's Hospital, Kurume 830-8543, Japan, ⁴Division of Social Medicine, Department of Preventive Medicine, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan and ⁵Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, Fukuoka 812-8581, Japan

*Corresponding author: Laboratory of Microbial Technology, Division of applied Molecular Microbiology and Biomass Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Tel: +81 92 642 3020; Fax: +81 92 642 3021; E-mail: nakayama@agr.kyushu-u.ac.jp

One sentence summary: We have shown that altered gut microbiotas during the lactation and weaning periods are associated with the development of food allergies.

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ABSTRACT

Bacterial colonization in infancy is considered crucial for the development of the immune system. Recently, there has been a drastic increase in childhood allergies in Japan. Therefore, we conducted a prospective study with 56 infants on the relationship between gut microbiota in the first year of life and the development of allergies during the first 3 years. In the lactation period, organic acid producers such as *Leuconostoc*, *Weissella* and *Veillonella* tended to be underrepresented in subjects who developed food allergies (FA, n = 14) within the first two years. In the weaning period, children in the FA group were highly colonized by unclassified Enterobacteriaceae and two *Clostridium* species closely related to *Clostridium paraputrificum* and *C. tertium*, and the whole tree phylogenetic diversity index was significantly lower in the FA group. All of these differences in the weaning period were statistically significant, even after adjusting for potential confounding factors. A higher abundance of unclassified Enterobacteriaceae was also found in the other allergic group (n = 15), whereas the two *Clostridium* species were highly specific to the FA group. The mode of action of these *Clostridium* species in childhood food allergies remains unknown, warranting further investigation.

Keywords: infant; fecal microbiota; food allergies; 16S rRNA gene; *Clostridium*

INTRODUCTION

Several hundred bacterial species colonize the human gastrointestinal tract in a mutualistic relationship with the host

body and, notably, its immune system (Round and Mazmanian 2009; Nicholson et al. 2012). A stable gut microbial community is established with two big transitions in the first year of life. The first occurs soon after birth during lactation, with the

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establishment of a *Bifidobacterium*-dominant microbiota, and the second occurs during the weaning period, with the establishment of an adult-type complex microbiota associated with adult diets (Bergström et al. 2014; Mitsuoka 2014; Cheng et al. 2016; Matsuki et al. 2016). These processes are believed to have profound effects on the development and physiology of the host immune system (Francino 2014). In particular, bacteria colonization is necessary for the normal development of the immune system, as indicated by the loss of immune function in germ-free mouse (Macpherson and Harris 2004; Atarashi et al. 2008; Purchiaroni et al. 2013). Development of oral tolerance is a crucial event mediated by bacterial colonization in infancy, and it is required for normal action of the digestive tract after weaning (Sudo et al. 1997). Alterations in this process are suspected to cause childhood food allergies (FA; Ling et al. 2014; Azad et al. 2015). Indeed, the development of FAs appears within the first two years of life, in most cases (Prescott et al. 2013).

Childhood FAs have emerged as a second wave of the allergy epidemic, following the first epidemic of asthma, atopic dermatitis and allergic rhinitis that appeared in association with industrialization in the past half century (Prescott et al. 2013). Although the cause of this second epidemic is yet to be identified, and many factors, including environmental, dietary and medical factors, are suspected to be driving this epidemic, the gut microbiota is considered linked to the hygiene hypothesis, which suggests that less exposure of an immature immune system to microorganisms as 'old friends' can lead to allergies (Feehley et al. 2012). Indeed, the association between gut microbial community changes and childhood FAs has been confirmed in some epidemiological studies (Thompson-Chagoyan et al. 2011; Ling et al. 2014; Azad et al. 2015; Cheng et al. 2016). A Canadian healthy infant longitudinal development (CHILD) study showed alterations in the gut microbial community in food-sensitized infants, in which Enterobacteriaceae were over-represented in a less diverse microbial community in infants at 3 months of age, whereas Bacteroidaceae were underrepresented at 1 year (Azad et al. 2015). A Chinese infant cohort study showed that infants with FAs had a higher abundance of Firmicutes and lower abundance of Bacteroidetes at 6 months, but no significant difference in total microbial diversity was found (Ling et al. 2014).

In Japan, 5%–10% of infants, 5% of preschool children and 1.5%–3% of school children develop FAs. According to a recent report by the Tokyo Metropolitan Institute of Public Health, the incidence of FAs in children at 3 years old in Tokyo has increased from 8.5% in 2004 to 16.7% in 2014, indicating that childhood FAs are a substantial and evolving public health issue (<http://www.fukushihoken.metro.tokyo.jp/allergy>). Here, we conducted a prospective study in Japan on the relationship between the gut microbiota in the first year of life and the development of FAs by 2 years of age.

We found several changes in the gut microbiota in both the pre- and post-weaning periods associated with childhood FAs. In the context of other cohort studies, we discuss the possible link between altered pre- and post-weaning gut microbiotas and the later development of FAs.

METHODS

Subjects and study design

The study protocol was approved by the Ethics Committees of the Faculty of Agriculture, Kyushu University (07–004), St. Mary's College (H19–027) and St. Mary's Hospital (8 April 2008). Informed

written consent was obtained from the parents of all participants prior to enrollment.

Sixty-nine subjects, who were born in St. Mary's Hospital in Kurume City in Japan between July 2008 and October 2009, were enrolled in this prospective study. The infants were born between week 36 and 42 of gestation (mean, 39 weeks). Fecal samples were collected at 1, 2 and 6 months and 1 year of age. Freshly evacuated feces in diapers were gathered into collection tubes and frozen for transport to the laboratory. All of the samples were stored at -80°C until further processing.

A questionnaire based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire was used for a follow-up investigation of medical diagnoses of allergies and symptoms of FAs, atopic dermatitis, allergic rhinitis and asthma during first 3 years of life (Asher et al. 1995). Fourteen subjects had symptom of FAs in the first 2 years and were categorized in the FA group of this study. We also evaluated total and specific IgE levels in subjects who developed any allergy according to the ISAAC questionnaire. Eleven subjects out of 14 FA subjects were subjected to the blood IgE test and 10 subjects were positive for specific IgE. Fifteen subjects had medical diagnoses of atopic dermatitis, allergic rhinitis and/or asthma during first 3 years of life and were categorized as the other allergy (OA) group. Twenty-seven subjects were assessed as infants without allergies who showed no evidence of an allergic disease by 3 years of age and were categorized as the non-allergy (NA) group. Thirteen subjects were excluded from this study because they suspended sample collection and/or follow-up before 3 years of age. Characteristics of each group are shown in Table 1 and Table S1 (Supporting Information). Forty one out of 56 subjects (73%) were vaginally delivered. Twenty nine out of 56 subjects (51.8%) were exclusively breastfed in the first 2 months. All of the other infants were fed by mixture of breast milk and formula milk. Antibiotics administered to 11% of subjects in the first 6 months and 64% of subjects in the first 1 year. There were no statistical differences in sex, caesarean section delivery, breastfeeding up to 2 months of age and use of antibiotic in the study period between these groups.

Bacterial composition analysis by 16S rRNA amplicon sequencing

Total DNA was extracted from fecal samples by the bead beating method as previously described (Nakayama et al. 2011). The V1–V2 region of the 16S rRNA was amplified from total DNA using the universal primer Tru 27F (5'-CCCTCTTCCGATCTCTGAGRGTTTGATYMGGCTCAG-3') and Tru 354R (5'-TGCTCTTCCGATCTGACCTGCCTCCCTAGGAGT-3') (Kim et al. 2013). PCR was performed in 25 μL of a solution containing 0.625 U of Takara Ex Taq HS polymerase (Takara Bio, Shiga, Japan), 2.5 μL of 10 $\times$ Ex Taq buffer, 2.5 mM of each dNTP mixture, 1 μL of 10 to 50 ng of the extracted DNA template and 5 pmol of each primer. The PCR conditions were as follows: 94 $^{\circ}\text{C}$ for 3 min; 18 cycles at 94 $^{\circ}\text{C}$ for 30 s, 50 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 30 s; and finally 72 $^{\circ}\text{C}$ for 5 min. A barcode sequence tag was added to each amplicon by a second PCR. This second PCR was performed in a 25- μL solution containing 0.625 U Takara Ex Taq HS polymerase, 2.5 μL of 10 $\times$ Ex Taq buffer, 2.5 mM of each dNTP mixture, 0.5 μL of the product from the first PCR and 5 pmol of each primer. The PCR conditions were as follows: 94 $^{\circ}\text{C}$ for 3 min; 10 cycles at 94 $^{\circ}\text{C}$ for 30 s, 60 $^{\circ}\text{C}$ for 30 s, 72 $^{\circ}\text{C}$ for 30 s; and finally 72 $^{\circ}\text{C}$ for 5 min. The amplicons were purified using a QIAquick PCR Purification Kit (Qiagen), and the DNA concentrations were measured using PicoGreen (Invitrogen) in

Table 1. Characteristics of the participants in this study.

Parameter	All (n = 56) no. (%)	NA ^a (n = 27) no. (%)	FA ^b (n = 14) no. (%)	OA ^c (n = 15) no. (%)
Proportion of males	31 (55.4)	15 (55.6)	9 (64.3)	7 (46.7)
Vaginal delivery	41 (73.2)	19 (70.3)	10 (71.4)	12 (80.0)
< 2 months only breast feeding	29 (51.8)	15 (55.6)	4 (28.6)	10 (66.7)
Use of antibiotic				
< 1 month	3 (5.4)	3 (11.1)	0 (0.0)	0 (0.0)
1–2 months	6 (10.7)	3 (11.1)	0 (0.0)	3 (20.0)
2–6 months	6 (10.7)	3 (11.1)	1 (7.1)	2 (13.3)
6 months–1 year	36 (64.3)	15 (55.6)	11 (78.6)	10 (66.7)
Item of food allergies ^d				
Egg		NT	12 (8)	NT
Cow's milk		NT	6 (6)	NT
Soy bean		NT	1 (1)	NT
Wheat		NT	4 (4)	NT

^aNA: Non-allergic infants were assessed as infants without allergies who showed no evidence of an allergic disease by 3 years of age.

^bFA: Food allergy infants had symptom of food allergies in the first 2 years of life. Two FA subjects also developed asthma in the first 3 years of life.

^cOA: Other allergy infants had medical diagnosis of atopic dermatitis, allergic rhinitis and/or asthma during first 3 years of life.

^dEleven out of 14 FA subjects were examined by the blood IgE test. The number in parenthesis indicates the number of subjects showing specific IgE level higher than 0.35 KU/L.

a Tecan M200 (excitation at 480 nm and emission at 520 nm). A pool of equal amounts (200 ng) of amplicons from each sample was purified using an ethanol precipitation method. Then, the amplicon mixture was subjected to paired-end sequencing by Illumina MiSeq. The raw sequence data were deposited at the DNA Data Bank of Japan sequence read archive (DRA005562) under BioProject no. PRJDB5316, which contains links and access to stool sampling data under BioSample SAMD00074601 to SAMD75161.

The obtained sequences were processed using the Uparse pipeline in Usearch (Edgar 2013). First, the pairs of sequence reads were merged using the `-fastq-mergepairs` command. After quality filtering, length trimming, dereplication and discarding of singletons, the merged sequences were clustered into 1169 operational taxonomic units (OTUs), each representing greater than 97% identity, using the UPARSE-OTU algorithm. A total of 7.99×10^6 reads were retained from 252 fecal samples (median, 3.1×10^4 reads per sample; range, 6.06×10^3 – 8.56×10^4 reads/sample).

The taxonomy of each OTU was assigned using the Greengenes reference sequence database (gg-13.5) (DeSantis et al. 2006) and the UCLUST algorithm (`assign_taxonomy.py`) in QIIME (Caporaso et al. 2010). The taxonomic composition of each fecal sample was determined based on the OTU table using the QIIME `summarize_taxa_through_plots.py` command.

The 16S rRNA V6–V8 region amplicon sequences were obtained and processed using a Roche 454 GS (FLX titanium) pyrosequencing platform as described previously (Nakayama et al. 2015).

Alpha diversity analysis

The alpha diversity of each sample was calculated using three standard indices—observed species (the number of detected OTUs), phylogenetic diversity (PD) whole tree and the Shannon–Weiner index—and the OTU table rarified to 5000 sequences per sample with 10 random interactions using the QIIME `alpha_rarefaction.py` command.

Beta diversity analysis

The pairwise-weighted Unifrac distance between samples was calculated based on the OTU table rarified to 5000 sequences per sample using `beta_diversity_through_plots.py` in QIIME. Differences in community structures between the NA and FA groups were analyzed statistically based on the weighted Unifrac metric using the `'adonis'` function with 999 permutations in the QIIME pipeline. We also performed a supervised classification of microbial community structures in the NA and FA groups using the partial least square discriminant analysis (PLS-DA) in SIMCA 14.1 (Umetrics, Umea, Sweden). The genus composition of each sample, as well as OTU composition, was subjected to a PLS-DA. Genera and OTUs with variable importance in projection values greater than 1.0 were eventually selected for the DA. We performed an analysis of variance of the cross-validated residuals (CV-ANOVA) for significance testing of the PLS-DA model (Eriksson, Trygg and Wold 2008).

Identification of *Clostridium* species using 16S rRNA V1–V8 sequences

To obtain nearly full-length 16S rRNA fragments from OTU37 and OTU84, specific PCR primers were designed based on the V1–V2 sequence (forward primer) and V6–V8 sequence (reverse primer) (Table 2). To identify counterpart OTUs from the V6–V8 region, two OTUs, each showing a high identity to *Clostridium paraputrificum* (99.8%) or *C. tertium* (99.3%), were selected and confirmed to be significantly more abundant in FA group than NA group ($P < 0.05$ in Mann–Whitney *U*-test). PCR was performed in 50 μ L of a solution containing 1.25 U of Takara Ex Taq HS polymerase (Takara Bio, Shiga, Japan), 5 μ L of $10 \times$ Ex Taq buffer, 5 mM of each dNTP mixture, 5 pmol of each primer and 1 μ L of 10 to 50 ng of DNA from the two OTUs extracted from the samples from 1-year-old infants in the FA and NA groups. The PCR conditions were as follows: 95°C for 5 min; 40 cycles at 95°C for 30 s, 53°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified fragments were cloned into a pGEM-T vector (Promega, Madison, USA), and the ligated DNAs were transformed into *Escherichia coli* DH5 α (Merck,

Table 2. Sequences of OTU-specific primers for *Clostridium* identification.

Target OTU	Primer name	Sequence (5'-3')	Size (bp)	Tm (°C)
OTU37	OTU37_F	AGTTCCTTCGGGAACGGATT	1155	55
	OTU37_R	GAGACCGGTTTATAGTTTTGCT		54
OTU84	OTU84_F	AGTTCCTTCGGGAACAAATC	1155	50
	OTU84_R	CTGAGACAAGTTTATAGTTTAGCTC		53

Table 3. Sequences and PCR conditions of species-specific primers for three species of *Clostridium*.

Target species	Primer name	Sequence (5'-3')	Size (bp)	PCR condition
<i>C. paraputrificum</i>	para_F2	ATTGTAGCTTCGCATGAAGT	256	95°C (5 s)–60°C (45 s)
	para_R2	ACAACCCGAAGGCCGTCA		
<i>C. neonatale</i>	neo_F2	ATTGTAGTATCGCATGATAT	256	95°C (5 s)–60°C (45 s)
	para_R2	ACAACCCGAAGGCCGTCA		
<i>C. tertium</i>	ter_F2	ATTACATTTTCGCATGAAGA	256	95°C (5 s)–55°C (30 s)–72°C (30 s)
	ter_R2	ACGATCCGAAAACCTTCA		

Darmstadt, Germany). Twenty-three and eight clones were selected for OTU37 and OTU84, respectively, and plasmids for sequencing were extracted using the FastGene Plasmid Mini Kit (NIPPON Genetics Co, Ltd, Tokyo, Japan). Plasmid inserts were sequenced by bidirectional Sanger sequencing employing vector-specific primers (M13 forward and reverse) by Fasmac Co. Ltd. (Kanagawa, Japan).

Twenty-three and eight sequences obtained for OTU37 and OTU84, respectively, were used in a search for 16S rRNA sequences of closely related type strain species in the Sequence Match program of Ribosomal Database Project II (<http://rdp.cme.msu.edu/seqmatch.intro.jsp>). All sequences, including the reference 16S rRNA sequences obtained in the search, were aligned using ClustalW 2.1 (<http://clustalw.ddbj.nig/>) and then analyzed using the neighbor-joining method (Saitou and Nei 1987). For the neighbor-joining tree, evolutionary distances were calculated based on a two-parameter model (Kimura 1980). A bootstrap analysis was calculated with 1000 resamplings to evaluate the tree topologies.

Quantitative PCR

Species-specific primers were designed based on the 16S rRNA sequences obtained from search for V1–V8 sequences. We performed quantitative real-time PCR on the three *Clostridium* species using an Mx3000P QPCR System (Stratagene, La Jolla, CA). Each reaction was performed with 20 µL of reaction mixture containing 4 µL of DNA from fecal samples according to the manufacturer's protocol (Takara Bio). The primer sequences and PCR conditions for each primer set are shown in Table 3.

Statistical analysis

We performed the Mann-Whitney *U*-test in R version 3.2.2 to test differences in the relative or absolute abundance of each taxonomic group and the alpha diversities of the FA and NA groups. Pairwise co-occurrences of genera across samples were calculated with Spearman's rank correlation of relative abundance in R version 3.2.2.

Stratified analysis was performed by using Mantel-Haenszel procedures in Stata 12.1 (StataCorp LP, Texas, USA). Multiple logistic regression analyses were used to test associations

between factors that generally affect the gut microbiota in infancy and characteristics of the gut microbiome in FA subjects using Stata 12.1. The following covariates were taken into account in the logistic models: use of antibiotics, caesarean section delivery, duration of breastfeeding and maternal history of allergies.

RESULTS

Development of allergies in our subjects

In our cohort of 56 subjects, 14 subjects experienced FAs during the first 2 years of life. The onset of FAs occurred in the first year for 10 subjects and in the second year for 4 subjects.

Ten out of 11 FA subjects who had a blood IgE test tested positive for specific IgE. These subjects were sensitized mainly to egg (*n* = 8) and then to cow's milk (*n* = 6) (Table 1 and Table S1, Supporting Information). Nine subjects in OA group developed asthma, which was another prevalent allergy in our cohort, during the second and third years.

Succession in the fecal bacterial community composition in the subjects' first year

Figure 1 shows changes in the relative abundance of four dominant phyla in subjects during the first year of life. First of all, we examined profiles of undisturbed microbiota (UM) using the subgroup of subjects who were vaginally delivered, exclusively breastfed in the first 2 months and were not administered antibiotics for the first 6 months (UM, left panel in each phylum). Although there was large individual variation in the abundance of these phyla, marked population shifts were observed at two points: one was an increase in Actinobacteria at ~6 months, and the other was an increase in Firmicutes and Bacteroidetes and a decrease in Actinobacteria and Proteobacteria between 6 months and 1 year. The first shift corresponded to the establishment of a *Bifidobacterium*-dominant microbiota in lactating infants. The latter shift corresponded to a decay in the *Bifidobacterium*-dominant microbiota that was generally observed after intake of solid foods began. These trends were also observed in both the NA (middle panel) and FA (right panel) groups, whereas Firmicutes and Proteobacteria were somewhat underrepresented in early stage in the UM group. Some

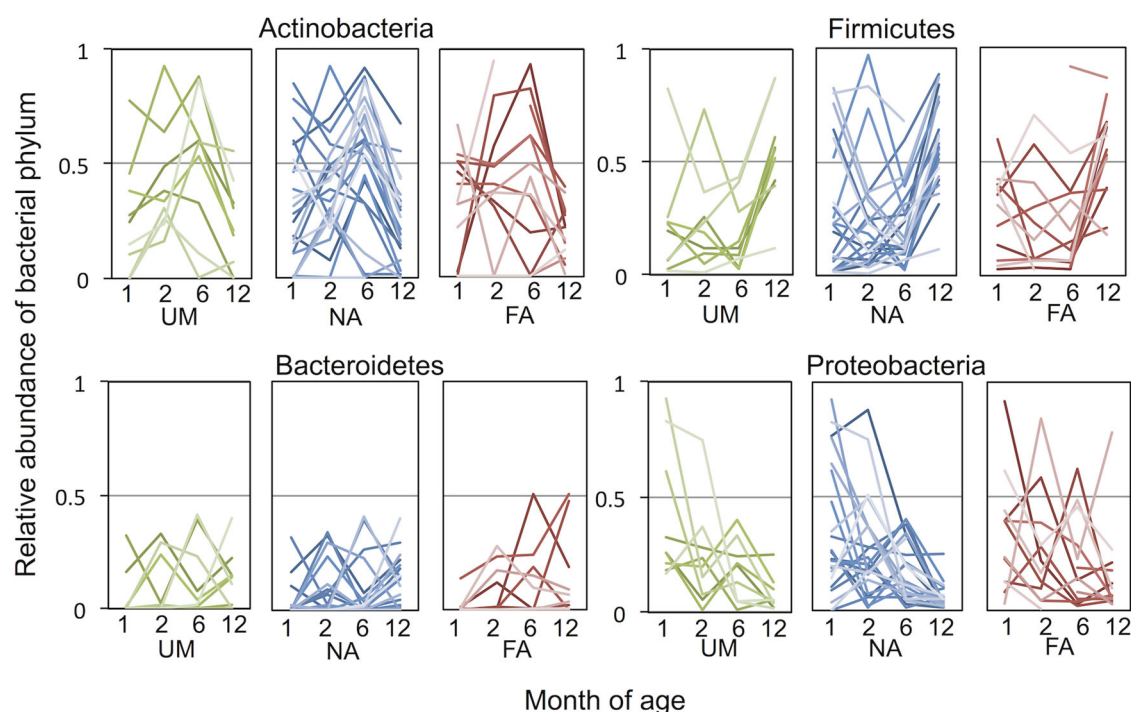


Figure 1. Changes in the relative abundance of four dominant phyla in the first year of life of infants in the undisturbed microbiota (UM), food allergy (FA) and non-allergy (NA) groups.

subjects did not follow the process of regular fecal microbiota development via *Bifidobacterium*-dominant microbiota but did not develop allergies.

Comparison of the structures of fecal bacterial communities in the first year of life of infants in the FA and NA groups

The alpha and beta diversities of the fecal microbiotas of infants in their first year of life were compared between the FA and NA groups (Tables 4 and 5). The alpha diversity of the bacterial community in each sample was evaluated according to the number of detected OTUs (observed species), PD whole tree, and richness and evenness of OTUs (Shannon-Wiener index), and these measures were compared non-parametrically between the two groups. At 1 and 6 months of age, these diversity indices were not markedly different between the two groups, whereas the observed species and PD whole tree measures were somewhat lower in the FA group than in the NA group at 2 months of age. At 1 year of age, these two indices differed more clearly.

According to beta diversities based on weighted Unifrac distances, overall structures of the gut microbiotas did not differ statistically between the FA and NA groups (Table 5). Therefore, we performed a PLS-DA using the OTU composition matrix data (Table 5). A permutation test in the PLS-DA indicated that there was a significant difference in fecal bacterial communities at 2 months and 1 year of age between the FA and NA groups.

Associations between fecal microbiota at the age of 2 months and later development of FAs

Because the PLS-DA at 2 months of age showed significant differences in gut microbial communities in the NA and FA groups (Table 5), a non-parametric Mann-Whitney *U*-test was performed to determine the statistically significant differences in

the relative abundance of taxonomic groups. The genera *Leuconostoc* and *Weissella*, which are lactic acid-producing bacteria, and *Veillonella*, which assimilates lactate to produce propionate and acetate, were significantly underrepresented in the FA group (Fig. 2). Other genera, namely the lactic acid bacteria *Lactococcus*, *Enterococcus* and *Lactobacillus*, were also underrepresented in the FA group, although the differences were not statistically significant.

To examine the community networks among these and other genera, we estimated pairwise co-occurrences with Spearman's rank correlation coefficient of the relative abundance of genera across samples (Fig. 3). The genus *Veillonella* was found to co-occur with the lactic acid bacteria, suggesting a metabolic link through lactate. Several lactic acid bacteria were also found to co-occur with the genus *Acinetobacter*.

The abundance of four genera, *Leuconostoc*, *Weissella*, *Veillonella* and *Acinetobacter*, was summed and the infant subjects were classified into two groups below and above its median. The odds ratio (OR) of this classification for FA development at 1 year was calculated to be 0.167 with $P = 0.02$. If limited to IgE-positive group, the OR value and significance did not change. Then, we performed stratified analysis to examine the effect of possible confounding factors, namely type of delivery, type of milk and use of antibiotics (Table S2, Supporting Information). The adjusted ORs ranged from 0.123 to 0.223 and were not statistically different confounding by these factors in our subjects.

Associations between fecal microbiota at 1 year of age and allergy development

Following the PLS-DA based on the OTU composition at 1 year of age (Table 5), we performed the same discriminant analysis at the genus level (Fig. 4). FA and NA subjects separated into discrete clusters (Fig. 4a), with some genera distinct between the two groups listed in Fig. 4b. The Mann-Whitney

Table 4. Comparison of α -diversity of fecal microbiota in the first year of life of infants in the FA and NA groups.

		Median %		P*
		NA	FA	
1 month	Number of detected OTU	34.0	32.0	0.91
	PD whole tree	3.10	3.13	0.94
	Shannon wiener index	2.23	2.54	0.35
2 months	Number of detected OTU	44.0	38.0	0.18
	PD whole tree	3.71	3.33	0.27
	Shannon wiener index	2.76	2.76	1
6 months	Number of detected OTU	41.0	44.0	0.8
	PD whole tree	3.76	3.48	0.37
	Shannon wiener index	2.63	2.55	0.97
1 year	Number of detected OTU	60.5	53.0	0.056
	PD whole tree	6.52	5.44	0.004
	Shannon wiener index	3.79	3.86	0.4

*Comparisons by non-parametric Mann-Whitney U-test.

Table 5. Analysis of variance result of β -diversity of fecal microbiota in the first year of life of infants in the FA and NA groups.

		β -Diversity	
		Adonis/weighted* Unifrac	CV-ANOVA** PLS-DA
1 month	F-score	0.64	1.72
	R ²	0.02	0.52
	P	0.61	0.17
2 months	F-score	0.46	10
	R ²	0.02	0.521
	P	0.79	2.85×10^{-5}
6 months	F-score	0.46	2.13
	R ²	0.01	0.79
	P	0.73	0.1
1 year	F-score	1.16	10.9
	R ²	0.03	0.791
	P	0.32	9.39×10^{-6}

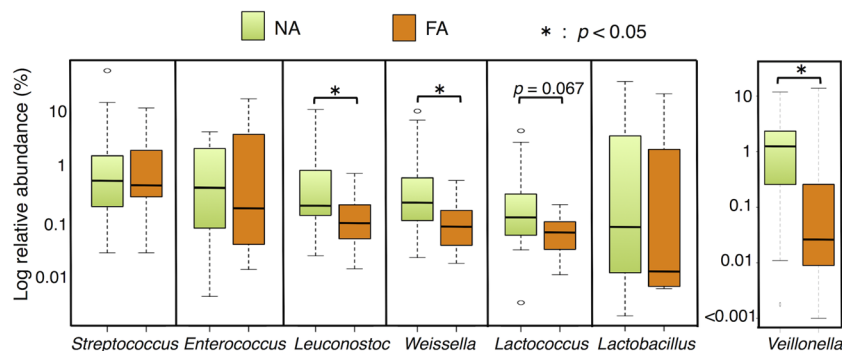
*For weighted Unifrac, the statistical significance was assessed by the adonis test with 999 permutations.

**For PS-DA, the statistical significance was assessed by the analysis of variance of cross-validated residuals (CV-ANOVA).

U-test indicated that two groups, *Clostridium* and unclassified Enterobacteriaceae, were significantly overrepresented in the FA group (Fig. 4c). The unclassified Enterobacteriaceae group consisted of OTUs closely and indistinguishably related to genera *Enterobacter*, *Erwinia*, *Citrobacter*, *Escherichia* and *Shigella*. On the other hand, no genera were significantly more abundant in the NA group than in the FA group. The FA cluster included 10 subjects that exhibited FA symptoms in this sampling period but also 4 subjects that had not yet developed an FA, suggesting that this gut microbial signature was not necessarily associated with the onset of FAs.

We performed another PLS-DA to discriminate between the three groups (NA, FA and OA) based on bacterial genera (Fig. 5). The genus *Clostridium* contributed to distinguishing the FA group from the other two groups, whereas unclassified Enterobacteriaceae contributed to separating the allergic groups, namely OA and FA, from the NA group.

At the OTU level, one dominant and one subdominant OTU, namely OTU37 and OTU84, respectively, were classified in the genus *Clostridium*. These OTUs were significantly more abundant in the FA group than the NA group (OTU37: average relative abundance of 0.11% vs 1.06%, $P = 0.02$; OTU84: 0.03% vs 0.29%, $P = 0.01$, by Mann-Whitney U-test). Both OTUs colonized not all but more than two-thirds FA subjects and their prevalences were noticeably higher than in NA group (OTU37: 79% vs 56%, $P = 0.09$; OTU84: 71% vs 41%, $P = 0.05$, in Fisher's exact test). In the

**Figure 2.** Comparison of the relative abundance of lactic acid bacteria and the genus *Veillonella* of infants at 2 months of age in the food allergy (FA) and non-allergy (NA) groups. Box plots showing the relative abundances of lactic acid bacteria and the genus *Veillonella*. A non-parametric Mann-Whitney U-test was used to test the significance of differences in the relative abundance of each genus between the FA and NA groups.

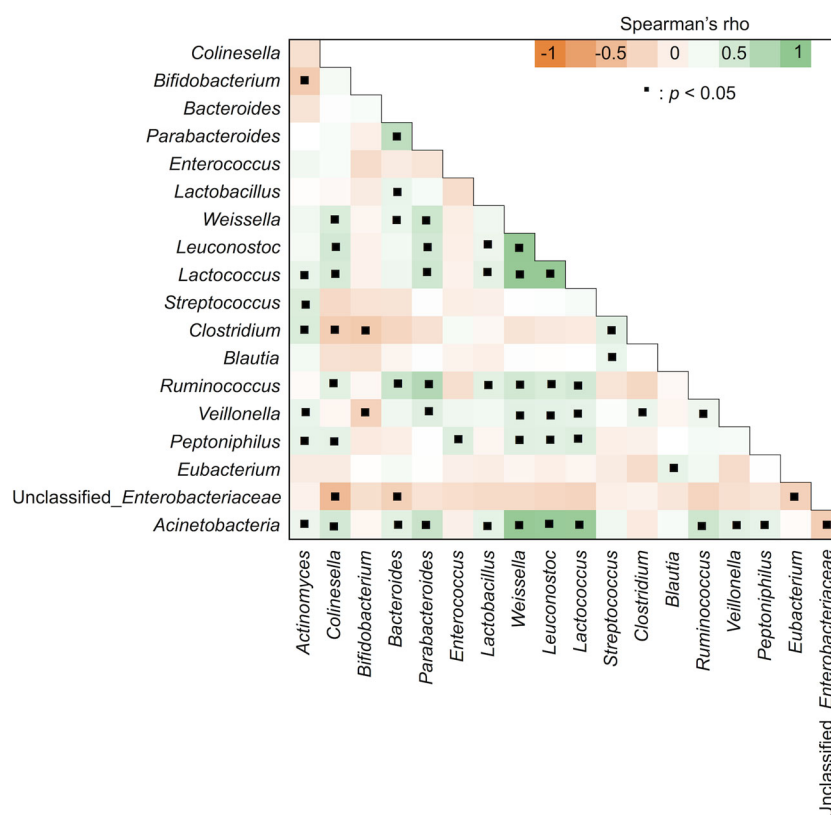


Figure 3. Pairwise Spearman's correlation coefficient of the relative abundance of genera in infants at 2 months of age in the food allergy (FA), non-allergy (NA) and other allergy (OA) groups. Genera with mean abundances greater than 0.1% were subjected to pairwise Spearman's rank test. The correlation coefficients of genera are presented on a color scale. Dotted cells indicate statistically significant correlations ($P < 0.05$).

phylogenetic analysis of nearly full-length 16S rRNA sequences from clones of these two OTUs (Fig. 6), two different sequences from the amplicon targeting OTU37, one was most closely related to *Clostridium paraputrificum* and other was most closely related to *C. neonatale*, whereas the amplicon targeting OTU84 was closely related to *C. tertium*. Results of the quantitative PCR specifically targeting these three species showed that *C. paraputrificum* and *C. tertium* were significantly overrepresented in the FA group at 1 year of age, whereas the abundances of *C. neonatale* were comparable between the FA and NA groups (Fig. 7).

Finally, in order to account for potential confounding factors that can affect the gut microbiota in infancy, we conducted a multivariate logistic regression analyses of FA development using the signatures found in infants in the FA group (Fig. 8). High abundances of the two *Clostridium* species were strongly and significantly associated with FAs (*C. paraputrificum*: OR = 3.03, 95% confidence interval (CI) = 1.17–7.81; *C. tertium*: OR = 3.92, 95% CI = 1.43–10.8). The group 'unclassified Enterobacteriaceae' was also positively and significantly associated with the development of FAs, although the OR was modest (OR = 1.17, 95% CI = 1.02–1.33). The PD whole tree index was negatively and significantly associated with the development of FAs (OR = 0.15, 95% CI = 0.14–0.81). Even though the FA group was limited to IgE-positive subjects, ORs for these microbiota features remained significant.

DISCUSSION

In this prospective study of 56 infants in Japan, we identified the bacterial lineages that related to the development of FA at both lactation and weaning. At the age of 2 months, within the lac-

tation period, infants in the FA group tended to have less lactic acid bacteria in their fecal microbiotas than infants in the NA group. In particular, two genera, *Leuconostoc* and *Weissella*, were remarkably underrepresented in the FA group. These two genera are frequently detected in human breast milk (Cabrera-Rubio et al. 2012), and some studies suggest that bacteria in a mother's breast milk originate in the mother's gut microbiota (Jost et al. 2014; Milani et al. 2015). In addition, some strains of *Leuconostoc* and *Weissella* produce exopolysaccharides that exhibit beneficial biological activities, including anti-allergy activity (Chabot et al. 2001; Ruas-madiedo, Hugenholtz and Zoon 2002; Matsuzaki et al. 2015). Although we did not evaluate a correlation between breast milk and formula milk and FA development, the maternal gut microbiota, representing a source of lactic acid bacteria for the gut microbiota of offspring, might contribute to the prevention of FA development in infancy.

Underrepresentation of lactic acid bacteria in subjects who developed FAs correlated with an underrepresentation of the genera *Veillonella* and *Acinetobacter*. The fact that these two genera assimilate lactate suggests a metabolic link between lactate-producing bacteria and lactate-assimilating bacteria. *Veillonella* is also known to convert lactate to acetic acid and propionic acid. In addition to butyrate, propionate induces regulatory T cells that play a pivotal role in control of immunity (Furusawa et al. 2013). Another paper reported that propionate impaired the T-helper type 2 cell effector function (Trompette et al. 2014), and a previous cohort study showed that *Veillonella* was reduced in infants at high risk of asthma (Arrieta et al. 2015). In addition, protection against allergies by *Acinetobacter lwoffii* has been reported (Debarry et al. 2010), and our previous cohort study showed that a reduction in this species was associated with high risk of

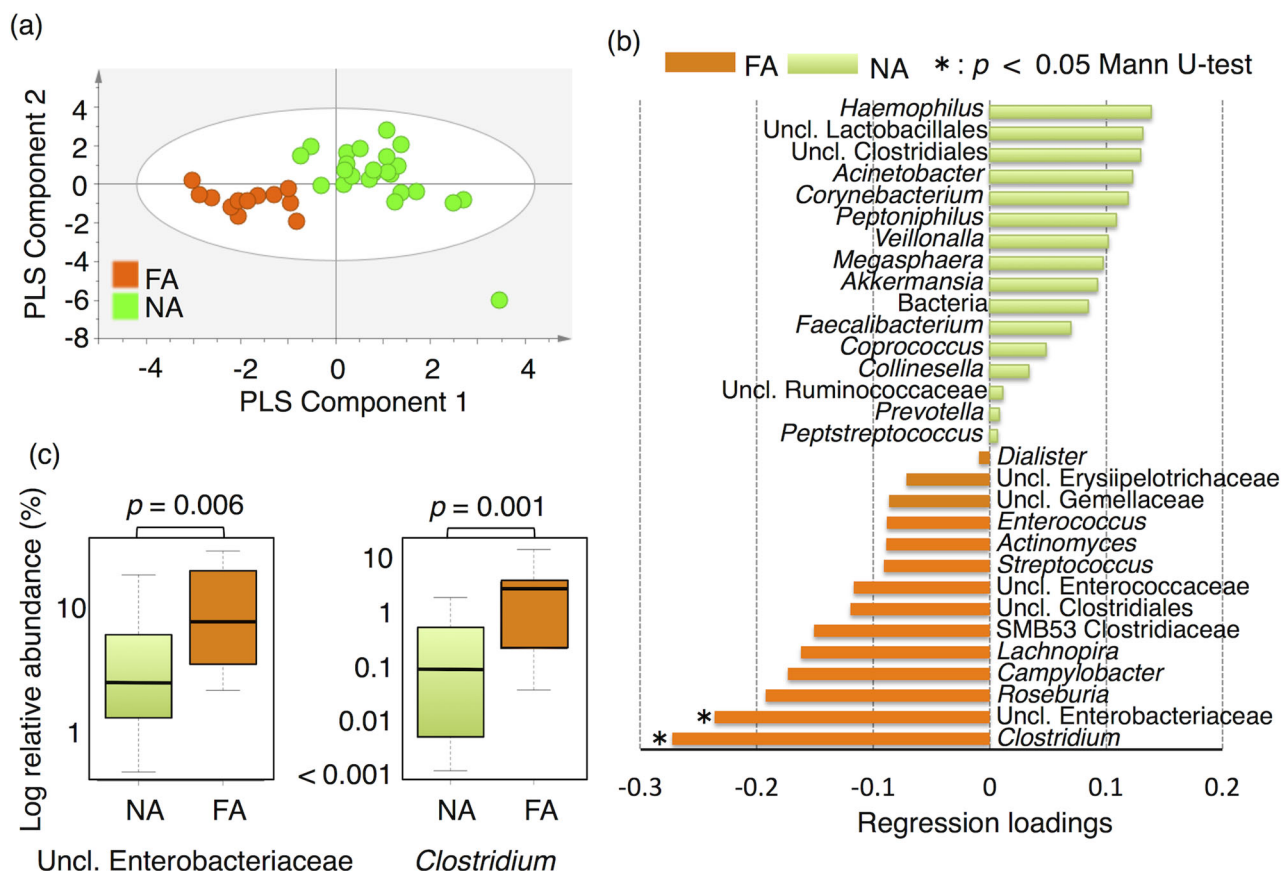


Figure 4. Differences in the fecal microbiotas of infants at 1 year of age in the food allergy (FA) and non-allergy (NA) groups. (a) Partial least squares discriminant analysis (PLS-DA) of the separation of FA (orange circles) and NA (green circles) groups based on bacterial genera. (b) Loading plot of the genera positively (orange) or negatively (green) associated with FAs. * $P < 0.05$ by Mann-Whitney U-test. (c) Box plot showing the relative abundances of genera which were statistically significant differences ($P < 0.05$ in Mann Whitney U-test) between the FA and NA groups.

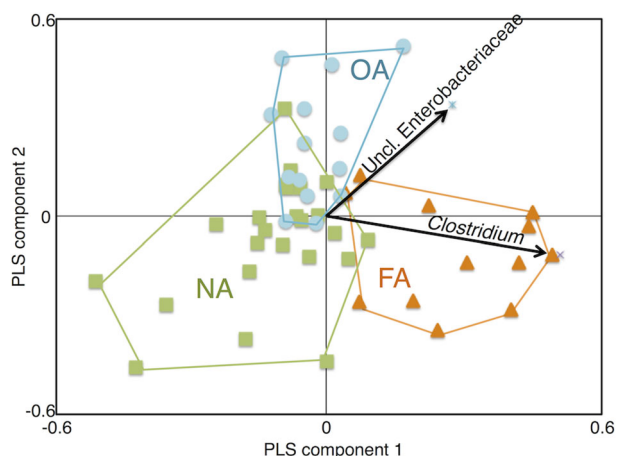


Figure 5. Partial least squares discriminant analysis (PLS-DA) classification of the food allergy (FA, orange triangles), other allergy (OA, blue circles), and non-allergy (NA, green squares) groups based on the relative abundance of bacterial genera. Black arrows indicate the bacterial genera associated with the different samples.

allergy development (Nakayama et al. 2011). The co-occurrence of *Acinetobacter* with lactic acid bacteria in the healthy infant group may also have contributed to the reduction in risk of FA development.

The fecal microbiotas of the FA group differed more remarkably at 1 year of age than at 2 months of age, based on the PLS-DA (Table 5). Alpha diversity was also significantly lower in the FA group than the NA group (Table 4). Whether these changes are the cause or effect of allergies remains a question. Indeed, 10 out of 14 subjects already had symptoms of FAs at 1 year, but 4 subjects without symptoms showed similar community compositions of their fecal microbiotas. Two taxonomic groups, namely the genus *Clostridium* and unclassified Enterobacteriaceae, were remarkably overrepresented in the FA group at 1 year of age. By phylogenetic analysis of nearly full-length 16S rRNA sequences, *Clostridium* spp. were identified as *C. paraputrificum*, *C. neonatale* and *C. tertium*. These species are frequently isolated from infant feces (Stark and Lee 1982) and belong to *Clostridium sensu stricto* or *Clostridium* cluster I, which has already been associated with childhood food or other allergies (Penders et al. 2013; Cheng et al. 2016). However, this is the first report to show an association with allergies of bacteria at the species level. *Clostridium paraputrificum* is known to produce chitinase, which degrades chitin (Morimoto et al. 1997), and mammalian chitinases are known to play a role in asthma by mediating T-helper 2 cell-driven inflammatory responses (Elias et al. 2005; Lee et al. 2011). Chitinase produced by *C. paraputrificum* may play a similar inflammatory function, although there is currently no information on the action of chitinase in intestinal inflammation. Furthermore, it is known that fungal infections, especially *Candida*, can promote allergies (Kim et al. 2014). The overgrowth of chitinase-producing *C.*

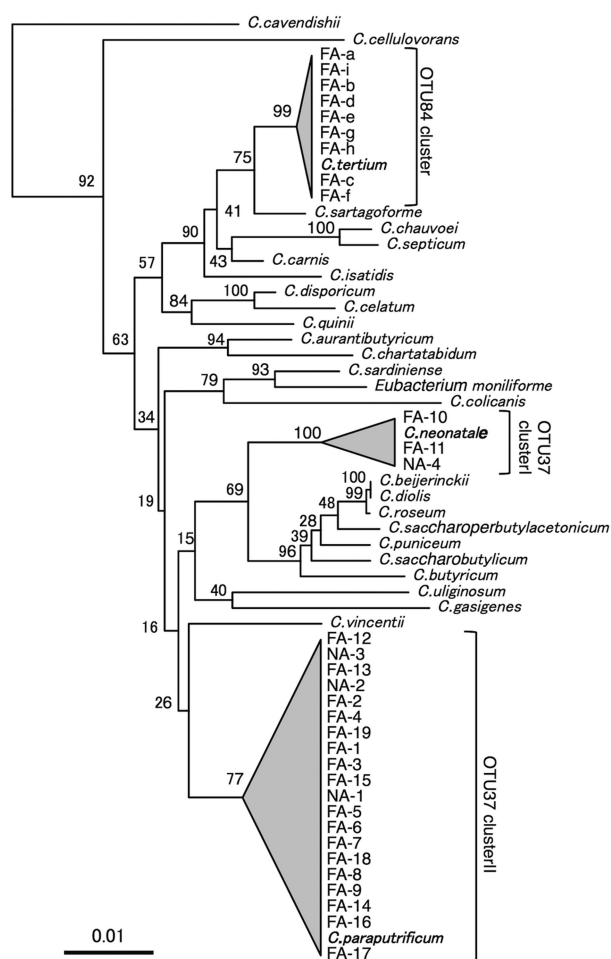


Figure 6. Phylogenetic tree of clones of OTU37 and OTU84 and related species. Clones FA-1 to -19 and clones NA-1 to -4 were derived from OTU37 and FA-a to -i were derived from OTU84. Sequences of the V1-V8 region of 16S rRNA from these clones were analyzed by the neighbor-joining method. Bootstrap values (%) from 1000 resamplings are shown at tree nodes. The three clades including the clones and the closest species were clustered with more than 99% sequence identities and high bootstrap values, respectively, and were then collapsed.

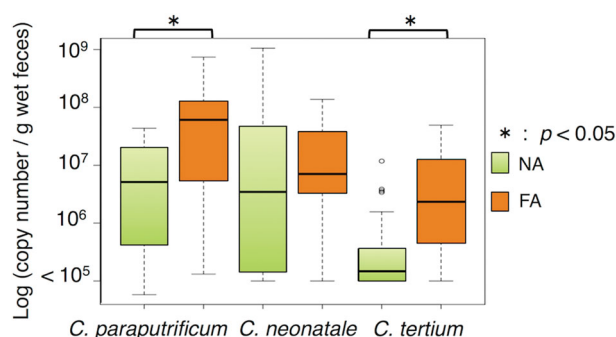


Figure 7. Comparison of absolute amounts of *C. parapatrificum*, *C. neonatale* and *C. tertium* in infants in the NA and FA groups at 1 year of age. Absolute amounts of these species were determined by quantitative PCR using primer sets specific to each species. * $P < 0.05$ by Mann-Whitney U-test.

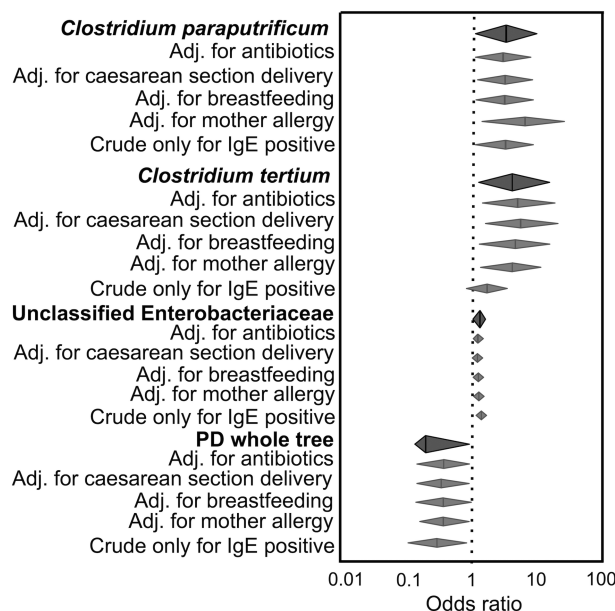


Figure 8. Adjusted likelihood of FA development based on gut microbiota in infants at 1 year of age with or without adjustment for potential confounding factors. Absolute abundances of *C. parapatrificum* and *C. tertium*, relative abundances of unclassified Enterobacteriaceae and PD whole tree indices were subjected to a multiple logistic regression analysis to estimate their associations with infant's development of FAs within 2 years. The following covariates were individually considered as confounding factors in the logistic models: use of antibiotics, caesarean section delivery, duration of breastfeeding and maternal history of allergies. Also, odds ratio was calculated between NA and only IgE-positive subjects. Adj. = Adjusted.

parapatrificum might co-occur with intestinal colonization of fungi having chitin cell walls.

Overrepresentation of unclassified Enterobacteriaceae was remarkable in both the FA and OA groups, whereas overrepresentation of *Clostridium* was specific to the FA group. This may reflect to differences in etiologies of food and other allergies. Overrepresentation of Enterobacteriaceae are often observed with reduced bacterial diversity in inflammatory diseases; the status of microbiota was so-called dysbiosis (Honda and Littman 2012; Petersen and Round 2014). Some other papers have reported a similar association in food-sensitized infants at 1 year (Azad et al. 2015) and in infants (Bisgaard et al. 2011) and schoolchildren (Candela et al. 2012) with atopic dermatitis. Furthermore, some studies have found reduced gut microbial diversity in early infancy (1 week, 1 month and 3 months of age) to be associated with atopic disease and FAs (Wang et al. 2008; Abrahamsson et al. 2012; Azad et al. 2015). Overrepresentation of unclassified Enterobacteriaceae and reduced bacterial diversity observed in our FA subjects may be signs of dysbiosis associated with impending allergy onset.

In conclusion, we have shown that altered gut microbiotas during the lactation and weaning periods are associated with the development of FAs. However, this study had limitations due to small sample size, which did not provide enough power for statistical analysis. Although there found no significant confounding in our dataset, new onset of allergies must result from complex interplay of genetic and environmental risk factors. Furthermore, this study did not treat non-IgE-mediated group. As observed in a previous study, microbiota would be differently involved in IgE-mediated FA and non-IgE mediated FA (Ling et al. 2014). Taken together, we definitely need more controlled and

large-scale study to address the function of infant developmental gut microbiota to prevent new onset of allergic diseases in infancy.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](http://femsec.oup.com) online.

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Conflict of interest. None declared.

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