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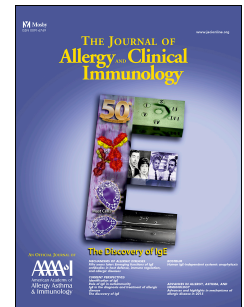
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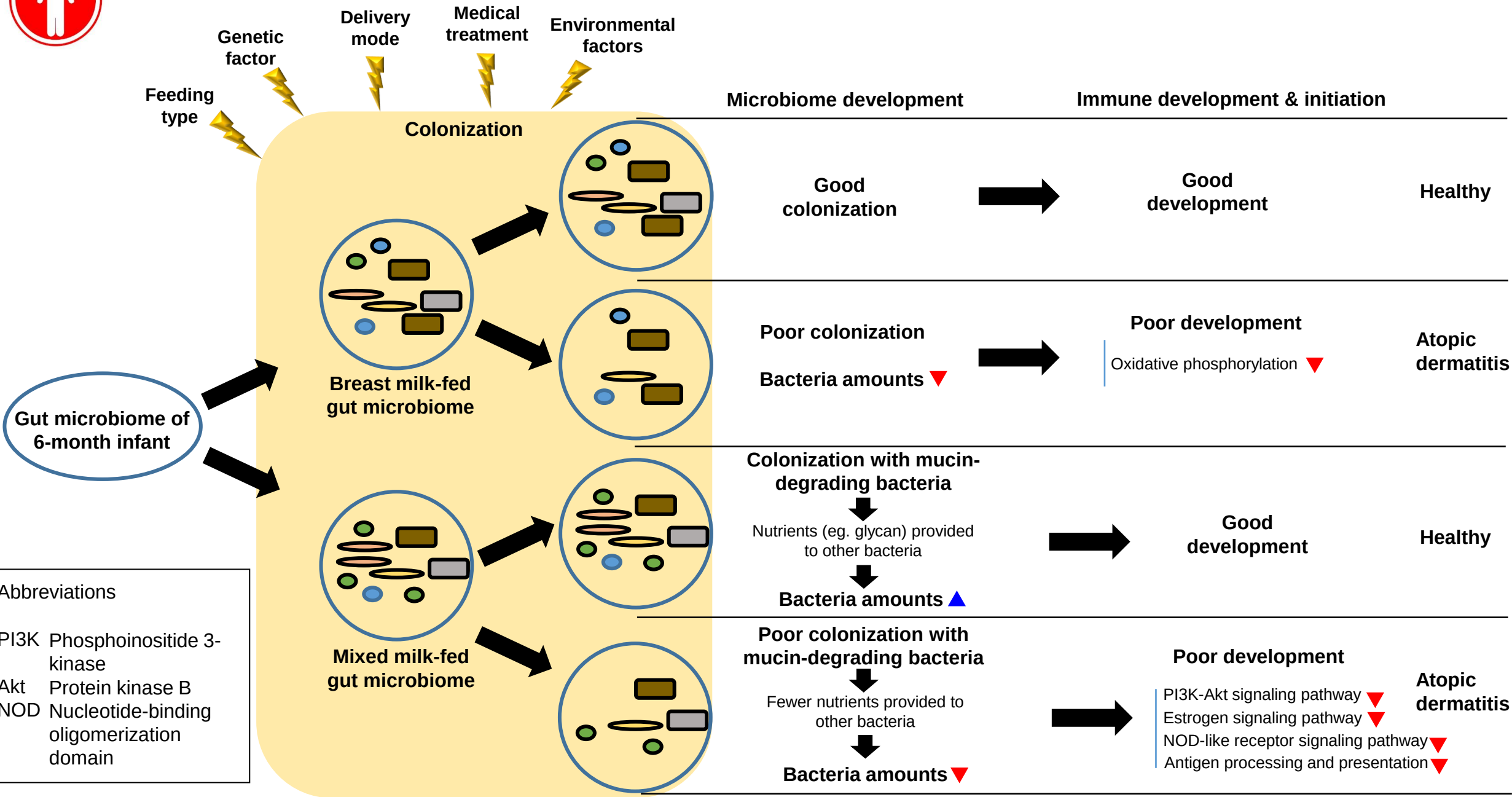
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Abstract

Background: Perturbations of the infant gut microbiota can shape the development of the immune system and link to the risk of allergic diseases.

Objective: To understand the role of the gut microbiome in atopic dermatitis, the metagenome of the infant gut microbiome was analyzed according to feeding types.

Methods: The composition of gut microbiota was analyzed in fecal samples from 129 infants (6-month-old) by pyrosequencing, including 66 healthy infants and 63 infants with atopic dermatitis. The functional profile of the gut microbiome was analyzed by whole metagenome sequencing (20 controls and 20 AD). In addition, the total number of bacteria in the feces was determined by real-time PCR.

Results: The gut microbiome of 6-month-old infants was different by feeding types, and two microbiota groups (*Bifidobacterium* dominated and *Escherichia/Veillonella* dominated group) were found in breast-fed and mixed-fed infants. The bacterial cell amounts in the feces were lower in infants with AD than in controls. Although no specific taxa directly correlated with AD in 16S rRNA gene results, whole metagenome analysis revealed differences in functional genes related to immune development. The reduction of genes for oxidative phosphorylation, PI3K-Akt signaling, estrogen signaling, NOD-like receptor signaling, and antigen processing and presentation induced by reduced colonization of mucin-degrading bacteria (*Akkermansia muciniphila*, *Ruminococcus gnavus*, and *Lachnospiraceae* bacterium 2_1_58FAA) was significantly associated with stunted immune development in the AD group compared to the control group ($P < .05$).

Conclusions: Alterations in the gut microbiome may be associated with atopic dermatitis due to different bacterial genes that can modulate host immune cell function.

Key Messages

- The gut microbiota of 6-month-old infants can be clustered into two enterotypes (*Bifidobacterium*-dominated and *Escherichia/Veillonella*-dominated) in both breast-fed and mixed-fed infants.
- Decreased functional genes related to host immune development in the gut microbiome was associated with atopic dermatitis.
- The presence of *Akkermansia muciniphila*, *Ruminococcus gnavus*, and *Lachnospiraceae* bacterium 2_1 58FAA was associated with the alteration of functional genes related to host immune development.

Capsule Summary

Perturbations of colonization of the gut microbiome in 6-month-old infants may increase the risk of atopic dermatitis by affecting immune development.

Key words: Atopic dermatitis, infant, gut, microbiome, immune

Abbreviations

AD: Atopic dermatitis

BLAST: Basic Local Alignment Search Tool

79 COCOA: COhort for Childhood Origin of Asthma and Allergic diseases

80 RR: relative risk

81 KO: KEGG orthology

82 OTUs: operational taxonomic units

83 BFGM1: breast-fed gut microbiota type 1

84 BFGM2: breast-fed gut microbiota type 2

85 MFGM1: mixed-fed gut microbiota type 1

86 MFGM2: mixed-fed gut microbiota type 2

87 PI3K: phosphatidylinositol 3-kinase

88 NOD: nucleotide-binding domain

89 SCORAD: Scoring of Atopic Dermatitis

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INTRODUCTION

The gut microbiome affects many aspects of human health, including metabolism, gastrointestinal health, and the immune response.¹ Infancy is a critical period for the colonization of the gut microbiota, which affects host immune development. The colonization of the gut microbiota in infancy is influenced by many factors, including host genotype, the mode of delivery, feeding type, and antibiotic/probiotic treatments.²⁻⁴ In particular, feeding type effectively regulate the diversity and composition of the gut microbiota in infancy, and disturbances of the gut microbiome in early life have been linked to several non-communicable diseases such as obesity, cardiovascular disease, and allergic diseases.⁵

Atopic dermatitis (AD) is a common and chronic, pruritic, inflammatory skin disease associated with cutaneous hyperactivity to environmental triggers, and it can affect at least 15% of children.^{6, 7} Although AD usually presents during infancy and early childhood, it can persist into adolescence and adulthood.⁸ Despite various clinical and experimental studies of AD, the etiology of this disease remains unclear. Several studies show that the microbiota of infants with AD differs from the microbiota of infants without AD.⁹⁻¹¹ The temporal associations of microbiota with multisensitized atopy, IgE-associated eczema, and asthma development have been studied.^{9, 12-14} Because the microbiota is linked to immune development and immune responses, it is reasonable to speculate that the microbiome may play an important role in the development of allergic diseases.^{12, 13, 15-17} Although differences in gut microbiota precede the manifestation of allergic diseases, the causes of the differences in gut microbiota between healthy infants and infants with allergic disease have been difficult to determine, due to a number of confounding factors that affect gut microbiome colonization.^{12, 14, 18, 19} Feeding type has the greatest influence on the colonization of the gut microbiome in early life, and it can affect the development of AD.²⁰ However, studies on the

complex relationship between the gut microbiome and AD according to feeding type in early life are lacking. In addition, whole metagenome sequencing provides a higher resolution of taxonomic profile with functional classification of microbiome than dose 16S rRNA gene sequencing; however, the application of this technique remains rare in gut microbiome studies of AD infant.^{17, 21}

In present study, we compared the composition of the gut microbiota between healthy 6-month-old infants and infants with AD according to feeding type. Furthermore, functional differences in the gut microbiome between healthy infants and infants with AD were analyzed to understand the role of the gut microbiome in AD in early life.

METHODS

Study subjects and sampling

Study subjects consisted of 129 6-month-old infants (66 healthy controls and 63 infants with AD) from the longitudinal Cohort for Childhood Origin of Asthma and Allergic Diseases (COCOA) birth cohort study.²² This study was approved by the Institutional Review Boards of Asan Medical Center (IRB no. 2008-0616), Samsung Medical Center (IRB No. 2009-02-021), Severance Hospital (IRB No. 4-2008-0588), the CHA Medical Center (IRB No. 2010-010), and the Seoul National University Hospital (IRB No. H-1401-086-550). Written informed consent was obtained from the parents of each infant.

AD in infants was diagnosed by a pediatric allergist according to Hanifin and Rajka's criteria²³ at 6 months and 12 months of age, and severity of physical symptoms were assessed by the SCORAD (Scoring Atopic Dermatitis) index at 6 months. Measurement of total IgE and egg- and milk-specific serum IgE at 12 months of age was performed by fluorescent enzyme immunoassay (AutoCAP system, Phadia AB, Uppsala, Sweden) on all subjects. Atopy was defined as the presence of specific IgE for eggs or milk. For microbiota analysis, subjects were selected by propensity score matching to contrast the microbiota profiles of AD samples and control samples that have similar propensities for atopic diseases.^{24, 25} The characteristics used to determine the propensity scores are shown in Table 1. Broad-spectrum antibiotics was not prescribed after birth in our study population. Fecal samples were collected and immediately stored at -80°C before being processed for DNA extraction. Details are provided in this article's online repository.

Metagenomic DNA extraction and pyrosequencing

Metagenomic DNA was extracted from 250 mg of feces using the Power Microbiome RNA/DNA Isolation kit (Mo Bio/Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was quantified on a BioPhotometer D30 with a μ Cuvette G1.0 (Eppendorf, Hamburg, Germany) and stored at -80°C until further processing.

For pyrosequencing, the extracted DNA was amplified using barcoded primers targeting the V1-V3 region of the 16S rRNA gene. Preparation for sequencing is detailed in this article's online repository. Sequencing was performed on a Roche/454 FLX Titanium system following the manufacturer's instructions.

Quantitative real-time PCR

The total amount of bacteria in fecal samples was determined by quantitative real-time PCR for 16S rRNA genes. Details are provided in this article's online repository.

Pyrosequencing analysis

Pyrosequencing reads were analyzed as described previously.²⁶ For enterotype clustering of gut microbiota, the Jensen-Shannon divergence and other distance metrics for the relative abundance of genera were calculated.^{27, 28} Details of the analysis process are provided in this article's online repository.

Whole metagenome sequencing and analysis

A total of 40 samples were randomly selected from each group (20 control and 20 AD

samples) according to feeding type and used for whole metagenome sequencing. Metagenomic DNA of selected samples was fragmented using a Covaris M220 sonicator (Covaris, Inc., Woburn, MA, USA), and metagenomic libraries were prepared using a TruSeq DNA sample preparation kit according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using the Illumina HiSeq 2500 system (250 bp paired ends). Further details of the whole metagenome sequencing and analysis are provided in this article's online repository.

Statistical analysis

Relative risk (RR) ratios and corresponding 95% confidence intervals were calculated by log-binominal regression using the maximum likelihood estimation in R. Significantly different bacterial taxa and KO categories between groups were determined using the Kruskal-Wallis rank-sum test in R. Detailed analyses are provided in this article's online repository.

RESULTS

Comparison of the diversity and composition of the gut microbiome between controls and AD infants

A total of 1,423,835 high-quality reads (719,168 reads with 436.1 ± 14.9 bp for controls and 704,667 reads with 429.6 ± 23.6 bp for AD samples) were analyzed from fecal samples of selected 129 infants by propensity score matching. The diversity indices of gut microbiota were compared after normalized read numbers in each sample (see Table E1 in this article's online repository). The numbers of observed operational taxonomic units (OTUs) and the Shannon diversity indices were not significantly different between control and AD samples ($P > .05$) (see Fig E1 in this article's online repository). However, the numbers of bacterial cells in feces were significantly higher in control than AD samples ($P < .01$). Actinobacteria was the predominant phylum in 6-month infant gut microbiota collected from both healthy ($45.35 \pm 3.71\%$) and AD ($43.69 \pm 3.45\%$) infants. At the genus level, seven genera were predominant in samples from both controls and AD infants. However, the prevalence of these genera was not significantly different between groups ($P > .05$).

The influence of feeding type on the gut microbiota of 6-month infants

Because feeding type is a major determinant of gut microbiota colonization in 6-month infants, samples were analyzed according to the infants' feeding type (breast milk, formula, or mixed). Within AD patients, the highest number of observed OTUs was found in samples from formula-fed infants (278.0 ± 33.8), whereas the lowest number of observed OTUs was found in samples from breast-fed infants (215.0 ± 13.7) ($P < .05$; Fig 1). Within healthy infants, the highest number of observed OTUs in the gut microbiota was found in mixed-fed

infants (263.0 ± 82.2), and the lowest number of observed OTUs was found in breast-fed infants (222.0 ± 78.9) ($P < .01$). The bacterial cell counts in fecal samples were higher in breast-fed and mixed-fed controls than in AD infants ($P < .05$; Fig 1C). The highest amounts were detected in mixed-fed controls ($10.36 \pm 0.10 \log_{10}$ cells/g), and the lowest amounts were detected in breast-fed AD infants ($9.51 \pm 0.14 \log_{10}$ cells/g). The bacterial counts differed according to feeding type more highly in the AD group than in the controls.

The phylum compositions of the gut microbiota were compared between controls and AD infants according to feeding type (Fig 1D, E, and F). The relative abundance of Actinobacteria was higher in breast-fed infants in both the control group ($59.31 \pm 6.29\%$) and the AD group ($54.26 \pm 5.44\%$). The lowest relative abundance of Actinobacteria was found in mixed-fed controls ($35.74 \pm 4.69\%$) and AD infants ($35.15 \pm 4.45\%$). However, the relative abundance of Proteobacteria was higher in the gut microbiota of mixed-fed infants ($31.96 \pm 4.68\%$ in controls and $27.38 \pm 3.12\%$ in AD infants) than in other feeding types. The differences in the relative abundances of Proteobacteria, Firmicutes, and Bacteroidetes between controls and AD infants were similar in mixed- and formula-fed infants. Although there were differences in the phylum composition of the gut microbiota according to feeding type, the differences between control and AD infants within the same feeding groups were not significant ($P > .05$). At the genus level, six genera were common in all feeding types (see Fig E3 in this article's online repository). *Bifidobacterium* was the predominant genus in both controls and AD infants of all feeding groups. However, there was no significant difference in genus composition between controls and AD infants ($P > .05$).

Different gut microbiota within the same feeding type

Enterotype clustering was performed to detail differences in the microbiota within each

feeding type. Formula-fed subjects were excluded from this analysis due to the limited number of subjects (healthy = 7 and AD = 6). The gut microbiota of breast-fed infants clustered into two enterotypes, which were driven mainly by the relative abundance of *Bifidobacterium* and *Veillonella* at the genus level ($P < .05$; Fig 2A). The relative abundance of *Bifidobacterium* was higher in breast-fed gut microbiota type 1 (BFGM1) ($74.8 \pm 19.4\%$) than in breast-fed gut microbiota type 2 (BFGM2) ($27.22 \pm 0.04\%$), whereas the relative abundance of *Veillonella* was higher in BFGM2 ($13.27 \pm 3.12\%$) than in BFGM1 ($0.87 \pm 0.33\%$). There were also differences in *Staphylococcus*, *Enterobacter*, *Klebsiella*, and *Raoultella* between BFGM1 and BFGM2 ($P < .05$; see Fig E4 in this article's online repository).

The gut microbiota of mixed-fed infants also clustered into two enterotypes, which were driven mainly by the relative abundance of *Bifidobacterium* and *Escherichia/Veillonella* at the genus level ($P < .05$; Fig 2B). The relative abundance of *Bifidobacterium* was higher in mixed-fed gut microbiota type 1 (MFGM1) ($46.68 \pm 3.73\%$) than in mixed-fed gut microbiota type 2 (MFGM2) ($12.76 \pm 1.88\%$), whereas *Escherichia* and *Veillonella* were higher in MFGM2 ($33.08 \pm 4.64\%$ and $16.17 \pm 3.53\%$, respectively) than in MFGM1 ($16.67 \pm 2.49\%$ and $3.34 \pm 2.49\%$). There were also differences in *Staphylococcus*, *Blautia*, and *Clostridium* between MFGM1 and MFGM2 (see Fig E5 in this article's online repository). Although the gut microbiota of breast-fed and mixed-fed infants was clustered according to their composition, the numbers of controls and AD infants were similar within each cluster.

The characteristics of subjects were compared between enterotypes within each feeding group (Table 2). Vaginal delivery was preferentially associated with an MFGM1 enterotype compared to MFGM2 ($P = .03$). However, there were no significant differences between enterotypes with respect to the other factors analyzed. In addition, there were no significant

differences in RR ratios between two enterotypes within the same feeding type (see Table E2 in this article's online repository). Thus, we analyzed functional genes of gut microbiota between control and AD infants using whole metagenome sequencing to identify the difference of gut microbiota roles between the two groups.

Different functional profiles between control and AD infants by whole metagenome analysis

A total of 231,010,091 reads (116.4 Gb) were analyzed after removal of human gene sequences. The dominant phyla and genera identified by the whole metagenome analysis were similar to those identified based on the 16S rRNA gene sequencing (see Fig E6 in this article's online repository). The relative abundance of genes associated with oxidative phosphorylation was higher in the control group than in the AD group for breast-fed infants ($P = .028$; see Fig E7 in this article's online repository). The number of species contributing to these genes was higher in the control group (62 species) than in the AD group (38 species).

In mixed-fed infants, four pathways were significantly different between the control group and the AD group ($P < .05$). The median numbers of genes associated with PI3K-Akt signaling and estrogen signaling were higher in the control group than in the AD group ($P = .0002$; Fig 3). The relative abundance of genes associated with NOD-like receptor signaling and antigen processing and presentation was also higher in controls than in AD infants ($P < .01$; Fig 4). The same species (*Akkermansia muciniphila*, *Ruminococcus gnavus*, and *Lachnospiraceae* bacterium 2_1_58FAA) contributed to the difference of these genes between control and AD samples ($P < .05$).

DISCUSSION

In this study, we compared the composition of the gut microbiota between healthy infants and those with AD at 6 months of age according to feeding type. A simple comparison between control and AD samples revealed no differences in the members of the gut microbiota, which may partially be due to the fact that the 6-month age is a dynamic period of microbiota colonization in gut.^{3, 29} However, the gut microbiota differed according to feeding type, even among infants with the same disease status. The gut microbiota was divided into *Bifidobacterium*-dominated microbiota and *Escherichia/Veillonella*-dominated microbiota in both the breast-fed and mixed-fed groups. Nevertheless, the relative abundance of *Bifidobacterium* was higher in breast-fed compared to mixed-fed groups. Although the composition of the gut microbiota in AD infants was not significantly different from controls in 16S rRNA gene results, differences in functional metabolic genes, including genes involved in oxidative phosphorylation and NOD-like receptor signaling, were found between control and AD samples within each feeding type. The relative abundances of genes associated with immune development were higher in controls compared to AD samples. These differences were associated with the depletion of *A. muciniphila*, *R. gnavus*, and *Lachnospiraceae* bacterium 2_1_58FAA in the AD group. These findings suggest that alterations of the gut microbiota affecting host immune development may be associated with AD. In addition, our results show that the association of gut microbiota with AD should be considered feeding type and analyzed by using deep analysis of species level identification or functional genes.

The total bacterial cell counts in fecal samples from controls were higher than in those from AD patients (Fig 1C). The gut immune system reacts to exposure to bacterial antigens, and repeated exposure enhances the development of immune responses. Therefore, fewer

bacteria in the gut during the maturation of the immune system may result in dysfunctional immune responses.

However, there were no significant differences in microbes between controls and infants with AD (Fig E1). Whether specific gut microbes are different between healthy controls and infants with allergic disease is a matter of debate. It was reported that *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* are less abundant in the gut microbiota of infants with AD, whereas *Clostridium* is more abundant.^{9, 10} However, some studies found no significant differences of gut microbiota between control and AD, and the high abundance of *Faecalibacterium prausnitzii* may lead to increased risk of eczema by reducing butyrate and propionate.^{17, 30} These inconsistencies could be due to differences in detection methods across studies, as well as differences in other factors affecting the gut microbiota such as host genotype, mode of delivery, age, and feeding type.^{3, 4} To reduce the effect of influencing factors on gut microbiota analysis, propensity score matching, which is an alternative technique to reduce selection bias, was used in the present study.^{24, 25} No differences in these factors were found between the two groups (Table 1). In particular, because the age of subjects is important for accurately analyzing the difference between groups, infants with the same age were analyzed in the present study.^{12, 31}

Different feeding types influence the developing gut microbiota.^{5, 32} Therefore, the samples were analyzed according to feeding type, and differences in gut microbiota by feeding type were found within both groups (Fig 1). Although the composition of the gut microbiota differed according to feeding type, there were no differences between controls and AD infants within each feeding type ($P > .05$). Therefore, the microbiota were examined in further detail by enterotype analysis.

The 6-month gut microbiota of breast-fed infants clustered into a *Bifidobacterium*-

abundant (>70% of gut microbiota) cluster (BFGM1) and a *Veillonella*-abundant (>10%) cluster (BFGM2) (Fig 2). In mixed-fed infants, the gut microbiota also clustered into a *Bifidobacterium*-abundant (>45%) cluster (MFGM1) and an *Escherichia/Veillonella*-abundant (>29%/>12%) cluster (MFGM2). The gut microbiota of 6-month infants in the present study developed into either a Gram-positive (*Bifidobacterium*)-dominated microbiota or a Gram-negative (*Escherichia/Veillonella*)-dominated microbiota in both breast-fed and mixed-fed infants. Increased levels of bile acids could be associated with a Gram-positive gut microbiota, whereas decreased levels of bile acids could be associated with a Gram-negative gut microbiota.³³ Further studies are necessary to clarify the association of bile acids with the gut microbiome in infants.

Although the differences in the composition of the gut microbiota were not found between control and AD infants in 16S rRNA gene results, differences in functional genes in the gut microbiota were investigated in the two groups. There were no differences in RR ratios and the number of AD patients between *Bifidobacterium*-dominated and *Escherichia/Veillonella*-dominated enterotypes within the same feeding type (Fig 2 and Table E2). Therefore, samples from each feeding type were randomly selected for whole metagenome analysis. The relative abundance of microbial genes associated with oxidative phosphorylation was higher in controls than in AD samples in breast-fed infants (Fig E7). Oxidative phosphorylation can be a function of the gut microbiota priming the host immune system and is associated with the activation of regulatory T cells,³⁴ which may affect the development of AD.

In mixed-fed infants, the relative abundance of genes related to PI3K-Akt signaling, estrogen signaling, NOD-like receptor signaling, and antigen processing and presentation was higher in the control group than in the AD group (Fig 3 and 4). The PI3K-Akt signaling pathway is important in cell growth and survival, particularly in intestinal epithelial cells and

dendritic cells (DC), and it contributes to the activation of mammalian target of rapamycin (mTOR), which regulates a variety of processes, including cellular metabolism.³⁵⁻³⁷ The mucosal immune system of the gut is also influenced by estrogen signaling in conventional as well as regulatory T cells.³⁸ NOD-like receptors play critical roles in the sensing of commensal microbiota, maintaining homeostasis, and regulating intestinal inflammation.³⁹ To communicate with the immune system, commensal microbes, similarly to pathogens, express microbial-associated molecular patterns that can be sensed by specialized receptors on a variety of cells, including immune, epithelial, and endothelial cells.⁴⁰ Stimulation of the immune system by antigens from colonizing microbiota is required to promote the development of the gut immune system.⁴¹ The lower abundance of these metabolic pathways in the AD group was associated with depletion of *A. muciniphila*, *R. gnavus*, and *Lachnospiraceae* bacterium 2_1_58FAA. These bacteria are known to degrade mucins in the gut mucus layer.^{42, 43} The ability of mucin-degrading bacteria to forage on diverse and abundant glycans present in the gut mucus may have a role in early colonization by providing bacteria with an endogenous source of nutrients before the introduction of dietary glycan.⁴⁴ Depletion of *A. muciniphila* in the gut microbiome of children with AD is reported in several studies.^{9, 45} Bacteria within *Lachnospiraceae* including *R. gnavus* can degrade complex polysaccharides into short-chain fatty acids that can be used for energy by the host.⁴⁶ Therefore, mucin-degrading bacteria provide nutrients to allow colonization of the gut by diverse microbes, and these bacteria can also influence immune development by influencing PI3K-Akt signaling, estrogen signaling, NOD-like receptor signaling, and antigen processing and presentation. These whole metagenome results clearly showed the roles of infant gut microbiome in AD according to feeding types with high resolution.²¹ A previous study also reported the importance of accurate identification of metabolic activities by subspecies level to understand the role of gut microbiome.¹⁷ Therefore, deep analyses such as whole

metagenome and metatranscriptome are needed for understanding the role of gut microbiome in the development of AD. Potential association of the colonizing gut microbiome with AD by present study was summarized in Fig. 5.

Generalization of the results in the present study could be difficult due to the relatively small number of samples used to generate microbiota and metagenomic data. However, the analyzed subjects were selected by propensity score matching to reduce sampling bias. In addition, this study analyzed 129 infants at the same age, and showed differences in gut microbiota according to feeding type in infants with AD using whole metagenome sequencing. Further studies will be necessary to clarify these findings, including the role of detected mucin-degrading bacteria in infants and longitudinal changes of gut microbiome according to alleviation or persistence of AD.

The present study suggests that the analysis of gut microbiome roles using functional gene analysis is more important than simple comparison of microbial community between control and AD. In conclusion, the colonization of mucin-degrading bacteria and their contribution to innate immune development in gut plays a crucial role in infants with AD. These studies will elucidate the interaction between the gut microbiome and the host immune system.

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516

517 **TABLE 1.** Clinical characteristics of control and AD infants.

Variables	Control (n=66)	Infants with AD (n=63)	<i>P</i> value
Sex, male, n (%)	36/66 (54.5)	33/63 (52.4)	.805
SCORAD	NA	23.5 ± 14.9 (n = 54)	NA
Delivery mode, vaginal delivery/caesarean delivery	47/19 (71.2/28.8)	45/18 (71.4/28.6)	.978
Administration of antibiotics in infancy, yes, n (%)	14/66 (21.2)	12/63 (19.0)	.759
Probiotics, yes, n (%)	10/66 (15.2)	10/63 (15.9)	.91
Parental history of allergic diseases, yes, n (%)	37/61 (60.7)	18/62 (29.0)	< .001
Atopy, n (%)	18/56 (32.1)	29/59 (49.2)	.064
Serum eosinophils (%)	2.7 ± 1.8 (n=54)	6.1 ± 4.4 (n=53)	< .001
Total serum IgE levels (kUA/L)	75.8 ± 166.8 (n=56)	73.1 ± 175.2 (n=11)	.934
Feeding			.956
Breast feeding, n (%)	25/66 (37.9)	23/63 (36.5)	
Mixed feeding, n (%)	34/66 (51.5)	34/63 (54.0)	
Formula feeding, n (%)	7/66 (10.6)	6/63 (9.5)	
Solid food intake (starting month)	5.59 ± 0.89	5.69 ± 0.65	.28

518 SCORAD: Scoring of Atopic Dermatitis.

TABLE 2. The comparison of subject characteristics according to enterotype.

Subject characteristic	BFGM1 (n=28)	BFGM2 (n=20)	<i>P</i> value	MFGM1 (n=41)	MFGM2 (n=27)	<i>P</i> value
Gender						
Male (%)	16 (57.1)	9 (45)	.16	21 (51.2)	17 (63.0)	.52
Female (%)	12 (42.9)	11 (55)	.83	20 (48.8)	10 (37.0)	.07
Delivery						
Vaginally (%)	25 (89.3)	17 (85)	.22	31 (75.6)	16 (59.3)	.03
C-section (%)	3 (10.7)	3 (15)	1	10 (24.4)	11 (40.7)	.83
Infant antibiotics (%)	5 (17.9)	5 (25)	1	10 (24.4)	5 (18.5)	.19
Infant probiotics (%)	4 (14.3)	3 (15)	.71	9 (22.0)	3 (11.1)	.08
Total IgE	48.19 ± 82.41	106.1 ± 208.87	.13	68.70 ± 92.73	81.02 ± 243.97	.36
Egg_IgE	2.08 ± 5.32	5.65 ± 13.45	.12	1.42 ± 3.96	0.59 ± 1.28	.39
Milk_IgE	0.43 ± 0.77	1.06 ± 3.00	.89	0.33 ± 0.56	0.51 ± 1.38	.24
Eosinophil	5.20 ± 4.81	4.79 ± 3.78	.61	4.24 ± 3.55	4.26 ± 2.43	.34
Family history (%)	18 (64.3)	10 (50)	.13	23 (56.1)	13 (48.2)	.09
Diagnosis of AD (%)	10 (35.7)	12 (60)	.67	15 (36.6)	7 (25.9)	.08
Solid food intake, n (%)						
Yes	19 (67.9)	14 (70)	.49	31 (75.6)	21 (77.8)	.21
No	7 (25)	5 (25)	.77	6 (14.6)	3 (11.1)	.51

Figure Legends

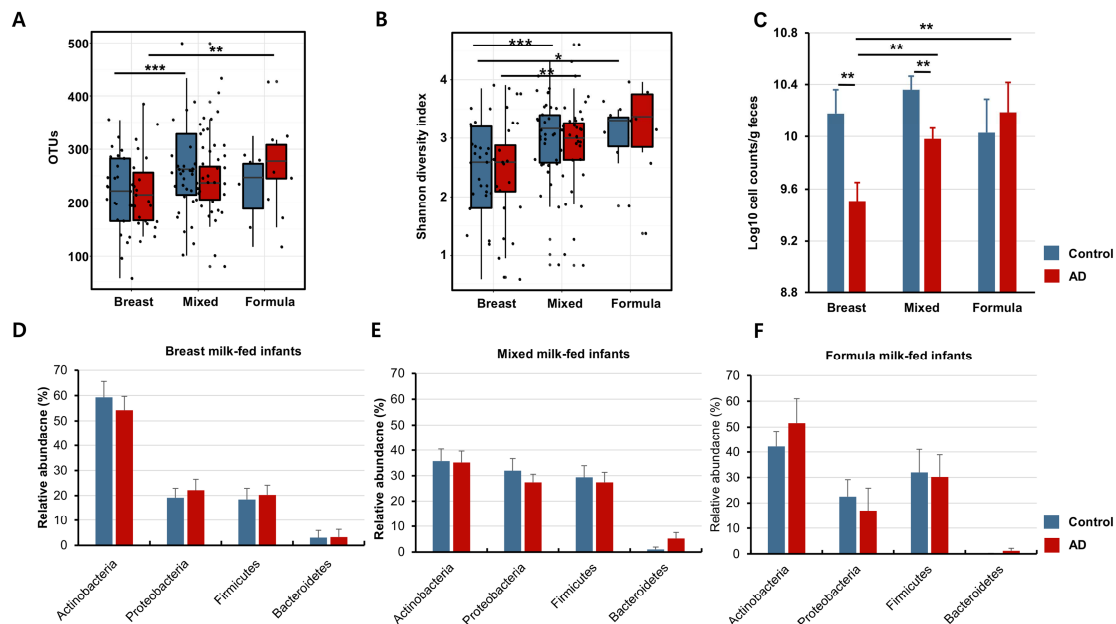
FIG 1. Comparison of bacterial diversity and phylum composition between control and AD infants according to feeding type. **(A)** The number of observed OTUs was compared after normalized read numbers for each sample. **(B)** The Shannon diversity indices were compared among groups. **(C)** The numbers of bacterial cells in feces was compared among groups. Cell numbers were calculated based on measurement of 16S rRNA gene copies, as described in the Methods. Phylum composition was compared between the control and AD group in **(D)** breast milk-fed infants, **(E)** mixed milk-fed infants, and **(F)** formula milk-fed infants. *** $P < .01$, ** $P < .05$, * $P < .1$.

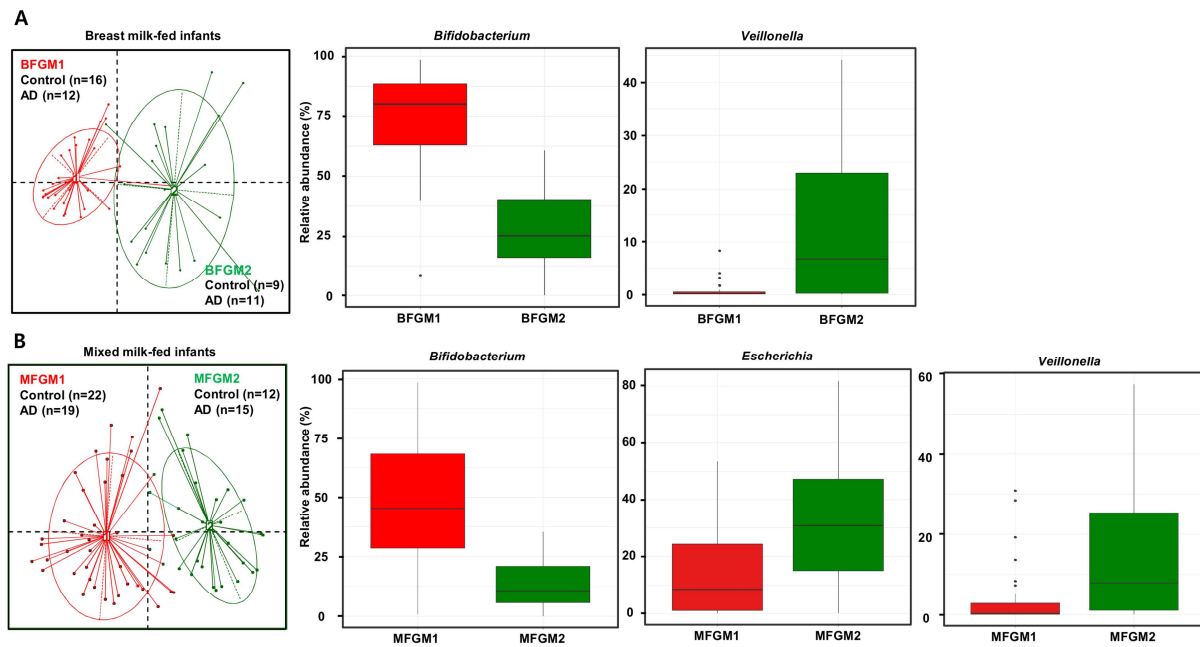
FIG 2. Enterotype clustering of gut microbiota from 6-month-old infants. **(A)** Two enterotypes were detected in the gut microbiota of breast milk-fed infants. *Bifidobacterium* was the dominant genus in BFGM1, whereas *Veillonella* was dominant in BFGM2. **(B)** Two enterotypes were detected in the gut microbiota of mixed milk-fed infants. *Bifidobacterium* was the dominant genus in MFGM1, whereas *Escherichia* and *Veillonella* were dominant in MFGM2.

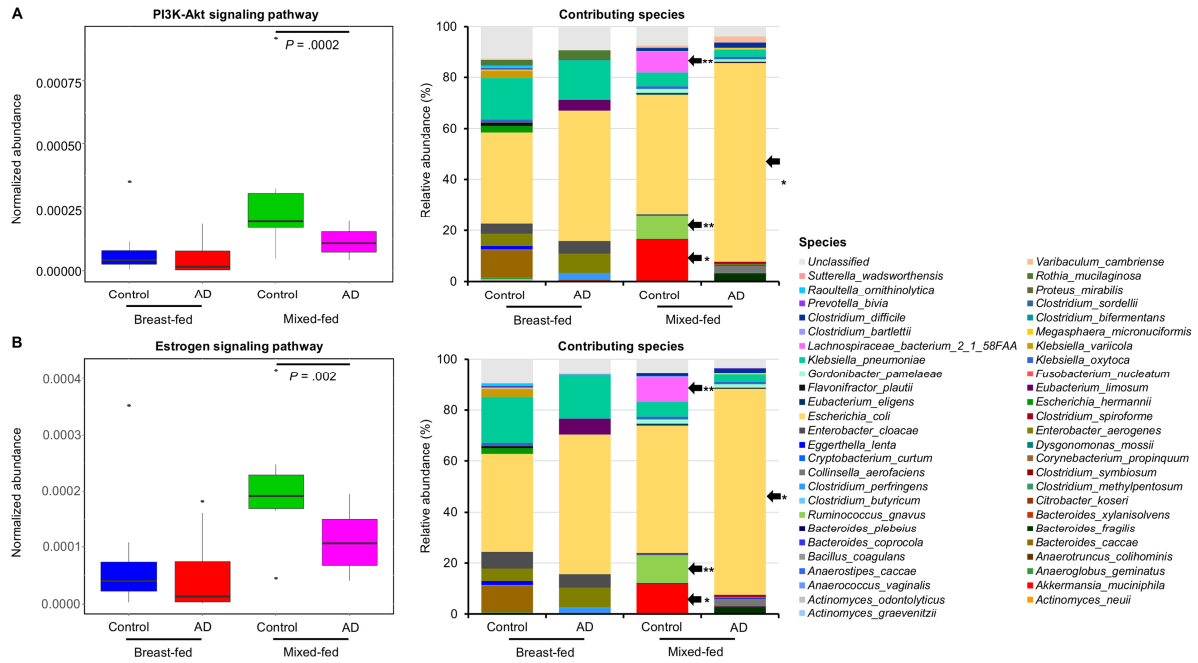
FIG 3. Comparison of metabolic pathways by whole metagenome analysis of gut microbiome in mixed milk-fed infants. **(A)** Comparison of functional genes related to PI3K-Akt signaling and their contributing species in control and AD infants. **(B)** Comparison of functional genes related to estrogen signaling and their contributing species in control and AD infants. ** $P < .05$, * $P < .1$.

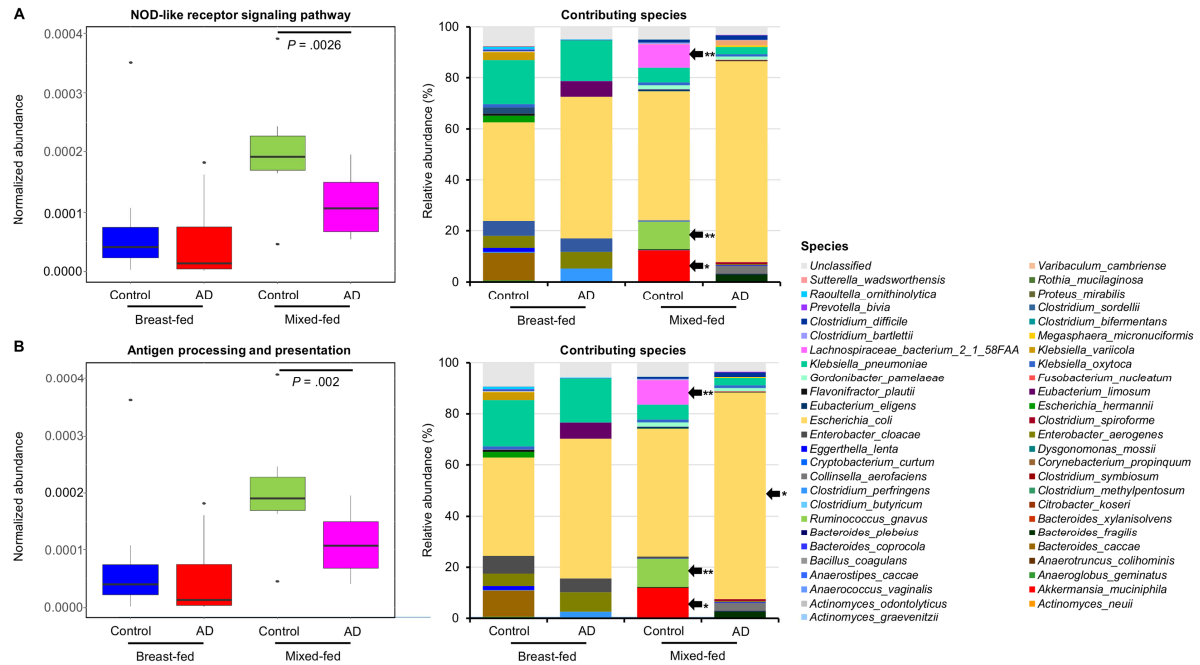
FIG 4. Comparison of significantly different pathways by whole metagenome analysis of gut microbiome in mixed milk-fed infants. **(A)** Comparison of genes related to NOD-like receptor signaling and their contributing species in control and AD infants. **(B)** Comparison of genes related to antigen processing and presentation and their contributing species in control and AD infants. $**P < .05$, $*P < .1$.

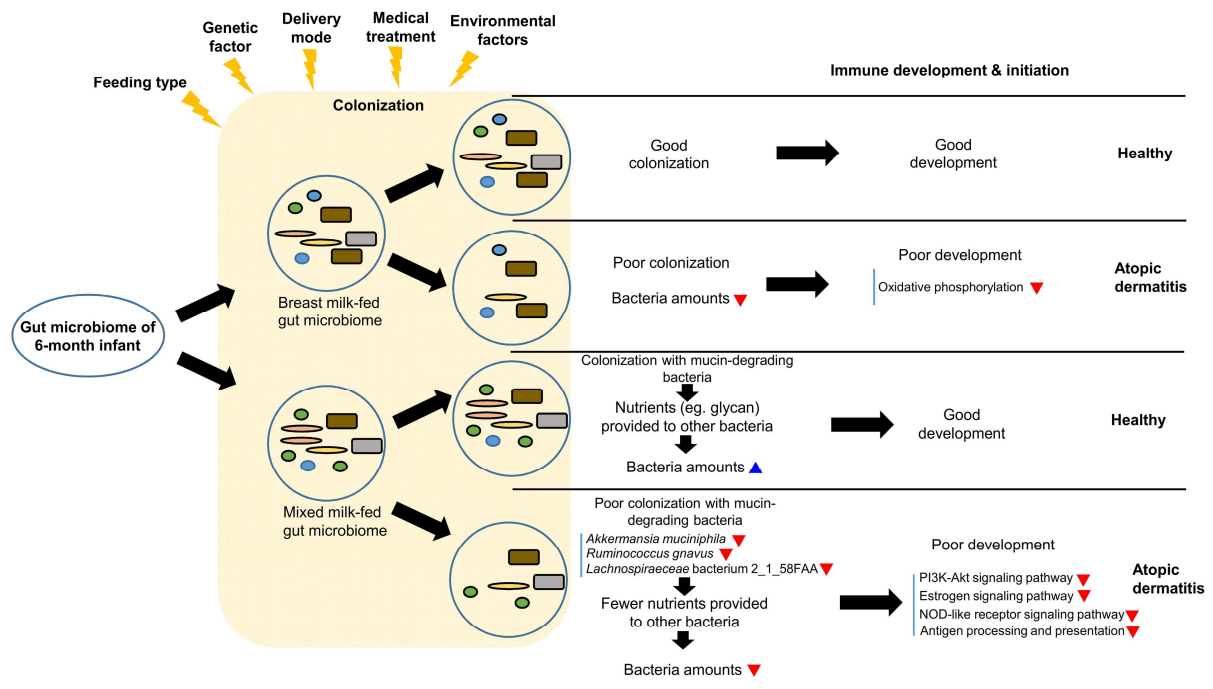
FIG 5. Potential model for the colonization of the gut microbiome and the development of atopic dermatitis.











Supplementary documents

Perturbations of the gut microbiome genes in infants with atopic dermatitis according to feeding type

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23

24 **Supplementary Methods**25 **Study subjects and sampling**

26 Study subjects consisted of 129 6-month-old infants (66 healthy controls and 63 infants
27 with AD) from the longitudinal Cohort for Childhood Origin of Asthma and Allergic Diseases
28 (COCOA) birth cohort study.^{E1} In COCOA, women in the third trimester of pregnancy were
29 recruited from 5 tertiary hospitals and 8 public health centers for prenatal care; all centers are
30 located in Seoul, Korea. Recruitment took place between August 2007 and August 2015.
31 A woman was recruited if she (1) lacked high-risk conditions that could affect the
32 development of allergic diseases in the child (e.g., diabetes, preeclampsia, anemia, and severe
33 infection), (2) planned to deliver at an affiliated medical center, and (3) was a resident of
34 Seoul. The eligibility of the baby was determined soon after delivery by collaborative efforts
35 of obstetricians and pediatricians. Babies were excluded at birth if (1) their gestational age
36 was less than 37 weeks or (2) they had any major congenital anomalies or birth asphyxia that
37 required oxygen supplementation.

38 This study was approved by the Institutional Review Boards of Asan Medical Center (IRB
39 no. 2008-0616), Samsung Medical Center (IRB No. 2009-02-021), Severance Hospital (IRB
40 No. 4-2008-0588), the CHA Medical Center (IRB No. 2010-010), and the Seoul National
41 University Hospital (IRB No. H-1401-086-550). Written informed consent was obtained from
42 the parents of each infant.

43 AD in infants was diagnosed by a pediatric allergist according to Hanifin and Rajka's
44 criteria^{E2} at 6 months and 12 months of age, and severity of physical symptoms were
45 assessed by the SCORAD (Scoring Atopic Dermatitis) index at 6 months. Measurement of
46 total IgE and egg- and milk-specific serum IgE at 12 months of age was performed by

fluorescent enzyme immunoassay (AutoCAP system, Phadia AB, Uppsala, Sweden) on all subjects. Atopy was defined as the presence of specific IgE for eggs or milk.

For microbiota analysis, subjects were selected by propensity score matching to contrast the microbiome profiles of AD samples and control samples that have similar propensities for atopic diseases.^{E3,4} A logistic regression model using gender, delivery type, nursing type, and intake of antibiotics or probiotics as independent variables and the presence or absence of AD as a response variable was used to calculate the propensity scores. This method was used to match AD samples with control samples with the most similar propensity scores. Only the samples with the same antibiotic and probiotic application status were used, because the effects of antibiotics and probiotics on microbiome profiles could be especially significant. The clinical characteristics of the groups are shown in Table 1. Fecal samples were collected from frozen diapers of each infant. Each center transported the diapers in ice-boxes to Asan Medical Center within 4 hours, which were immediately stored at -80°C before being processed for DNA extraction.

Metagenomic DNA extraction and pyrosequencing

Metagenomic DNA was extracted from 250 mg of feces using the Power Microbiome RNA/DNA Isolation kit (Mo Bio/Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions, with additional glass bead-beating steps on a vortex adapter. The extracted DNA was quantified using a BioPhotometer D30 with a μ Cuvette G1.0 (Eppendorf, Hamburg, Germany) and stored at -80°C until further processing.

For pyrosequencing, the extracted DNA was amplified using barcoded primers targeting the V1-V3 region of the 16S rRNA gene (Table E3). The amplification was conducted using a

C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) as follows: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 7 min. The amplified products were purified using an AMPure XP bead kit (Agencourt Bioscience, Beverly, MA, USA) and quantified using the PicoGreen dsDNA assay kit (ThermoFisher Scientific, Waltham, MA, USA). Equimolar concentrations of samples were pooled and then amplified on sequencing beads by emulsion PCR. Beads recovered from the emulsion PCR were deposited on a 454 picotiter plate and sequenced on a Roche/454 FLX Titanium system following the manufacturer's instructions.

Quantitative real-time PCR

The total amount of gut bacteria in fecal samples was determined by quantitative real-time PCR for 16S rRNA genes. The 16S rRNA gene was amplified with the primers 340F (5'-TCC TAC GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') using a Thermal Cycle Dice Real-Time System III (Takara Bio, Inc., Shiga, Japan). Each sample was measured triplicate in a 25 µL reaction containing 12.5 µL of SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, Japan), 2 µM of each primer, and 1 µL of DNA template (a ten-fold dilution series of sample DNA). The amplification conditions were as follows: 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing at 60°C for 30 sec. Standard curves were generated from a parallel reaction of serial dilutions (1×10^2 – 1×10^8) of the 16S rRNA gene from the *Escherichia coli* K12 w3110 strain. Regression coefficients (r^2) for all standard curves were ≥ 0.98 . The measured rRNA gene copy numbers were divided by 4.2 (the average copy number of the 16S rRNA gene in bacteria)

for estimation of the total cell number.^{E5} Differences between samples were analyzed by the Mann-Whitney test using R software (ver. 3.2.0). Results with *P*-values < .05 were considered statistically significant.

Pyrosequencing analysis

Pyrosequencing reads were analyzed as previously described.^{E6} The raw sequencing reads were de-multiplexed and low-quality sequences (average quality score < 25 or read length < 300 bp) were trimmed for further analysis. Chimeric sequences were removed using the UCHIME software.^{E7} The trimmed sequences were clustered, and representative sequences in each cluster were identified using a Basic Local Alignment Search Tool (BLAST) search of the EzTaxon-e database.^{E8} The sequence reads were clustered into operational taxonomic units (OTUs) based on a 97% nucleotide similarity. The diversity indices, Good's coverages, and rarefaction curves were calculated using MOTHUR after random subsampling.^{E9} Principal coordinate analysis (PCoA) was performed to compare the microbiota among samples based on the Fast-UniFrac distance. The Mann-Whitney U-test was used to identify statistically significant differences using R software (Version 3.3.2).

For enterotype clustering of gut microbiota, Weighted UniFrac, Unweighted UniFrac, Bray-Curtis, Jensen-Shannon divergence, and Root Jensen-Shannon divergence for the relative abundance of genera was calculated.^{E10,11} Then, the Partitioning Around Medoids method was used to cluster the abundance profiles using the R package 'cluster.' The optimal number of clusters and the cluster quality were determined by the Calinski-Harabasz pseudo F-statistic (CH) index and the Rousseeuw's Silhouette internal cluster quality index (SI) for each distance matrix using the R package 'clusterSim.' We compared index scores among different distance metrics (Table E4). Clusters of gut microbiota were plotted by PCoA using the R

package ‘ade4.’ Detailed analysis was conducted following the manual at <http://enterotype.embl.de/index.html>. Significantly different microbial members between clusters were analyzed again by the linear discriminant analysis effect size (LEfSe).^{E12} A significant alpha of 0.05 and an effect size threshold of 2 were used to identify significant differences between groups.

Whole metagenome sequencing and analysis

Forty samples were randomly selected (20 control and 20 AD samples) according to feeding type for whole metagenome sequencing. Metagenomic DNA from selected samples was fragmented using a Covaris M220 sonicator (Covaris, Inc., Woburn, MA, USA), and metagenomic libraries were prepared using the TruSeq DNA sample preparation kit according to the manufacturer’s instructions (Illumina, San Diego, CA, USA). The size of the prepared libraries was confirmed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Equimolar concentrations of each library (2 nM) were calculated by quantitative real-time PCR using a CFX 96 Real-Time System (Bio-Rad, Hercules, CA, USA) with Illumina adapter sequence primers. Sequencing was performed using the Illumina HiSeq 2500 system (250 bp paired ends).

Sequences from the HiSeq 2500 sequencer were trimmed using the Nsoni clip tool within the Nsoni high-throughput sequencing data analysis toolset (ver. 0.127, <http://www.vicbioinformatics.com/software.nsoni.shtml>) to remove the Illumina adapter sequences and sequences with short read lengths (< 150 bp in each pair) and low Q scores (< 15). Contaminating human genes were removed using BMap (<http://sourceforge.net/project/bbmap>) by mapping to a reference human genome. Functional profiling of metagenomic sequences was performed using HUMAnN2, which uses pan-

genome mapping sequences to functionally annotate species pangenomes determined by taxonomic profiling from MetaPhlAn2.^{E13,14} The resulting functional profiles were obtained in terms of gene family abundance at the community level in reads per kilobase to normalize for gene length. The resulting UniRef50 IDs were converted to the KO (KEGG Orthology) based on the matching K numbers (<http://www.genome.jp/kegg/kegg2.html>). Converted KO categories were compared among groups.

Statistical analysis

Relative risk (RR) ratios and corresponding 95% confidence intervals were calculated using R software. Unadjusted and adjusted RRs were calculated by log-binomial regression using maximum likelihood estimation when prevalence ratios were near 1.0 or when the log-binomial model did not converge. *P*-values were calculated by one-way ANOVA.

Significantly different KO categories among groups were identified using the Kruskal-Wallis rank-sum test using the 'kruskal.test' function in R.^{E15} Significant pathways had a *P*-value < .05. Furthermore, differences between bacterial taxa among groups were tested by Kruskal-Wallis rank-sum test using the 'kruskal.test' function in R. The Kruskal-Wallis rank-sum test was used to compare the frequency of each taxa among groups, and *P*-values were calculated using the approximate chi-squared distribution. After identifying taxa with significantly different frequencies among groups, Dunn's test was used to identify pairwise differences between groups within the same feeding type.^{E16} Dunn's test was performed using the 'dunn.test' package in R, and the approximate normal distribution was used to calculate *P*-values. Corrections were made using the Benjamini-Hochberg False Discovery Rate

multiple testing correction.^{E17}

Supplementary Results

Comparison of the diversity and composition of the gut microbiome between controls and AD infants

The gut microbiomes of 6-month-old infants did not cluster by disease status in a PCoA plot based on the Fast-UniFrac distance (Fig E2). The variations in gut microbiota within and between groups were next compared by the unweighted UniFrac distance (Fig. E2). This analysis revealed that the microbiota in control infants was more highly varied than in AD infants. The inter-group variation in microbiota between control and AD infants was higher than the intra-group variation in AD infants ($P < .001$).

The influence of feeding type on gut microbiota in 6-month-old infants

At the genus level, six genera were common in at least two feeding types in both controls and AD infants (Fig E3). *Bifidobacterium* was the predominant genus in both controls and AD infants within all feeding groups. Nevertheless, the proportion of *Bifidobacterium* was higher in the gut microbiota of breast-fed infants ($56.78 \pm 6.52\%$ in controls and $53.25 \pm 5.56\%$ in AD infants) than in other feeding types. The relative abundance of *Escherichia* was higher in the gut microbiota of mixed-fed infants ($21.29 \pm 4.05\%$ in controls and $22.13 \pm 3.22\%$ in AD infants) than in other feeding groups. However, there were no significant differences in microbe composition between control and AD infants at the genus level in any

feeding group ($P > .05$).

Different gut microbiota within the same feeding type

Enterotype clustering based on weighted UniFrac distance was performed to compare with enterotypes by Jensen-Shannon divergence (Fig E8).^{E11} Two enterotypes were also found in both breast-fed and mixed-fed infants in enterotype clustering by weighted UniFrac distance, and the dominant genera in each cluster were the same as the result of enterotype clustering based on Jensen-Shannon divergence (Fig. 2).

The characteristics of subjects were compared between enterotypes within each feeding group (Table 2). There were no significant difference in total IgE, egg IgE, and milk IgE measurements between enterotypes within each feeding group ($P > .1$). Furthermore, there were no significant differences of gut microbiome diversity and composition between sensitized infants with AD and healthy non-sensitized infants (Fig E9). However, vaginal delivery was preferentially associated with *Bifidobacterium*-abundant MFGM1 enterotype compared to MFGM2 ($P = .03$). Although the proportion of *Bacteroides* tended to be higher in gut microbiota of infants delivered vaginally than those delivered with C-section, the difference between groups was not statistically significant ($P > .05$; Fig E10). In addition, there were no significant differences in RR ratios between two enterotypes within the same feeding type (Table E2).

Functional profiles of gut microbiota in control and AD infants by whole metagenome analysis

The characteristics of subjects for whole metagenome analysis are shown in Table E5. The

average proportions of functional genes among groups were compared by KO category, and KO categories with a P -value $< .05$ (obtained by Kruskal-Wallis test and by pairwise comparison with the Mann-Whitney test) were considered significantly different between groups. The relative abundances of the KO categories differed according to feeding type.

Supplementary Discussion

A simple comparison between control and AD samples revealed no significant differences in the members of the gut microbiota (Fig E1). It was reported that *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* are less abundant in the gut microbiota of infants with AD, whereas *Clostridium* is more abundant.^{E18,19} The lower abundance of *Ruminococcaceae* in gut microbiome of 1 week age infant was associated with the onset of IgE-associated eczema over 2.5 years in predisposed children.^{E20} Lower richness of gut microbiota and an elevated *Enterobacteriaceae/Bacteroidaceae* ratio in early infancy were associated with development of food sensitization.^{E20,21} However, the high abundance of *Faecalibacterium prausnitzii* could be associated with eczema by leading to reduced butyrate and propionate, and some studies have found no significant differences of gut microbiota between control and AD.^{E22,23} In addition, the gut microbiota obtained from infants of age 3 to 6 months did not significantly differed by atopic dermatitis severity in a milk allergy study.^{E24} These inconsistencies could be due to the differences in detection methods across studies, as well as differences in other factors affecting the gut microbiota such as host genotype, mode of delivery, age, and feeding type.^{E25,26} In particular, the age of studied subjects is important for accurately analyzing the difference between groups. *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* were depleted in samples obtained at 3 months of age in infants who exhibited wheezing and positive allergen skin prick testing at 12 months. However, this

perturbation was not detected in fecal samples obtained at the 12-month time point.^{E27,28}

Therefore, infants with the same age were analyzed in present study.

Differences in nutrition due to feeding type may be a key factor that shapes the developing gut microbiota.^{E29,30} Therefore, samples were divided according to feeding type, and differences in the gut microbiota by feeding type were found within both the control and the AD group (Fig 1). The relative abundance of *Bifidobacterium* within Actinobacteria was higher in the gut microbiota of controls than in AD infants, and was higher in breast-fed samples than in mixed-fed and formula-fed samples (Fig E3). The predominance of *Bifidobacterium* in the gut microbiota of breast-fed infants has been linked to breast milk oligosaccharides, and this bacteria has been shown to correlate with a reduced risk of allergic disease.^{E31} Although the composition of the gut microbiota differed according to feeding type, no significant differences in microbial composition were found between control and AD infants ($P > .05$). Therefore, differences in microbiota within each feeding group were detailed by enterotype analysis.

Bifidobacterium was dominant in both breast-fed and mixed-fed infants, since the human milk oligosaccharides were also present in mixed-fed infants. Although *Staphylococcus* was more abundant in the *Bifidobacterium*-dominated enterotypes BFGM1 and MFGM1, the proportions were relatively low (0.04-0.12%). *Staphylococcus* can adhere to human colonic mucus, but can be displaced by lactic acid bacteria during colonization of the gut microbiota.^{E32} *Escherichia/Veillonella* are dominant in the microbiota during early infancy. *Escherichia* can remain highly abundant during the first 6 months of life, but decreases after 6 months.^{E33} *Veillonella* obtains energy from lactic acid produced by *Streptococcus* as an end product of carbohydrate fermentation.^{E34,35} Species belonging to the genus *Veillonella* use lactate to produce propionate rather than butyrate. Propionate decreases mucin synthesis and

increases tight junction permeability.^{E36} The interaction between *Streptococcus* and *Veillonella* can influence human immune homeostasis by immunomodulatory mechanisms; thus, *Klebsiella* and *Clostridium* were more abundant in *Escherichia/Veillonella*-dominant gut microbiota.^{E37,38} Increased colonization of the gut by *Klebsiella* and *Clostridium* has been found in infants with colic.^{E39} Although association of vaginal delivery was found between MFGM1 and MFGM2 enterotype, there were no differences of gut microbiota between controls and AD infants (Table 2 and Fig E10). Therefore, we analyzed the differences of functional genes in gut microbiome between control and AD groups using whole metagenome sequencing.

The present study demonstrates a potential association between the composition of the infant gut microbiome at 6 months and atopic dermatitis (Fig. 5). The gut microbiota of 6-month-old infants is dynamic and can be affected by various factors including host genotype, delivery mode, feeding type, and medical history. The composition of the gut microbiota did not differ in patients with or without AD, but did differ according to feeding types. The gut microbiota could be categorized into a Gram-positive *Bifidobacterium*-dominated composition and a Gram-negative *Escherichia/Veillonella*-dominated composition in breast-fed and mixed-fed infants. In breast-fed infants, reduced bacterial colonization in the AD group may have influenced the level of oxidative phosphorylation, which is related to priming the immune system. In mixed-fed infants, reduced colonization by mucin-degrading bacteria (particularly *A. muciniphila*, *R. gnavus*, and *Lachnospiraceae* bacterium 2_1_58FAA) may have led to a deficiency in nutrients generated from mucin degradation and utilized by other gut bacteria, resulting in reduced colonization of the gut in the AD group. In addition, these differences in colonization by mucin-degrading bacteria were associated with differences in the genes involved in PI3K-Akt signaling, estrogen signaling, NOD-like receptor signaling, and antigen processing and presentation, all of which may affect immune

development in infants with AD.

In the present study, the difference of gut microbiome and their function between control and AD groups were clearly observed in whole metagenome analysis. The composition analysis based on 16S rRNA gene is appropriate for comparing large number of samples, but provides low resolution of taxonomic profiles.^{E40} This analysis cannot be classified at the species level. However, whole metagenome sequencing can provide significantly higher resolution of specific taxonomic (at strain level) and functional classification of sequences.^{E41} A previous study also reported that the dysbiosis of *Faecalibacterium prausnitzii* subspecies level in gut microbiome was associated with AD.^{E22}

In addition, the temporal association of gut microbiota with disease development was important for identifying the causative factors. Several studies analyzed the temporal association of gut microbiota with the development of AD with T cell differentiation and metabolic alteration.^{E18,20,27} Although these studies identify the association of gut microbiome with disease development, they used low resolution analysis based on 16S rRNA gene analysis without functional gene analysis of microbiota. Therefore, further studies with high resolution analytic methods such as whole metagenome and metatranscriptome are needed to provide sufficient detail on the temporal association of gut microbiome with the development of AD.

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Supplementary Figure Legends

FIG E1. Comparison of bacterial diversity and bacterial composition between control and AD samples regardless of feeding type. **(A)** The number of observed OTUs was compared after normalized read number for each sample. **(B)** The Shannon diversity indices were compared between groups. **(C)** The number of bacterial cells in the feces was compared between groups. Cell numbers were calculated based on 16S rRNA gene copy numbers, as described in the Methods. **(D)** Comparison of phylum composition between control and AD samples. **(E)** Comparison of genus composition between control and AD samples. *** $P < .01$.

FIG E2. Principal coordinate analysis (PCoA) of 6-month gut microbiota from control and AD infants. Intra-group and inter-group differences in microbiota composition were compared according to unweighted UniFrac distances. *** $P < .01$.

FIG E3. Comparison of genus composition between control and AD samples according to feeding type.

FIG E4. Different taxa between BFGM1 and BFGM2 enterotypes identified by linear discriminant analysis coupled with effect size (LEfSe). **(A)** Differences in detected taxa between enterotypes. **(B)** Comparison of different genera between enterotypes by boxplot based on the relative abundance.

FIG E5. Different taxa between MFGM1 and MFGM2 enterotypes identified by linear discriminant analysis coupled with effect size (LEfSe). **(A)** Differences in detected taxa

between enterotypes. **(B)** Comparison of different genera between enterotypes by boxplot based on the relative abundance.

FIG E6. Comparison of results on the composition of the gut microbiota obtained by 16S rRNA gene pyrosequencing and whole metagenome sequencing.

FIG E7. Comparison of the relative abundance of functional genes related to oxidative phosphorylation and their contributing species between breast-fed control and AD infants.

FIG E8. Enterotype clustering of gut microbiota based on weighted UniFrac distance. **(A)** Two enterotypes were detected in the gut microbiota of breast-fed infants. *Bifidobacterium* was the dominant genus in Group B1, whereas *Veillonella* was dominant in Group B2. **(B)** Two enterotypes were detected in the gut microbiota of mixed-fed infants. *Bifidobacterium* was the dominant genus in Group M1, whereas *Escherichia* and *Veillonella* were dominant in Group M2.

FIG E9. Comparison of gut microbiota between sensitized infants with AD and healthy non-sensitized infant for detected IgE to egg/milk. **(A)** Comparison of diversity index between sensitized infants with AD and healthy non-sensitized infants. **(B)** Comparison of diversity between sensitized infants and healthy non-sensitized infants according feeding types. **(C)** Comparison of phylum composition between groups. **(D)** Comparison of genus composition between groups. All analyzed results were not statistically significant ($P > .05$).

501 **FIG E10.** Comparison of *Bacteroides* proportions between gut microbiota of infants
502 delivered vaginally and those of infants delivered with C-section. **(A)** Comparison of
503 *Bacteroides* proportion between infants delivered vaginally and infants delivered with C-
504 section in MFGM1 and MFGM2. **(B)** Comparison of *Bacteroides* proportion between control
505 and AD groups within MFGM1 and MFGM2.

TABLE E1. Summary of diversity indices obtained from gut microbiota

Sample	Group	Analyzed	Normalized	Observed	Estimated	Shannon	Good's
		reads	reads			diversity index	
CB_1	control	13,643	5,000	305	651.92	3.24	0.97
CB_2	control	11,737	5,000	318	695.08	3.25	0.97
CB_3	control	5,107	5,000	199	461.65	3.13	0.98
CB_4	control	10,465	5,000	355	834.89	3.26	0.96
CB_5	control	17,940	5,000	288	669.49	2.84	0.97
CB_6	control	15,248	5,000	268	543.65	3.2	0.97
CB_7	control	20,154	5,000	193	411.5	1.2	0.98
CB_8	control	15,963	5,000	222	532.56	1.8	0.97
CB_9	control	16,048	5,000	243	696.25	1.81	0.97
CB_10	control	13,599	5,000	298	841.1	2.19	0.96
CB_11	control	19,279	5,000	125	550.38	0.59	0.98
CB_12	control	8,234	5,000	95	164.46	1.28	0.99
CB_13	control	3,127	3,127	58	71	2.82	0.99
CB_14	control	13,131	5,000	127	318.15	1.83	0.99
CB_15	control	10,006	5,000	301	571.09	3.86	0.97
CB_16	control	11,545	5,000	246	548.88	2.74	0.97
CB_17	control	13,334	5,000	161	396.59	1.25	0.98
CB_18	control	6,464	5,000	207	401.13	2.3	0.98
CB_19	control	7,959	5,000	195	442.5	2.56	0.98
CB_20	control	7,452	5,000	248	469.43	2.6	0.98
CB_21	control	9,249	5,000	135	325.59	1.19	0.98
CB_22	control	7,454	5,000	194	400.65	2.67	0.98

CB_23	control	12,744	5,000	165	255.23	2.1	0.99
CB_24	control	10,401	5,000	224	456.87	3.52	0.98
CB_25	control	15,007	5,000	283	457.49	3.76	0.98
CM_1	control	11,611	5,000	101	255	1.27	0.99
CM_2	control	8,442	5,000	288	594.15	3.18	0.97
CM_3	control	10,761	5,000	371	794.4	3.57	0.96
CM_4	control	11,416	5,000	222	456.19	3.02	0.98
CM_5	control	10,908	5,000	374	712.43	3.06	0.96
CM_6	control	13,465	5,000	296	639.18	2.56	0.97
CM_7	control	14,857	5,000	337	802	3.34	0.96
CM_8	control	23,034	5,000	346	940.16	3.33	0.96
CM_9	control	9,598	5,000	335	603.33	3.72	0.97
CM_10	control	8,198	5,000	273	726.91	3.3	0.97
CM_11	control	13,999	5,000	406	979.57	4.07	0.96
CM_12	control	11,262	5,000	102	194.81	0.84	0.99
CM_13	control	10,607	5,000	180	353.25	1.83	0.98
CM_14	control	6,917	5,000	262	502.17	3.05	0.97
CM_15	control	5,687	5,000	307	648	3.35	0.97
CM_16	control	9,982	5,000	123	378.6	1.01	0.99
CM_17	control	11,787	5,000	281	625.56	2.9	0.97
CM_18	control	5,757	5,000	304	558.29	4.07	0.97
CM_19	control	6,054	5,000	356	606.28	4.32	0.97
CM_20	control	7,721	5,000	145	239.92	1.83	0.99
CM_21	control	6,284	5,000	245	557.62	3.06	0.97
CM_22	control	11,938	5,000	259	533.31	3.29	0.97
CM_23	control	5,335	5,000	205	433.39	2.64	0.98
CM_24	control	7,747	5,000	288	538.28	3.4	0.97

CM_25	control	7,301	5,000	263	547.69	3.52	0.97
CM_26	control	7,403	5,000	254	496.45	3.33	0.97
CM_27	control	12,246	5,000	254	417.88	3.14	0.98
CM_28	control	11,298	5,000	194	297.78	2.1	0.98
CM_29	control	8,707	5,000	212	563.48	2.55	0.98
CM_30	control	7,721	5,000	359	690.9	3.82	0.96
CM_31	control	18,329	5,000	231	507.12	2.98	0.98
CM_32	control	18,089	5,000	238	562.55	3.31	0.98
CM_33	control	8,918	5,000	433	734.04	3.78	0.96
CM_34	control	19,307	5,000	175	271.28	2.56	0.98
CF_1	control	12,500	5,000	289	491.52	3.39	0.97
CF_2	control	9,594	5,000	257	478.43	3.33	0.98
CF_3	control	4,686	4,686	117	134	3.5	0.99
CF_4	control	11,107	5,000	324	776.5	3.3	0.96
CF_5	control	9,261	5,000	171	353	1.85	0.98
CF_6	control	6,867	5,000	247	450.5	3.15	0.98
CF_7	control	7,177	5,000	207	425.4	2.57	0.98
AB_1	AD	12,188	5,000	215	482.96	2.19	0.98
AB_2	AD	14,182	5,000	385	940.66	3.91	0.96
AB_3	AD	11,111	5,000	322	808.75	3.5	0.96
AB_4	AD	12,625	5,000	231	587.44	2.6	0.97
AB_5	AD	14,379	5,000	286	746.29	2.89	0.96
AB_6	AD	12,486	5,000	284	602	2.64	0.97
AB_7	AD	13,793	5,000	230	500.93	2.06	0.97
AB_8	AD	22,559	5,000	172	437.79	0.95	0.98
AB_9	AD	9,917	5,000	346	740.53	2.79	0.96
AB_10	AD	13,119	5,000	203	331.56	2.19	0.98

AB_11	AD	6,860	5,000	164	403.25	2.58	0.98
AB_12	AD	10,319	5,000	165	380.53	2.03	0.98
AB_13	AD	8,266	5,000	195	393.68	2.74	0.98
AB_14	AD	13,860	5,000	160	370	2.07	0.98
AB_15	AD	14,711	5,000	231	461	3.54	0.98
AB_16	AD	17,927	5,000	198	498.13	2.11	0.98
AB_17	AD	11,184	5,000	136	291.08	2.25	0.99
AB_18	AD	8,621	5,000	234	399.37	3.76	0.98
AB_19	AD	12,425	5,000	139	354	0.63	0.98
AB_20	AD	12,637	5,000	168	350.29	2.61	0.98
AB_21	AD	13,114	5,000	247	493.03	2.86	0.98
AB_22	AD	19,219	5,000	153	348	1.34	0.98
AB_23	AD	18,959	5,000	265	525.04	3.28	0.98
AM_1	AD	8,008	5,000	357	1,145.43	3.52	0.96
AM_2	AD	12,959	5,000	223	465.03	2.63	0.98
AM_3	AD	14,866	5,000	265	587	2.64	0.97
AM_4	AD	8,824	5,000	238	506.89	2.38	0.98
AM_5	AD	9,767	5,000	216	639.67	2.8	0.98
AM_6	AD	6,366	5,000	265	569.94	3.27	0.97
AM_7	AD	16,158	5,000	226	605.62	1.87	0.97
AM_8	AD	13,738	5,000	273	579	2.63	0.97
AM_9	AD	18,111	5,000	303	717.55	2.87	0.96
AM_10	AD	7,338	5,000	233	377.29	2.98	0.98
AM_11	AD	6,977	5,000	389	791.02	3.86	0.96
AM_12	AD	5,107	5,000	80	155.25	0.83	0.99
AM_13	AD	9,098	5,000	185	347	2.9	0.98
AM_14	AD	14,522	5,000	203	485.44	2.93	0.98

AM_15	AD	11,335	5,000	154	248.67	2.43	0.99
AM_16	AD	6,617	5,000	236	507.44	2.94	0.98
AM_17	AD	5,552	5,000	166	309.64	3.01	0.98
AM_18	AD	7,729	5,000	307	541.5	3.79	0.97
AM_19	AD	11,365	5,000	172	333	2.02	0.98
AM_20	AD	10,109	5,000	218	453.44	3.02	0.98
AM_21	AD	7,608	5,000	250	474.29	3.08	0.98
AM_22	AD	6,273	5,000	499	1,112.04	4.61	0.95
AM_23	AD	7,344	5,000	260	530.19	3.13	0.97
AM_24	AD	5,341	5,000	268	522.09	3.46	0.97
AM_25	AD	9,485	5,000	260	576.55	3.22	0.97
AM_26	AD	6,974	5,000	328	724.08	3.54	0.97
AM_27	AD	13,288	5,000	203	393	2.36	0.98
AM_28	AD	6,942	5,000	246	442.18	3.15	0.98
AM_29	AD	7,736	5,000	182	364.05	2.21	0.98
AM_30	AD	14,631	5,000	268	520.39	3.53	0.97
AM_31	AD	16,572	5,000	182	315.61	2.54	0.98
AM_32	AD	12,686	5,000	227	530.18	3.15	0.98
AM_33	AD	20,377	5,000	257	525.89	3.63	0.98
AM_34	AD	10,802	5,000	237	489.12	3.2	0.98
AF_1	AD	6,007	5,000	280	600.04	3.97	0.97
AF_2	AD	7,880	5,000	317	639.14	2.76	0.97
AF_3	AD	9,339	5,000	275	541.03	3.63	0.97
AF_4	AD	6,680	5,000	427	1,193.41	3.79	0.95
AF_5	AD	7,405	5,000	235	526.67	3.1	0.97
AF_6	AD	12,290	5,000	153	267	1.37	0.98

OTUs: operational taxonomic units; CB: controls in breast feeding; CM: controls in mixed feeding;

CF: controls in formula feeding; AB: AD in breast feeding; AM: AD in mixed feeding;

AF: AD in formula feeding

TABLE E2. Comparison of relative risk ratios between enterotypes.

	Gut Microbiota Type				RR (95% CI), <i>P</i> value	
	BFGM1	BFGM2	MFGM1	MFGM2	BFGM1 versus	MFGM1 versus
	(n=28)	(n=20)	(n=41)	(n=27)	BFGM2	MFGM2
Vaginal delivery	25 (89.3)	17 (85)	31 (75.6)	16 (59.3)		
C-section	3 (10.7)	3 (15)	10 (24.4)	11 (40.7)	0.34 (0.31-6.24) <i>P</i> =0.66	0.51 (0.83-3.38) <i>P</i> =0.15
Infant antibiotics	5 (17.9)	5 (25)	10 (24.4)	5 (18.5)	0.34 (0.45-4.20) <i>P</i> =0.55	-0.23 (0.29-1.98) <i>P</i> =0.60
Infant probiotics	4 (14.3)	3 (15)	9 (22.0)	3 (11.1)	0.05 (0.26-4.18) <i>P</i> =0.95	-0.68 (0.15-1.70) <i>P</i> =0.30
Egg_IgE (>0.35 IU ml ⁻¹)	7 (25)	9 (45)	15 (36.6)	4 (14.8)	0.64 (0.87-4.17) <i>P</i> =0.11	-0.90 (0.15-1.09) <i>P</i> =0.07
Milk_IgE (>0.35 IU ml ⁻¹)	7 (25)	6 (30)	7 (17.1)	4 (14.8)	0.18 (0.47-3.03) <i>P</i> =0.70	-0.14 (0.28-2.68) <i>P</i> =0.81
Family history	18 (64.3)	10 (50)	23 (56.1)	13 (48.2)	-0.25 (0.46-1.31) <i>P</i> =0.34	-0.15 (0.53-1.38) <i>P</i> =0.53
Diagnosis of AD	10 (35.7)	12 (60)	15 (36.6)	7 (25.9)	0.52 (0.91-3.10) <i>P</i> =0.09	-0.34 (0.33-1.51) <i>P</i> =0.40

BFGM1, breast-fed gut microbiota type 1; BFGM2, breast-fed gut microbiota type 2; MFGM1, mixed-fed gut microbiota type 1; MFGM2, mixed-fed gut microbiota type 2

TABLE E3. Primer information used for pyrosequencing in present study.

Sample	Barcode	Forward Linker	Forward Primer (5'-3')	Reverse Linker	Reverse Primer (5'-3')
CB_1	ACTCGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_2	AGAGAGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_3	AGTCACTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_4	AGCTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_5	AGAGAGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_6	AGCTATCGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_7	CAGCATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_8	AACTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_9	CACTACGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_10	ACTCACAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_11	TAGCAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_12	TCAGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_13	AGCTATCGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_14	TCATATACGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_15	ACGTGCAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_16	ATCACGTGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_17	CTAGAGACACT	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_18	TCGCATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_19	TCTGCAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_20	CACTATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_21	TAGCAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_22	TAGATAGTGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_23	ACTGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG

CB_24	TACAGCAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CB_25	ACTGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_1	ATGCTGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_2	AGTGTAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_3	ATACGTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_4	TAGCAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_5	CTCGATAGACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_6	TGACTCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_7	TACATCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_8	ACACACGCATC	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_9	AGTCTGACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_10	ATGCTGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_11	CTCGATAGACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_12	ATCGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_13	TCAGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_14	TGACTCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_15	AGCTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_16	AGACAGTACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_17	AGTCACTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_18	ACACTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_19	CTGTACTCACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_20	ACGTCTCTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_21	TCGAGTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_22	ACTCACAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_23	CTCGATAGACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_24	TAGCTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_25	ACTCACAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG

CM_26	ATACACGAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_27	CGTGTACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_28	AGTCTGACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_29	ATGTCGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_30	TACATCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_31	ATACGTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_32	CACTATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_33	AGCTATCGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CM_34	ACACACGCATC	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_1	CTAGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_2	AGCTCACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_3	AGTGAGCTCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_4	CTAGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_5	CTCAGACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_6	CGATGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
CF_7	AGAGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_1	TAGCAGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_2	CACTATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_3	ATCGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_4	ACTGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_5	AGACTCAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_6	AGACAGTACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_7	AGACTCAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_8	ATGTCGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_9	ACTGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_10	ATACGTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_11	TCTGCAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG

AB_12	CGATGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_13	ATCACGTGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_14	AGTCTGACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_15	CTCAGACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_16	TCGCATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_17	ATCGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_18	ATGCTGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_19	ACGTGCAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_20	ATAGCTCTCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_21	CTGTGTCTATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_22	TAGCTCTATC	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AB_23	TCGAGTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_1	CTGTACTCACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_2	TGACTCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_3	ATACACGAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_4	ACGTCTCTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_5	TCTGCAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_6	TCAGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_7	AGATGCTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_8	CTAGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_9	CACTATGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_10	ATGTCGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_11	AGCTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_12	TAGATAGTGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_13	TCATATACGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_14	AGCTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_15	AGTGAGCTCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG

AM_16	AGCGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_17	AGAGAGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_18	CTACACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_19	TAGATAGTGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_20	AGCTCACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_21	CGATGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_22	CTGTCTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_23	CACTACGAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_24	CTGTCTACG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_25	CTGTGTCTATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_26	CTACACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_27	AGACTCAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_28	AGATGCTAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_29	CAGCATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_30	ATACACGAGCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_31	CAGCATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_32	AGAGAGTGTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_33	AGCGATG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AM_34	CTAGCTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_1	ACACACGCATC	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_2	TAGCTCTATC	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_3	ATAGCTCTCG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_4	AGACAGTACAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_5	TACAGCAG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG
AF_6	CGTGTACTG	AC	GAGTTTGATCMTGGCTCAG	AC	WTTACCGCGGCTGCTGG

CB: controls in breast feeding; CM: controls in mixed feeding; CF: controls in formula feeding;

AB: AD in breast feeding; AM: AD in mixed feeding; AF: AD in formula feeding

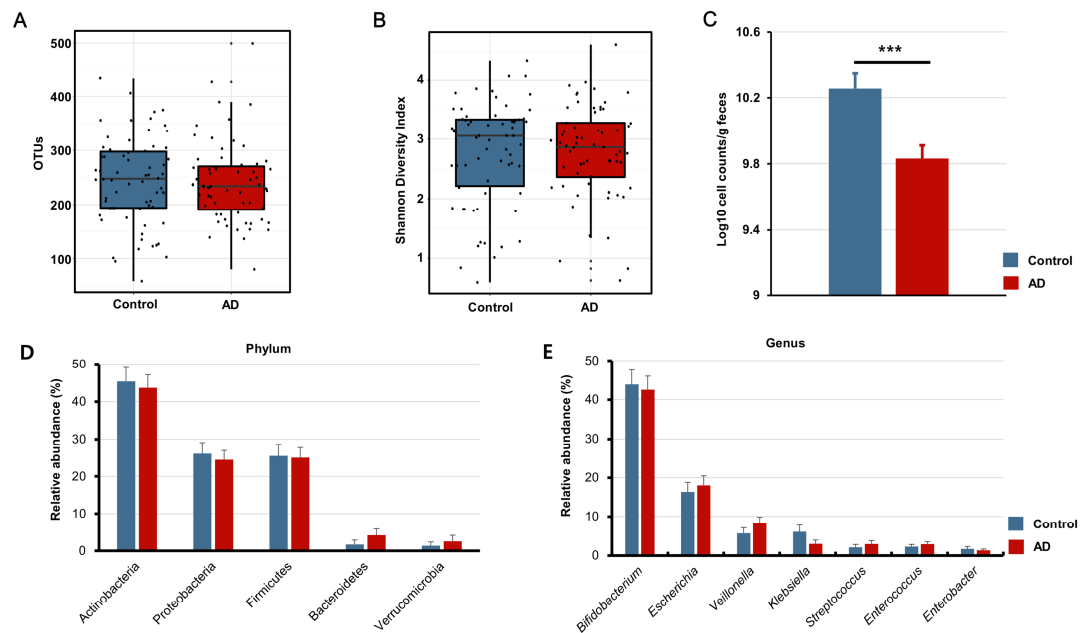
TABLE E4. Comparison of distance metrics for enterotype analysis.

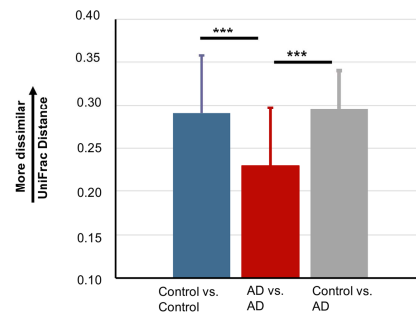
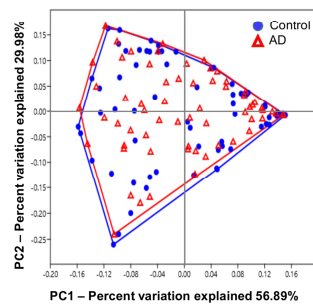
Distance matrix (PAM clustering)	Breast milk-fed infants		Mixed milk-fed infants	
	Calinski-Harabasz score		Calinski-Harabasz score	
	Average silhouette width	Prediction strength	Average silhouette width	Prediction strength
Weighted UniFrac	2 (0.28)	1 (0.67)	2 (0.25)	1 (0.56)
Unweighted UniFrac	2 (0.04)	2 (0.80)	2 (0.04)	1 (0.60)
Bray-Curtis	2 (0.19)	1 (0.75)	2 (0.12)	1 (0.56)
Jensen-Shannon divergence	3 (0.25)	2 (0.94)	2 (0.16)	2 (0.82)
Root Jensen-Shannon divergence	2 (0.16)	2 (0.85)	3 (0.09)	1 (0.64)

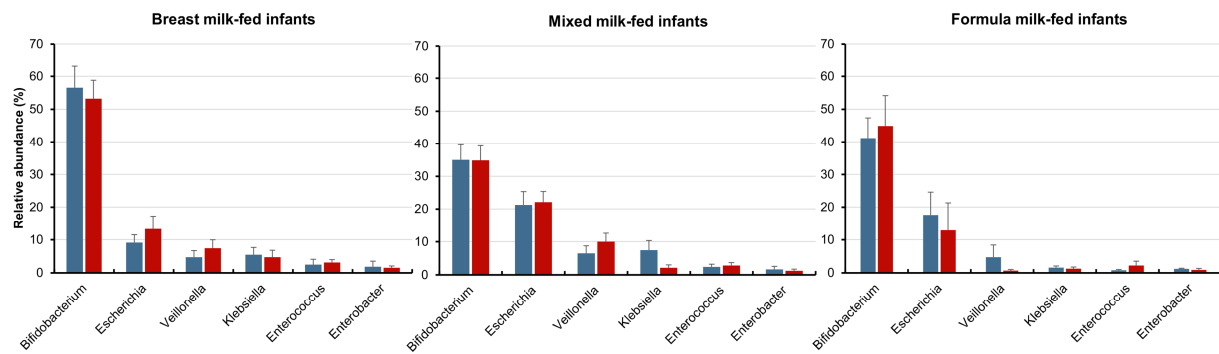
TABLE E5. Clinical characteristics of control and AD infants used in whole metagenome sequencing.

Variables	Control (n = 20)	Infants with AD (n = 20)	<i>P</i> value
Sex, male, n (%)	6/20 (30)	13/20 (65)	.027
SCORAD	NA	27.03 ± 21.16	NA
Delivery mode, vaginal delivery/caesarean delivery (%)	12/8 (60/40)	17/3 (85/15)	.077
Administration of antibiotics in infancy, yes, n (%)	0/20 (0)	2/20 (10)	.147
Probiotics, yes, n (%)	0/20 (0)	1/20 (5)	.311
Parental history of allergic disease, yes, n (%)	7/13 (53.8)	12/19 (63.2)	.598
Atopy, n (%)	3/14 (21.4)	9/17 (52.9)	.073
Serum eosinophils (%)	2.8 ± 1.3 (n = 13)	7.2 ± 5.6 (n = 18)	.001
Total serum IgE levels (kUA/L)	34.7 ± 41.3 (n = 14)	33.0 ± 55.5 (n = 17)	.552
Feeding			.301
Breast feeding, n (%)	8/20 (40)	8/20 (40)	
Mixed feeding, n (%)	10/20 (50)	12/20 (60)	
Formula feeding, n (%)	2/20 (10)	0/20 (0)	

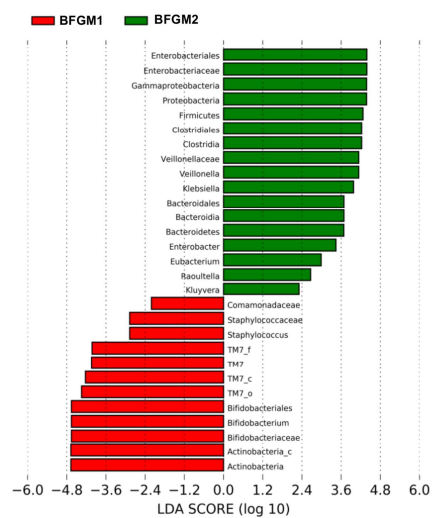
SCORAD: Scoring of Atopic Dermatitis







A



B

