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Dysbiosis involving methionine and PPAR-γ pathways is associated with early onset atopic dermatitis and food allergy

Anchalee Senavonge,[1,2] Massalin Nakphaichit,[3,4]* Wanwipa Vongsangnak,[5,6] Sittiruk Roytrakul,[7] Preecha Patumcharoenpol,[5] Amornthep Kingkaw,[8] Chantha Wongoutong,[9] Wanlapa Weerapakorn,[1] Natapol Pornputtapong,[10,11] Orawan La-ongkham,[12] Yong Poovorawan,[13] Nasamon Wanlapakorn,[13] Pannipa Kittipongpattana,[1] Sunee Nitisinprasert,[3,4] Pantipa Chatchatee,[1] Narissara Suratannon[1]*

Abstract

Background: Atopic dermatitis (AD) and food allergy (FA) often originate early in life. Gut microbiota interactions with the host immune system influence allergy development, yet the distinct gut microbiome and functional profiles in individuals with AD, FA, or both AD+FA remain underexplored.

Objective: We investigated microbial colonization and proteomic profiles in infants with AD, FA, and AD+FA compared to age- and sex-matched controls from the Allergy Development in Early Life and Associated Factors in the Thai Birth Cohort (ALICE).

Methods: Gut microbiomes from stool samples were analyzed using 16S sequencing, and proteomic analysis was conducted by liquid chromatography–tandem mass spectrometry.

Results: The study included 16 AD, 5 FA, 5 AD+FA subjects, and 26 controls. AD+FA group exhibited the most severe dysbiosis. Enrichment of proteins involved in methionine biosynthesis in *Bifidobacterium scardovii* and high *Erysipelotrichaceae* colonization suggest a link to high-fat diets, known to reduce intestinal short-chain fatty acid and serotonin levels, contributing to allergies. *Erysipelotrichaceae* in AD+FA groups also expressed proteins related to histidine degradation. Low *Bifidobacteriaceae* levels were noted in FA and AD+FA, with more pathogenic strains colonized. Increased *Bacteroidaceae* in FA and AD+FA and *Enterobacteriaceae* in FA were detected. Pathways involving vitamin B1, a ligand for proliferator-activated receptor-γ (PPAR-γ) from *Enterobacteriaceae* could promote TH2 cells, type 2 innate lymphoid cells, and M2 macrophages, likely contribute to allergic inflammation.

Conclusion: AD+FA phenotype exhibited the most distinctive gut microbiome alterations, highlighting unique dysbiosis patterns. Microbiome biosynthesis pathways involving metabolism of methionine, histidine, serotonin, and vitamin B1 point to new targets for modifying or treating AD and FA.

Key words: proteomics, gastrointestinal microbiome, cohort studies, dermatitis, atopic, food hypersensitivity, methionine, PPAR-γ, vitamin B1

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Affiliations:
[1] Center of Excellence for Allergy and Clinical Immunology, Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Bangkok, Thailand
[2] BNH Asthma and Allergy center, BNH Hospital, Bangkok, Thailand
[3] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand
[4] Center of Excellence for Microbiota Innovation, Kasetsart University, Bangkok, Thailand
[5] Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand

Affiliations (Continued):
[6] Omics Center for Agriculture, Bioresources, Food, and Health, Kasetsart University (OmiKU), Bangkok, Thailand
[7] Functional Proteomics Technology Laboratory, Functional Ingredients and Food Innovation Research Group, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand
[8] Interdisciplinary Graduate Program in Bioscience, Faculty of Science, Kasetsart University, Bangkok, Thailand
[9] Department of Statistics, Faculty of Science, Kasetsart University, Bangkok, Thailand
[10] Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
[11] Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[12] Department of Applied Microbiology, Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand
[13] Center of excellence in clinical virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

*These authors shared position of the first author

Corresponding author:
Narissara Suratannon
Center of Excellence for Allergy and Clinical Immunology, Division of Allergy and Immunology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, the Thai Red Cross Society, Bangkok, Thailand
1873, Praram 4 Road, Pathumwan, Bangkok, 10330, Thailand
E-mail: narissara.su@chula.ac.th

Abbreviations:

AD	atopic dermatitis
FA	food allergy
DEPs	differentially expressed proteins
NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit

Background

Atopic dermatitis (AD) and food allergy (FA) generally develop during early childhood, which reflects the importance of both genetic susceptibilities and early-life environmental factors. In this regard, evidence indicates that from birth to the third year of life, the infant immune system matures concomitant with progressive changes of the gut microbiome, and gut dysbiosis has been established to be associated with allergic diseases. However, whether gut microbial composition and function differ among participants with AD, those with FA, and those characterized by both allergic phenotypes (AD+FA) has yet to be elucidated. In this study, we sought to identify differences in gut microbiota based on a comparative analysis of healthy participants, those with either AD or FA, and those with both AD and FA, based on 16S rRNA sequencing and a shotgun metaproteomic approach.

Methods

Study design

This study was a general population hospital-based birth cohort study conducted during July 2015 and July 2019 at King Chulalongkorn Memorial Hospital (KCMH) in Bangkok, Thailand named "Allergy Development in Early Life and Associated Factors in the Thai Birth Cohort (ALICE). The inclusion criteria were healthy full-term infants who were born from healthy pregnant women. Informed consent was obtained from all parents. Six hundred women were screened, 500 mother-children's pairs were eligible, in which 369 pairs were enrolled. Only cesarean-section mothers would receive one day of intravenous ampicillin as a routine prophylaxis. Neither a mother with prolonged antibiotic administration nor a mother with infection-related complications that needed prolonged therapeutic antibiotics was recruited in our study. We performed longitudinal assessments of the subjects until the child reached 4 years of age. The part of the microbiome analysis was a cross-sectional study. The study was performed according to the Helsinki Guidelines and approved by the Ethics Committee of KCMH (IRB No. 358/58).

Clinical data collection

Clinical data during the perinatal and postnatal period were collected through interviews by physicians and study nurses. These include family history of atopic diseases (atopic dermatitis, allergic rhinitis, or asthma in a parent or siblings), family income, delivery mode, sex, duration of exclusive breastfeeding, timing to solid food introduction, household pet exposure, types of housing, smoke exposure, daycare attendance, history of illnesses & antibiotic use. A history taking and physical examination were performed by allergy specialists to reassure the diagnosis of allergy.

Definition of physician-diagnosed allergic diseases

Atopic dermatitis (*AD*) was diagnosed according to the criteria from the American Academy of Dermatology.[1] *IgE-mediated food allergy* (*FA*) was diagnosed by an oral food challenge test.[2] *Allergic proctocolitis* was diagnosed based on National Institute of Allergy and Infectious Diseases (NIAID) criteria.[3] *Food protein-induced enterocolitis syndrome* (*FPIES*) was diagnosed based on NIAID criteria,[3] and the international consensus guidelines for the diagnosis and management of FPIES.[4]

Fecal sample collection and preparation

Fecal samples were collected during the age of 9 to 12 months. The children must be in good health condition and did not receive antibiotics for at least one month at the time the samples were collected. A fecal sample of 20 g was collected from the diaper and placed into a 30 × 117 mm sterile container and immediately placed on ice for transfer to storage at -80°C. Stool samples were prepared according to Kisuse et. al.[5] The fecal sample was diluted 10-fold with phosphate-buffered saline (pH 8.0) using a stomacher blender (Stomacher® 80 Biomaster, Seward, Worthing, UK) for 5 minutes. 1 ml of fecal slurry was placed into a 1.5 ml centrifuge tube and then stored at -80°C.

Microbial DNA extraction and 16S rRNA gene sequencing

Microbial DNA was extracted according to method of Kisuse et al 2018,[5] which combined the bead meter method and a QIAamp® DNA stool mini kit (Qiagen GmbH, Germany). Qualification and quantification of DNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Microbial DNA samples were immediately stored at -20°C.

Fecal microbiotas were analyzed using 16S rRNA gene sequencing following the method described by Sathikowitchai et al.[6] The 16S rRNA genes was amplified at variable region of V3-V4 using the forward primer Imina-V3-V4-F (5′-TCGT CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGG GNGGC WGCAG-3′) and the reverse primer Imina-V3-V4-R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGACTACTA CHVGGGTATCTAATCC-3′). The cycling conditions consisted of an initial denaturation at 94°C for 2 minutes, followed by 25 cycles of denaturation at 94°C for 20 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR products were purified using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL Inc., USA) according to the manufacturer's protocol. The sequencing was performed using the Illumina MiSeq platform (USA) at the Omics Sciences and Bioinformatics Center, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

The pair reads were right trimming the last 10 nucleotides and the primers at the 5′ of reads were checked and trimmed as a quality control measure. The pair reads were then denoised and merged into amplicon sequence variants (ASVs) using DADA2 pipeline (v.1.10),[7] with pseudo-pooling mode. We assigned a taxonomy label to each ASV with QIIME2's sklearn classifier (v.2020.2),[8] and Greengenes database (v.13.8).[9] The singleton ASV and ASV with no phylum label were subsequently removed from the analysis to reduce a potential artifact from sequencing error. The functional genomic potential of the microbiota was estimated with PICRUSt2.[10] We converted PICRUSt2's result into KEGG pathway base on the instruction from PICRUSt2's wiki page (https://github.com/picrust/picrust2/wiki). All samples were normalized into relative abundance for later analysis.

Real-time PCR

Four bacterial groups including *Bifidobacterium spp.*, *Enterobacteriaceae*, *Bacteroides fragilis* group and *Erysipelotrichaceae* were quantified by real-time PCR (LightCycler 480; Roche, The Netherlands). The oligonucleotide primers, annealing temperatures and obtained PCR products are summarized in **Table S1**. The q-PCR reaction mixture consisted of 2 µl of microbial DNA template (50-100 ng), 10 µl of 2X SYBR Green I Mastermix (Roche Applied Science, Germany), 0.8 µl of specific primers (10 µM) and dH_2O added to a final volume of 20 µl. The amplification program contained an initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 10 s, annealing for 10 s and extension at 72°C for 10 s. To confirm specific amplification of the target DNA, a dissociation curve was created in the following cycles: a denaturation step at 95°C for 5 s, a decrease to 65°C for 1 min and a continuous increase from 65 to 97°C every 12 s of signal measurement. In addition, specific sizes of the PCR products were determined using gel electrophoresis.

Standard curves of four groups of bacteria were constructed using specific primers to amplify the genomic DNA of *Bacteroides fragilis* ATCC 25285, *Bifidobacterium bifidum* JCM 1255, *Salmonella* Typhimurium TISTR 292 and *Absiella dolichum* JCM 10413. Each PCR product was cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega, Madison, USA). The cloned plasmids were prepared by ten-fold serial dilution of the 16S rRNA gene to 10^2-10^9 copy number. The serial dilution series in each group was used as a template for the standard curve, which was created using LightCycler® 480 software (Roach Applied Science Mannheim, Germany). The diversity analysis and visualization were done with R (v. 3.6.3) (Team R Core., 2013), vegan package (v. 2.5-7),[11] and ggplot2 (v. 3.3.4).[12]

Proteomic analysis

Sample Preparation

All fecal samples performed 16S rRNA gene sequencing were performed proteomic analysis. Sample preparation was conducted as previously described.[13] Briefly, frozen fecal samples were reconstituted in 50 mM phosphate buffer pH 7.0 and then vortexed well. After centrifugation for 10 min at 12,000 rpm to remove debris and some large particles,[14] the solubilized protein remaining in the clear supernatant was collected. Total soluble protein was measured with a Lowry assay using bovine serum albumin as a standard.[15] In 5 mg protein samples, disulfide bonds were reduced using 5 mM dithiothreitol in 10 mM ammonium bicarbonate at 60°C for 1 h, followed by the alkylation of sulfhydryl groups by 15 mM iodoacetamide in 10 mM ammonium bicarbonate for 45 mins in the dark at room temperature. For digestion, the protein samples were mixed with sequencing-grade trypsin (ratio of 1:20) (Promega, Germany) and incubated at 37°C overnight. Prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the digested protein (tryptic peptide) samples were dried and protonated with 0.1% formic acid before injection into the LC-MS/MS system.

Liquid chromatography-tandem mass spectrometry

LC-MS/MS was conducted as previously described.[11] Specifically, the tryptic peptide samples (100 ng) were injected in triplicates into an Ultimate™ 3000 Nano/Capillary LC System (Thermo Fisher Scientific, USA) coupled to a Hybrid quadrupole Q-TOF impact II™ (Bruker Daltonics, Germany) equipped with a Nano-captive spray ionization (CSI source). Here, peptides were enriched on a m-Precolumn 300 mm i.d. × five mm C18 PepMap™ 100, 5 mm, 100 Å (Thermo Fisher Scientific, USA) and separated on a 75 mm I.D. × 15 cm and packed with Acclaim™ PepMap™ RSLC C18, 2 mm, 100 Å, nanoViper (Thermo Fisher Scientific, USA). A mobile phase of solvent X (0.1% formic acid) and solvent Y (80% acetonitrile and 0.1% formic acid) were applied on the analytical column. A linear gradient of 5-55% solvent Y was used to elute the peptides at a constant flow rate of 0.30 ml/min for 30 min. Electrospray ionization was performed at 1.6 kV using CaptiveSpray. Mass spectra (MS)

and MS/MS spectra were achieved in the positive-ion mode over the range (m/z) 150–2,200 (Compass 1.9 software, Bruker Daltonics, Germany).

Quantification and identification of proteins

For the quantification of proteins, MaxQuant (version 1.6.6.0) was used to quantify individual samples, and their MS/MS spectra were matched to the UniProt bacterial database by using the Andromeda search engine.[16] Labelfree quantitation with MaxQuant settings was performed, which included (1) a maximum of two missed cleavages, (2) mass tolerance of 0.6 Daltons for the main search, (3) trypsin as the digestion enzyme, (4) carbamidomethylation of cysteine residues as a fixed modification, and (5) oxidation of methionine and acetylation of the protein N-terminus as variable modifications. Notably, peptides with a minimum of 7 amino acids and at least one unique peptide were required for protein identification. The protein false discovery rate (FDR) was set at 1% and estimated from the reverse searches of sequences. The maximal number of modifications per peptide was set to 5. For searches in FASTA files, a protein database of 12 candidate bacterial families selected from our 16S rRNA gene sequencing results and earlier reports of gut microbiome data from Thailand,[5,17] which included *Bacteroidaceae*, *Bifidobacteriaceae Enterococcaceae*, *Enterobacteriaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Prevotellaceae*, *Streptococcaceae and Veillonellaceae*, was downloaded from UniProt. Database with potential contaminants included in MaxQuant was automatically added. The MaxQuant ProteinGroups.txt file was subsequently obtained in conjunction with the use of Perseus software (version 1.6.6.0) for importing peptide sequences into the metaproteome dataset.[16] Exact peptides for which a unique protein sequence was matched to a single bacterial strain were classified as bacterial strain-specific sequences for taxonomic classification.[18,19] The remaining peptides for which a unique protein sequence was not matched to a single bacterial strain were discarded. The protein sequences assigned with protein IDs with know/putative functions from the UniProt bacterial database were denoted as annotated proteins. In contrast, the protein sequences assigned an ID corresponding to a hypothetical protein/uncharacterized protein were designated as unannotated proteins. Maximum peptide intensities were log2 transformed in Microsoft Excel, providing the protein expression levels (PELs) for DEPs analysis.

Statistical analysis:

The statistical significance of differences among the three phenotypic groups was determined using one-way ANOVA and the Kruskal–wallis test for parametric and non-parametric data, respectively. Comparisons between two groups were performed using an independent t-test and the Mann-Whitney U-test for parametric and non-parametric data, respectively. The alpha diversity was estimated using Chao index, Shannon's index, and Simpson's index. Beta diversity was calculated from bray distance. To visualize the distance, we performed an ordination using non-metric multidimensional scaling (NMDS). Linear mixed modeling of bacterial relative abundance was performed using MaAslin[2,20] to model associations between bacterial abundance at the family and genus levels and the assessed clinical variables. Clinical variables were as follows: having AD, having FA, a maternal history of allergic diseases, cesarean delivery, use of antibiotics during infancy, having siblings, family income, and use of infant formula containing probiotics or prebiotics. Normalization of bacterial abundance was based on cumulative sum scaling (CSS), and associations were modeled using compound Poisson linear models. Associations were significant at q-value [false discovery rate (FDR) adjusted p-value] < 0.25.

Regarding proteomic analysis, to compare the Differentially Expressed Protein (DEP) among controls, AD, FA, and AD+FA, the Wilcoxon rank-sum test and multiple testing via false discovery rate (FDR) correction were used to identify significant proteins between the groups (adjusted p-value < 0.05). The significant protein expression was intended to serve as a potential protein to classify the control and allergic disease in infant. The Wilcoxon rank sum-test was selected in this study because it is a nonparametric test that compares medians between two groups of independent samples.

Result

In total 369 mother-children pairs enrolled, 42 infants with allergies were diagnosed within the period of 19 months of age. At ages between 9 and 12 months, fecal samples from 26 infants with allergies (16 AD, 5 FA, and 5 AD+FA) and 26 matched control infants were collected. Among 5 participants with isolated FA, two had urticaria induced by hen's egg and wheat respectively, one had banana-induced food protein-induced enterocolitis, and two had cow's milk allergic proctocolitis. Of 5 participants with AD+FA, 4 had urticaria (1 from wheat, 2 from hen's egg, 1 from both wheat and hen's egg), and another participant had challenge-proven cow's milk induced-eczema. The onset of allergic manifestations was as follows; AD during 2-4 months old, FA during 4-7 months old, and for those with both AD+FA, the onset of AD was during 2-7 months and for FA was during 7-8 months respectively.

Demographic data are shown in **Table 1**. Sixty percent of the participants were male and 42% of the infants were delivered by cesarean section. The duration of breastfeeding in the allergic and control groups was 11 and 12 months, respectively, whereas for both groups, the age at which solid food was introduced was 6 months. Compared with the controls, infants in the allergic group had a two-fold higher history of maternal allergic diseases, higher socioeconomic status and less likely to have siblings. In addition, one-third of the participants in both groups came from households with furry pets, while nearly half had received antibiotics during infancy. None of the participants had attended daycare centers. No probiotic/prebiotic supplementation in all infants by the time of stool collection. Demographic data of the allergic participants who were included in the study and those who dropped out were not different and are shown in **Table S2**.

Table 1. Demographic data.

Characteristic	Allergy group (N = 26)	AD (N = 16)	FA (N = 5)	AD+FA (N = 5)	Healthy control (N = 26)
Age at stool collection (months), median (IQR)	9 (9-12)	9 (9-12)	9 (9-12)	9 (9-12)	9 (9-12)
Age at onset of allergic manifestation (months), median (IQR)	4 (2.5-6)	4 (2-4)	6 (4-7)	Onset AD; 3 (2-4) Onset FA; 7 (7-8)	-
Male sex, n (%)*	16 (61.5)	10 (62.5)	2 (40)	4 (80)	15 (57.7)
Mode of delivery, n (%)*					
- Normal labor	15 (57.7)	11 (68.8)	2 (40)	2 (40)	15 (57.7)
- Cesarean delivery	11 (42.3)	5 (31.2)	3 (60)	3 (60)	11 (42.3)
Duration of breastfeeding (months), median (IQR)	11 (4-12)	7.7 (3-12)	13 (9-19)	10 (6-14)	12 (4-14)
Age of solid food introduction (months), median (IQR)*	6 (5-6)	5.3 (4-6)	6.4 (5-9)	5.6 (5-6)	6 (5-6)
Family history of allergic diseases, n (%)					
- None	13 (50)	9 (56.3)	1 (20)	3 (60)	13 (50)
- Father	4 (15.4)	2 (12.5)	2 (40)	0	3 (11.5)
- Mother	7 (26.9)	5 (31.2)	0	2 (40)	4 (15.4)
- Both parents	1 (3.9)	0	1 (20)	0	2 (7.7)
- Sibling(s)	1 (3.9)	0	1 (20)	0	4 (15.4)
Having siblings, n (%)					
- Yes	17 (65.4)	6 (37.5)	5 (100)	4 (80)	13 (50)
Family income, n (%)#					
- Low	12 (46.2)	8 (50)	3 (60)	1 (20)	11 (42.3)
- Medium	10 (38.4)	4 (25)	2 (40)	4 (80)	14 (53.9)
- High	4 (15.4)	4 (25)	0	0	1 (3.9)
Household furry pets, n (%)*					
- 0	17 (65.4)	11 (68.8)	3 (60)	3 (60)	19 (73.1)
- 1	3 (11.5)	2 (12.5)	0	1 (20)	3 (11.5)
- ≥ 2	6 (23.1)	3 (18.7)	2 (40)	1 (20)	4 (15.4)
Antibiotic usage during infancy, n (%)	12 (46.2)	9 (56.2)	0	3 (60)	12 (46.2)
Daycare attendance	0	0	0	0	0

*matching factors, #Categorized according to Thai GDP 2016, low income defined as income < 11,500 USD/year,middle income 11,500–28,500 USD/year, high income > 28,500 USD/year

Regarding microbial diversity and richness, we found no significant difference among participants with allergies compared to controls (**Figure 1A**). In addition, we detected no significant clustering among samples within each group (**Figure 2**). With respect to microbiome composition, we observed distinct patterns of microbial colonization associated with the different allergic phenotypes (**Figure 1B**). Specially, participants with AD and AD+FA phenotypes were characterized by significant colonization of *Erysipelotrichaceae* (**Figure 1B**, light navy-blue column), while those with FA and AD+FA phenotypes had a low abundance of *Bifidobacteriaceae* (**Figure 1B**, orange column) but a high abundance of *Bacteroidaceae* (**Figure 1B**, sky blue column). Additionally, *Enterobacteriaceae* were prominently observed in the microbiota of FA individuals (**Figure 1B**, grey column). These four bacterial families were selected for quantification using real-time PCR, where their identifications were confirmed (**Figure 3**), except for *Erysipelotrichaceae* in AD and AD+FA subjects. Although not statistically significant, real-time PCR results suggested a similar trend of higher

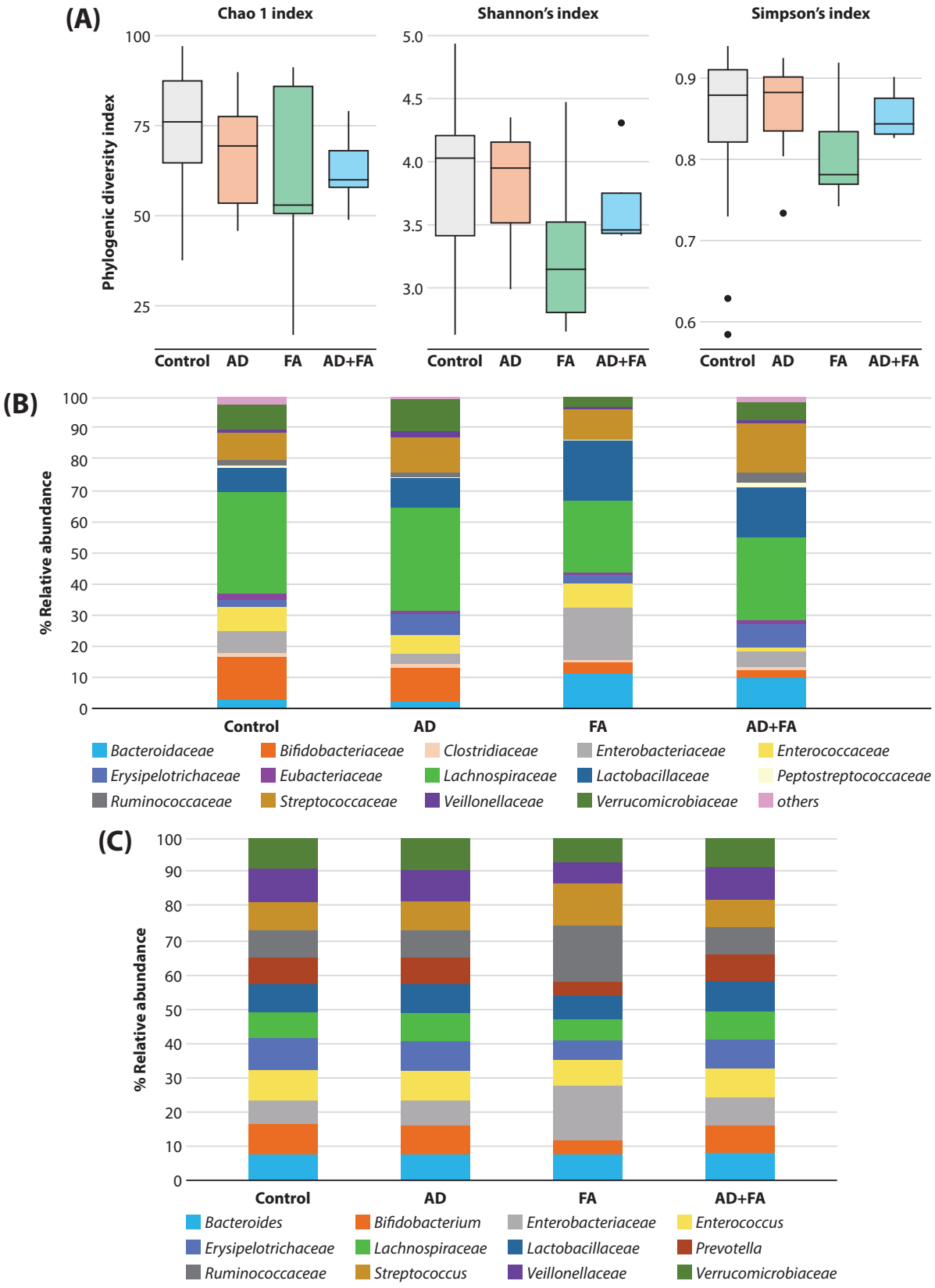

Figure 1. (A) Fecal microbial richness and diversity determined based on the Chao, Shannon, and Simpson indices. Boxplots display the median and 25th and 75th percentiles of index value. Dot plots denote outlier values. (B) Relative abundance of the gut microbiome based on 16S rRNA gene analysis. Bar charts represent the average operational taxonomic unit (out) distribution according to bacterial family. **P*-value < 0.05 compared with the control, as determined using the Mann–Whitney test. (C) Relative abundance of the protein expression of 12 selected bacterial families. (D) Comparison of differentially expressed proteins (DEPs) between different subgroups. AD, atopic dermatitis, FA, food allergy. Wilcoxon rank-sum test and multiple testing via false discovery rate (FDR) correction were used to identify proteins showing significantly different expression between groups, adjusted *P* value < 0.05.

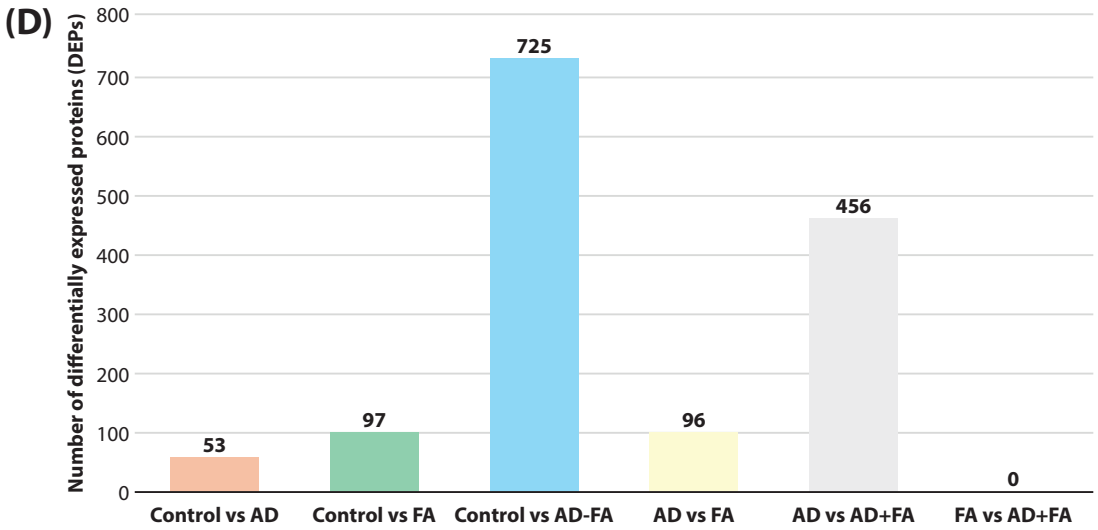

Figure 1. (Continued)

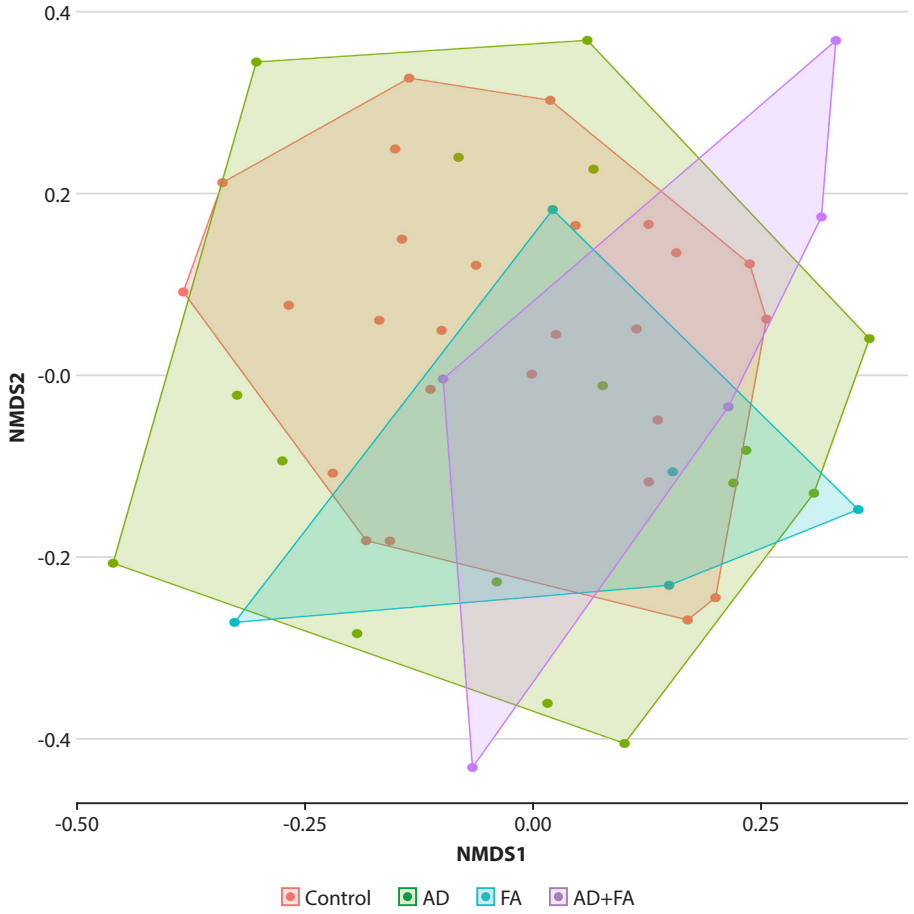

Figure 2. Beta diversity is determined by non-metric multidimensional scaling analysis. No significant differences between groups were detected bases on permutational multivariate analysis of variance using distance matrices (Adonis). NMDS, non-metric multidimensional scaling; AD, atopic dermatitis; FA, food allergy.

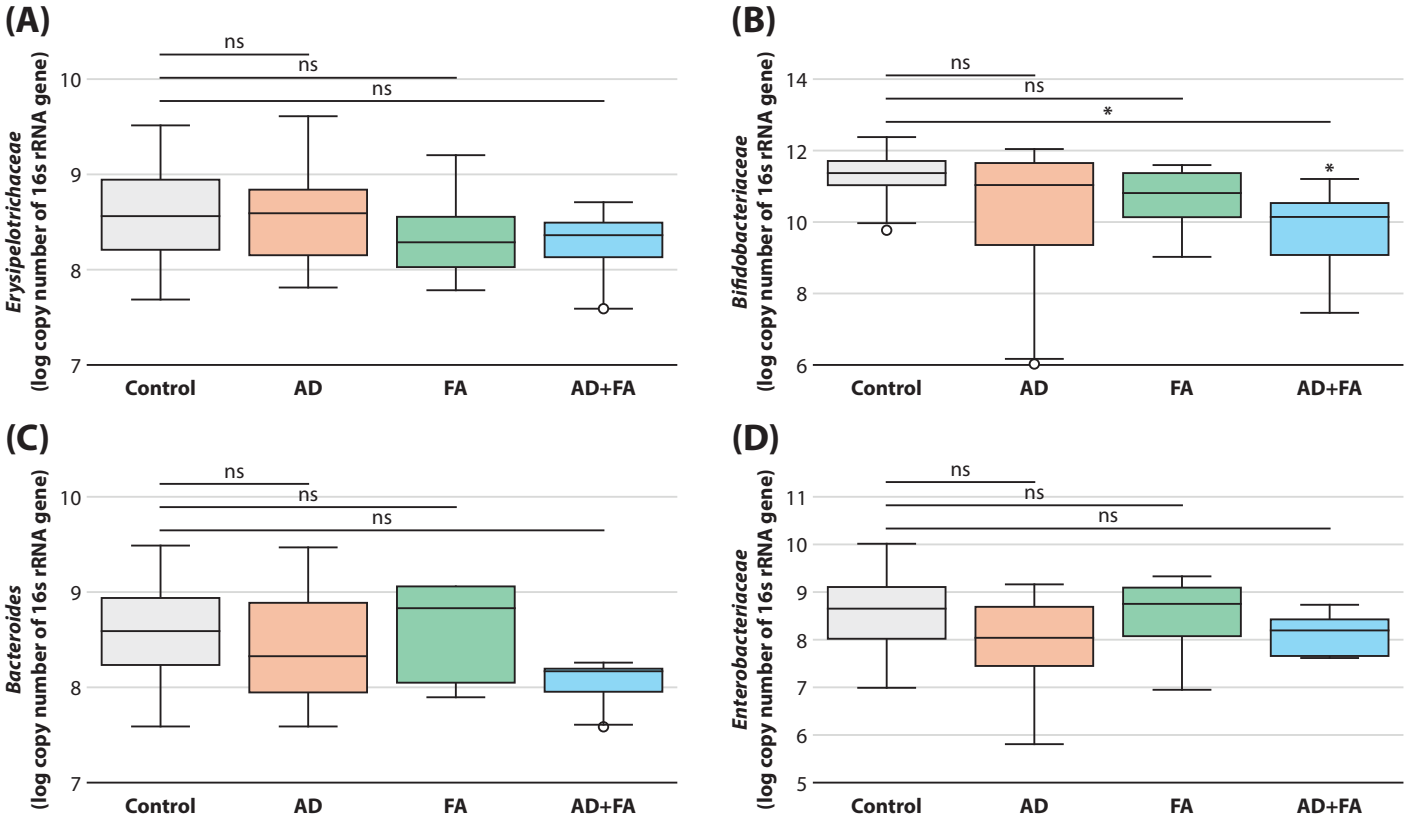

Figure 3. Real-time PCR analysis of (A) *Erysipelotrichaceae*, (B) *Bifidobacteriaceae*, (C) *Bacteroidaceae*, and (D) *Enterobacteriaceae*. Boxplots display the median and 25th and 75th percentiles values for the control, AD, FA, and AD+FA groups. *P value < 0.05 compared with the control, as determined using the Mann–Whitney test. AD, atopic dermatitis, FA, food allergy.

Erysipelotrichaceae abundance in AD patients compared to controls. However, the higher *Erysipelotrichaceae* abundance observed in the AD+FA group via 16S rRNA gene sequencing was not reflected in the real-time PCR results, which may be attributed to the low abundance of *Erysipelotrichaceae* in individuals with FA. Discrepancies between 16S rRNA gene sequencing and real-time PCR results could be due to the distinct nature of the techniques, as 16S rRNA gene sequencing is qualitative, while real-time PCR is quantitative and calibrated against a standard. Linear mixed-effect modeling (**Table 2**) revealed a significant association between *Erysipelotrichaceae* and AD at the family level, while associations between *Enterobacteriaceae* and both FA and AD phenotypes were confirmed at the genus level. We identified maternal history of allergic diseases, cesarean delivery, and antibiotic use during infancy as factors associated with the colonization of *Enterobacteriaceae*, whereas the mode of delivery and antibiotic use during infancy did not correlate with the other three bacterial taxa (**Table 2**, **Table S3**). Consuming infant formula containing

Table 2. Linear mix factor model results for *Erysipelotrichaceae* and *Enterobacteriaceae* with interactions between clinical factors.

Family	Genus	Variable	coef	stderr	pval	qval	odd ratio
Erysipelotrichaceae	N/A	having AD	1.6801	0.5310	0.0028*	0.1844	5.3662
Enterobacteriaceae	*Escherichia.Shigella*	having AD	-1.3848	0.3992	0.0012*	0.0472*	0.2504
	Klebsiella	having FA	-1.9078	0.5289	0.0008*	0.0450*	0.1484
	Klebsiella	maternal history of allergic diseases	-1.8305	0.5474	0.0017*	0.0593	0.1603
	Klebsiella	delivered by cesarean section	1.0974	0.3898	0.0073*	0.1785	2.9964
	Klebsiella	having AD	1.1073	0.3978	0.0079*	0.1785	3.0262
	Morganella	antibiotics usage during infancy period	1.3127	0.6236	0.0410*	0.3867	1.8939
	Proteus	antibiotics usage during infancy period	-2.8272	1.2447	0.0280*	0.3274	-4.0788

N/A not applicable, AD, atopic dermatitis, FA, food allergy, Coef; coefficient. Stderr; standard error. pval; p-value. qval; q-value, * p-value and q-value < 0.05.

probiotics or prebiotics was also not associated with colonization of any of the four bacterial taxa studied at the family level (p-value > 0.05) (**Figure S3**). Additionally, our FA populations included both non-IgE-mediated and IgE-mediated FA. It has not yet been determined whether the gut microbiomes of these two FA types are similar or distinct. To explore this, we performed beta diversity analysis using Bray-Curtis distance metrics to compare non-IgE-mediated and IgE-mediated FA. PERMANOVA statistical analysis revealed no significant difference between the two groups (p-value = 0.29).

For proteomic analysis, the independent mass spectral counts were shown in **Table S4**. From all 52 samples, spectral counts could be detected in 43 samples. These samples included 21 control samples, 14 AD samples, 3 FA samples and 5 AD+FA samples. Within these samples, mass spectral counts of 86,894 was identified, including 43,310 spectral counts from control samples, 31,171 spectral counts from AD, 181 spectral counts from FA and 12,232 spectral counts from AD+FA. Spectral counts per sample of control, AD and AD+FA groups are comparable, while spectral counts of FA group were remarkably low. When spectral counts assigned to the protein sequences on the basis of the spectral library and proteomic resources,[21] a greatest number of total protein sequences with annotations were identified in the control samples (21,297 sequences) than in the allergy samples (17,370 sequences in AD, 150 sequences in FA, and 8,415 sequences in AD+FA). After Assignment of spectral counts to protein sequences, based on the UniProt bacterial database,[16] the unique protein sequences of 37,579 total proteins were identified for further analysis.

Relative abundances of microbial protein expression for the 12 selected bacterial families are shown in **Figure 1C**, and those of each individual are shown in **Figure S1**. Among these proteins, we identified 1,179 that were significantly differentially expressed (DEPs) among the four study groups. **Figure 1D** shows the numbers of DEPs between each pair of groups. Compared with the controls, we detected the largest number of DEPs in the AD+FA group, followed by those in the FA and AD groups, thereby implying that compared those individuals with only AD or FA phenotypes, the functional properties of the microbiota in AD+FA participants were most distinct from those of the control microbiota. Interestingly, irrespective of allergic phenotype, all these DEPs were more highly expressed in participants with allergies than the controls, (**Figure S2**).

By applying manual curation, we selected 37 functionally assigned proteins from four bacterial taxa (*Bifidobacterium* spp., *Enterobacteriaceae*, *Bacteroides fragilis* group, and *Erysipelotrichaceae*), based on the results of 16S bacterial sequencing (**Table 3**). In the AD+FA group, we identified proteins involved in thiamine, riboflavin, and menaquinone synthesis from three genera of *Enterobacteriaceae*, while proteins involved in carbohydrate metabolism were identified from *Bifidobacterium pseudocatenulatum*, *Enterobacter huaxiensis*, *Enterobacter cloacae*, *Escherichia coli*, and *Faecalitalea cylindroides*. These findings indicate heightened gut microbiome activity in these individuals. Notably, we detected the expression of *O-acetylhomoserine aminocarboxypropyltransferase* (EC 2.5.1.49), a protein involved in the cysteine and methionine metabolism pathway, from *Bifidobacterium scardovii*, as well as aminotransferase class I/II from *Faecalitalea cylindroides* ATCC 27803 (family *Erysipelotrichaceae*). The roles of these microbial pathways will be discussed further. For the AD group, we identified demethylmenaquinone methyltransferase, a protein involved in menaquinone synthesis, from *Bacteroides* sp., and 6-phospho-beta-glucosidase from *Erysipelotrichaceae*, whereas in the FA group, a protein involved in the pyruvate kinase pathway was identified from *Bifidobacterium longum* subsp. longum.

Table 3. Lists of differentially expressed proteins (DEPs) in allergic subgroup compared to controls.

Protein ID	Protein names	Families	Species	Sub-functional categories
AD + FA group				
A0A286BQV6	1-deoxy-D-xylulose-5-phosphate synthase (EC 2.2.1.7) (1-deoxyxylulose-5-phosphate synthase); thiamine (vitamin B1) synthesis pathway	*Enterobacteriaceae*	*Enterobacteriaceae bacterium JKS000234*	Metabolism of cofactors and vitamins
A0A2K9PCZ4	Riboflavin biosynthesis protein [Includes: Riboflavin kinase (EC 2.7.1.26) (Flavokinase); FMN adenylyltransferase (EC 2.7.7.2) (FAD pyrophosphorylase); riboflavin (vitamin B2) synthesis pathway	*Enterobacteriaceae*	*Citrobacter freundii complex sp. CFNIH2*	Metabolism of cofactors and vitamins
J1GBX2	Ubiquinone biosynthesis O-methyltransferase (2-polyprenyl-6-hydroxyphenol methylase); menaquinone (vitamin K2) synthesis pathway	*Enterobacteriaceae*	*Enterobacter sp. Ag1*	Metabolism of cofactors and vitamins
A0A072N8I9	Phosphoenolpyruvate carboxylase (EC 4.1.1.31); pyruvate metabolism pathway	*Bifidobacterium*	*Bifidobacterium pseudocatenulatum IPLA36007*	Carbohydrate metabolism
A0A3R9PQU3	Cellulose synthase catalytic subunit [UDP-forming] (EC 2.4.1.12); cell wall formation synthesis pathway	*Enterobacteriaceae*	*Enterobacter huaxiensis*	Carbohydrate metabolism

Table 3. Lists of differentially expressed proteins (DEPs) in allergic subgroup compared to controls.

Protein ID	Protein names	Families	Species	Sub-functional categories
AD + FA group (Continued)				
A0A377LPN6	6-phosphofructokinase (EC 2.7.1.11); glycolysis/gluconeogenesis pathway	*Enterobacteriaceae*	*Enterobacter cloacae*	Carbohydrate metabolism
A0A377DQK3	dTDP-D-glucose 4,6-dehydratase rmlB (EC 4.2.1.46); glycan biosynthesis and metabolism pathway	*Enterobacteriaceae*	*Escherichia coli*	Carbohydrate metabolism
U2PN91	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (EC 2.4.1.227) (Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc GlcNAc transferase); glycan biosynthesis and metabolism pathway	*Erysipelotrichaceae*	*Faecalitalea cylindroides ATCC 27803*	Carbohydrate metabolism
A0A087DIW2	O-acetylhomoserine aminocarboxypropyltransferase (EC2.5.1.49); cysteine and methionine metabolism pathway	*Bifidobacterium*	*Bifidobacterium scardovii*	Amino acid metabolism
U2NVC4	Aminotransferase, class I/II	*Erysipelotrichaceae*	*Faecalitalea cylindroides ATCC 27803*	Amino acid metabolism
A0A087BMC1	Fabd (EC 1.1.1.100) (EC 2.3.1.41) (EC 2.3.1.86); fatty acid biosynthesis pathway	*Bifidobacterium*	*Bifidobacterium minimum*	Lipid metabolism
AD				
R6ZSA4	Demethylmenaquinone methyltransferase (EC 2.1.1.163); menaquinone (vitamin K2) synthesis pathway	*Bacteroides*	*Bacteroides sp. CAG:714*	Metabolism of cofactors and vitamins
A0A3D2W7G8	6-phospho-beta-glucosidase; glycolysis/gluconeogenesis pathway	*Erysipelotrichaceae*	*Erysipelotrichaceae bacterium*	Carbohydrate metabolism
FA				
A0A075QNH1	Pyruvate kinase (Fragment)	*Bifidobacterium*	*Bifidobacterium longum subsp. longum*	Carbohydrate metabolism

AD, atopic dermatitis, FA, food allergy

Discussion

Analyzing bacterial proteomes using a metaproteomic approach enables the direct measurement of bacterial proteins, thereby providing opportunities to determine the functional status associated with gut bacterial activity and metabolism. In the present study, we adopted a metaproteomic approach combined with 16S rRNA gene sequencing to characterize the distinct patterns of gut microbiome colonization and microbial activities in individuals with different allergic phenotypes. We confirmed the microbial abundance of significantly different families using real-time PCR. Although the real-time PCR results for *Erysipelotrichaceae* did not show statistically significant differences between groups, a similar trend of higher abundance in AD patients compared to controls was observed. Additionally, while the abundance of *Erysipelotrichaceae* in the AD+FA group could not be confirmed by real-time PCR, proteomic analysis indicated high activity and a potentially pathogenic role of *Erysipelotrichaceae* in AD+FA individuals, as described in the results and discussed later in this paper.

The human gut microbiome is significantly influenced by environmental factors, particularly diet. When dietary methionine is consumed, it is converted into homocysteine by the gut microbiome, which possesses enzymes related to the methionine biosynthesis pathway.[22] These enzymes and proteins are present in prokaryotes but not in vertebrates, indicating that humans cannot metabolize methionine independently. Evidence suggests that high-fat diets, which are rich in methionine, can lead to a significant decrease in the abundance of short-chain fatty acid (SCFA)-producing bacteria,[23] and serotonin-producing bacteria,[24] resulting in lower intestinal and blood SCFA and serotonin levels. These reductions have been observed in patients with obesity,[25] dementia-like neurodegeneration and Alzheimer's disease.[26] The influence of Westernized diets—high in fats and low in fibers—on allergy development is increasingly recognized, with such diets contributing to gut dysbiosis and reduced short-chain fatty acid (SCFA) levels.[27] Recent studies also underscore the protective role of serotonin-producing gut bacteria in newborns against allergies.[28] While the impact of methionine on allergic diseases remains underexplored,

the presence of *O-acetylhomoserine aminocarboxypropyl-transferase* in *Bifidobacterium scardovii*, a key enzyme in the methionine biosynthesis pathway (KEGG PATHWAY: bsca00270) identified in this study, raises the possibility that individuals with AD+FA may consume more high-fat diets compared to other groups.

Despite its limited presence in allergy-related studies, many reasons reassured the possibility of potentially pathogenic role of *Erysipelotrichaceae*. Firstly, *Erysipelotrichaceae* has been implicated in several other conditions, including inflammatory bowel diseases,[29] colorectal cancer,[30] and type 2 diabetes in mice.[31] Only one previous study has reported an increased abundance of *Erysipelotrichaceae* in individuals with food allergy and histamine intolerance compared to controls and those with food intolerance.[32] To the best of our knowledge, our cohort is the first to identify *Erysipelotrichaceae* colonization in individuals with AD,[33] and those with both AD and food allergy (AD+FA). Secondly, research has shown that *Erysipelotrichaceae* abundance increases in individuals consuming high-fat diets,[34] supporting our earlier hypothesis that the dietary patterns of the AD+FA group may differ from those of other groups. These findings further highlight the co-localization of specific gut microbiota and the influence of dietary habits, contributing to the development of allergic phenotypes.

Furthermore, beyond its abundance, our proteomic analysis confirmed that *Erysipelotrichaceae* was functionally active, as indicated by the enrichment of proteins involved in carbohydrate metabolism. Finally, *Erysipelotrichaceae* expressed proteins related to amino acid metabolism, specifically aminotransferase classes I and II within the histidine metabolism pathway (KEGG PATHWAY: Histidine metabolism – *Erysipelotrichaceae* bacterium I46), which are known to play a role in histamine production. While further experiments are required, indirect evidence from other organisms, such as *Candida glabrata*, is particularly relevant. In the absence of a gene coding for histidine ammonia-lyase, *Candida glabrata* can degrade histidine via an alternative aromatic amino acid aminotransferase pathway.[35] These findings highlight the potential role of the aminotransferase pathway and *Erysipelotrichaceae* in the development of allergic diseases, particularly in early life, warranting further investigation in larger populations.

Certain members of the gut microbiome could synthesize vitamins B and K. The findings of our proteomic analyses revealed the presence in AD+FA group infants of proteins involved in the vitamin B_1 synthesis pathway in *Enterobacteriaceae bacterium* JKS000234 and in the vitamin B_2 synthesis pathway in *Citrobacter freundii* complex sp. CFNIH2. It is assumed that these two vitamins might play important roles in the maintenance of immune homeostasis by mediating naïve B cell differentiation in the intestine.[36,37] Moreover, these vitamins are known to function as cofactors for enzymes involved in the TCA cycle, which naïve B cells preferentially use for efficient energy generation.[37] Vitamin B_1 supplementation was found to be related to eczema worsening.[38] Evidently, vitamin B_1 is proven to be a ligand for proliferator-activated receptor-γ (PPAR-γ).[39] PPAR-γ has been found to promote the functions of TH2 cells, type 2 innate lymphoid cells, M2 macrophages, and dendritic cells, regulate lipid metabolism and directly induce effector gene expression which drives allergic inflammation.[40] We also identified proteins associated with vitamin K_2 synthesis, including those in the menaquinone biosynthesis pathway (in *Enterbacter* sp. Ag1) in the AD+FA group samples and the DMM pathway (in *Bacteroides* sp. CAG 714) in those from the AD group. This vitamin was found to be a growth promotor of microbiome.[41] We accordingly speculate that the upregulated vitamin K_2 pathway may reflect the active state of such a microbiome.

In the present study, we detected a low abundance of *Bifidobacterium* spp. in the FA and AD+FA patients. Moreover, proteomic analysis revealed that among these bacteria, it is the pathogenic species *Bifidobacterium pseudocatenulum*,[42] and *Bifidobacterium scardovii*,[43] that colonize the guts of AD+FA patients, whereas *Bifidobacterium longum*, a beneficial member of the gut microbiome, was detected in FA participants. We hypothesize that the colonization pattern found in FA participants reflects a compensatory mechanism of the gut microbiota associated with the regulation of immune homeostasis in patients with allergies.

The limitation of this study is the small number of study participants and the cross-sectional study design for microbiome analysis. More patient numbers and further prospective studies will strengthen the findings. However, to decrease bias we selected matched controls with matching factors that influenced the microbiome change and might relate to allergic manifestations. These factors include the age at stool collection, sex, delivery mode, age at solid food introduction, socioeconomic status of the family, and household furry pets. Moreover, we used non-parametric tests for statistical analysis, which were suitable for a study with small sample size. Finally, to confirm our findings, we use two methods (16S rRNA gene sequencing and real-time PCR) to confirm the existence of these microbiomes in each subgroup. For microbial protein expression, the actual metaproteomic data were measured by LC-MS and analyzed using Wilcoxon rank-sum test. These results are related to the functional genomic potential of the microbiota (PICRUSt2).

In conclusion, participants with different allergic phenotypes were found to be characterized by gut microbiota with distinct patterns and functions. Compared with AD and AD+FA group infants, the gut microbiota of FA individuals was found to be remarkably less diverse. Participants with combined AD+FA phenotypes displayed the most severe dysbiosis and a predominantly FA-like gut microbiome pattern. The detection of protein profiles indicative of gut microbiota-derived vitamin B and K metabolism, as well as biosynthesis pathways involving methionine and histidine, may elucidate the mechanisms of regulation and balance of host immunity in participants with allergy, which could potentially lead to new targeted therapies for AD and FA in the future.

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Conflicts of interest

All authors have no relevant financial relationships to disclose.

Authors' contributions

- AS contributed on writing the first draft of manuscript, data collection and data analysis.
- MN wrote the first draft of manuscript, and performed 16S sequencing, real-time PCR.
- WV and PP performed bioinformatic analysis and wrote the manuscript.
- AK and SR performed proteomic analysis and wrote the manuscript.
- CW performed the statistical analysis and wrote the manuscript.
- WW, NP, OL, PK, NW, YP, SN, and PC provided valuable advice, reviewed and edited the manuscript.
- NS wrote the manuscript and took care overall of this work.
- All authors read and approved of the final manuscript.

Ethics approval

The study was approved by the Ethics Committee of King Chulalongkorn Memorial Hospital in Bangkok, Thailand (IRB No. 358/58).

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Data Deposition

Sequence data generated and analyzed during this study are available on the NIH Sequence Read Archive (SRA) under Bioproject ID PRJNA716451. Raw MS/MS spectra data are available in ProteomeXchange: JPST001282 and PXD027692.

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Supplementary Materials & Methods

Table S1. Primer sequence, annealing temperature, and size of PCR products.

Target	Sequence	Size (bp)	Tm (°C)	Reference
Bacteroides fragilis	F: CTGAACCAGCCAAGTAGCG R: CCGCAAACTTTCACAACTGACTTA	500	56	Matsuki et al.[1]
Bifidobacterium spp.	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	243	60	Rinttilä et al.[2]
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	57	Bartosh et al.[3]
Erysipelotrichaceae	F: GGCAGCAGTAGGGAAT R: CACGTAGTTAGCCGTGG	168	55	This Study

Table S2. Demographic data compared between 26 allergic children included in the study and the dropped out 16 allergic children

Characteristic	Allergy group (N = 26)	Drop-out allergy group (N=16)
Age at onset of allergic manifestation (months), median (IQR)	4 (2.5-6)	4 (3-4)
Male sex, n (%)	16 (61.5)	9 (56.3)
Mode of delivery, n (%)		
- Normal labor	15 (57.7)	9 (56.3)
- Cesarean delivery	11 (42.3)	7 (43.7)
Duration of breastfeeding (months), median (IQR)	11 (4-12)	7.5 (3-13.7)
Age of solid food introduction (months), median (IQR)	6 (5-6)	6 (5-6)
Family history of allergic diseases, n (%)		
- None	13 (50)	9 (56.3)
- Father	4 (15.4)	2 (12.5)
- Mother	7 (26.9)	4 (25)
- Both parents	1 (3.9)	0
- Sibling(s)	1 (3.9)	1 (6.3)
Having siblings, n (%)		
- Yes	17 (65.4)	12 (75)
Family income, n (%)[£]		
- Low	12 (46.2)	7 (43.7)
- Medium	10 (38.4)	7 (43.7)
- High	4 (15.4)	2 (12.5)
Household furry pets, n (%)		
- 0	17 (65.4)	10 (62.5)
- 1	3 (11.5)	2 (12.5)
- 2	6 (23.1)	4 (25)
Antibiotic usage during infancy, n (%)	12 (46.2)	7 (43.8)
Daycare attendance	0	0

[£]Categorized according to Thai GDP 2016, low income defined as income < 11,500 USD/year, middle income 11,500–28,500 USD/year, high income > 28,500 USD/year

Table S3. Linear mix factor model results *Bifidobacteriaceae*, *Erysipelotrichaceae*, *Bacteroidaceae* and *Enterobacteriaceae* with interactions between cesarean section and antibiotic usage during infancy period.

Clinical factor	Family	Genus	coef	Stderr	pval	qval	odd-ratio
Cesarean section	*Bifidobacteriaceae*	N/A	-0.3664	0.2480	0.1467	0.8006	-0.5287
	Erysipelotrichaceae	N/A	-0.7658	0.54055	0.1635	0.8220	-1.1049
	Bacteroidaceae	N/A	0.6162	0.5175	0.2401	0.8715	0.8890
	Enterobacteriaceae	*Klebsiella*	1.0974	0.3898	0.0073*	0.1785	2.9964
Antibiotic usage during infancy period	*Bifidobacteriaceae*	N/A	0.0862	0.23808	0.7188	0.9312	0.1244
	Erysipelotrichaceae	N/A	-0.3105	0.51004	0.5457	0.9102	-0.4479
	Bacteroidaceae	N/A	-0.1145	0.51404	0.8247	0.9312	-0.1652
	Enterobacteriaceae	*Morganella*	1.3127	0.6236	0.0410*	0.38669	1.8939
	Enterobacteriaceae	*Proteus*	-2.8272	1.2447	0.0280*	0.3274	-4.0788

Coef; coefficient. Stderr; standard error. pval; *p*-value. qval; q-value
*p-value and q-value < 0.05

Table S4. Total spectral proteins, spectral counts per samples and spectral counts assigned to protein sequences comparing between each group.

Item	Total spectral protein (Spectral counts per sample)				Spectral count assigned to protein sequences				Unique proteins
	Control	AD	FA	AD+FA	Control	AD	FA	AD+FA	
Annotated protein⁺	35,757 (1,702)	25,806 (1,843)	150 (50)	10,083 (2,016)	21,297	17,370	150	8,415	31,084
Unannotated protein	7,553 (360)	5,362 (383)	31 (10)	2,149 (430)	4,512	3,546	31	1,780	6,495
Total	43,310 (2,062)	31,171 (2,226)	181 (60)	12,232 (2,446)	25,809	20,916	181	10,195	37,579

AD, atopic dermatitis, FA, food allergy
⁺Annotated proteins are based on protein ID from Uniprot database

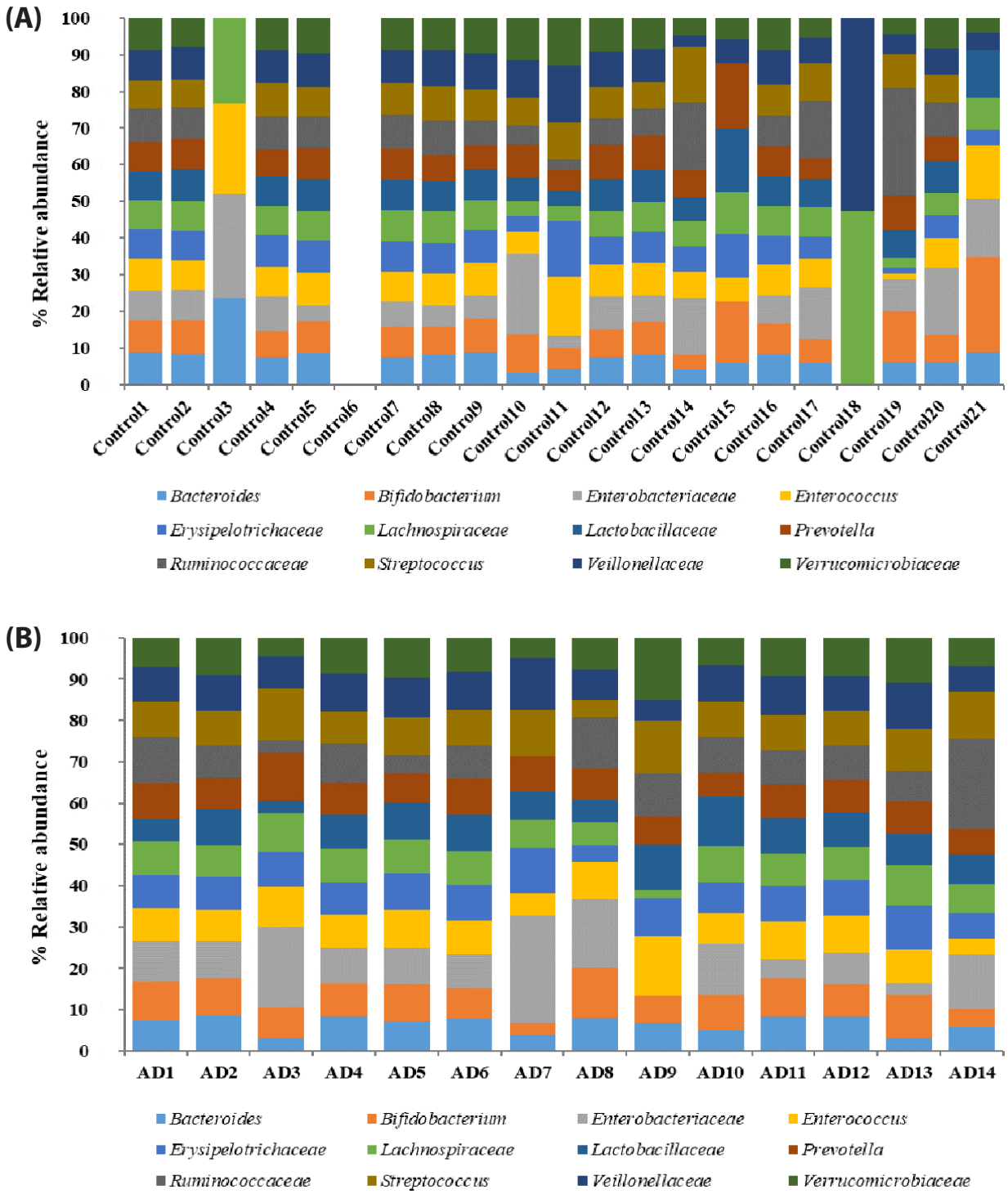

Figure S1. Relative abundance of protein expression of 12 selected bacterial families showing in individuals. Bar charts represented the average metaproteomic distribution by family in controls, AD, and AD+FA groups. AD, atopic dermatitis, FA, food allergy.

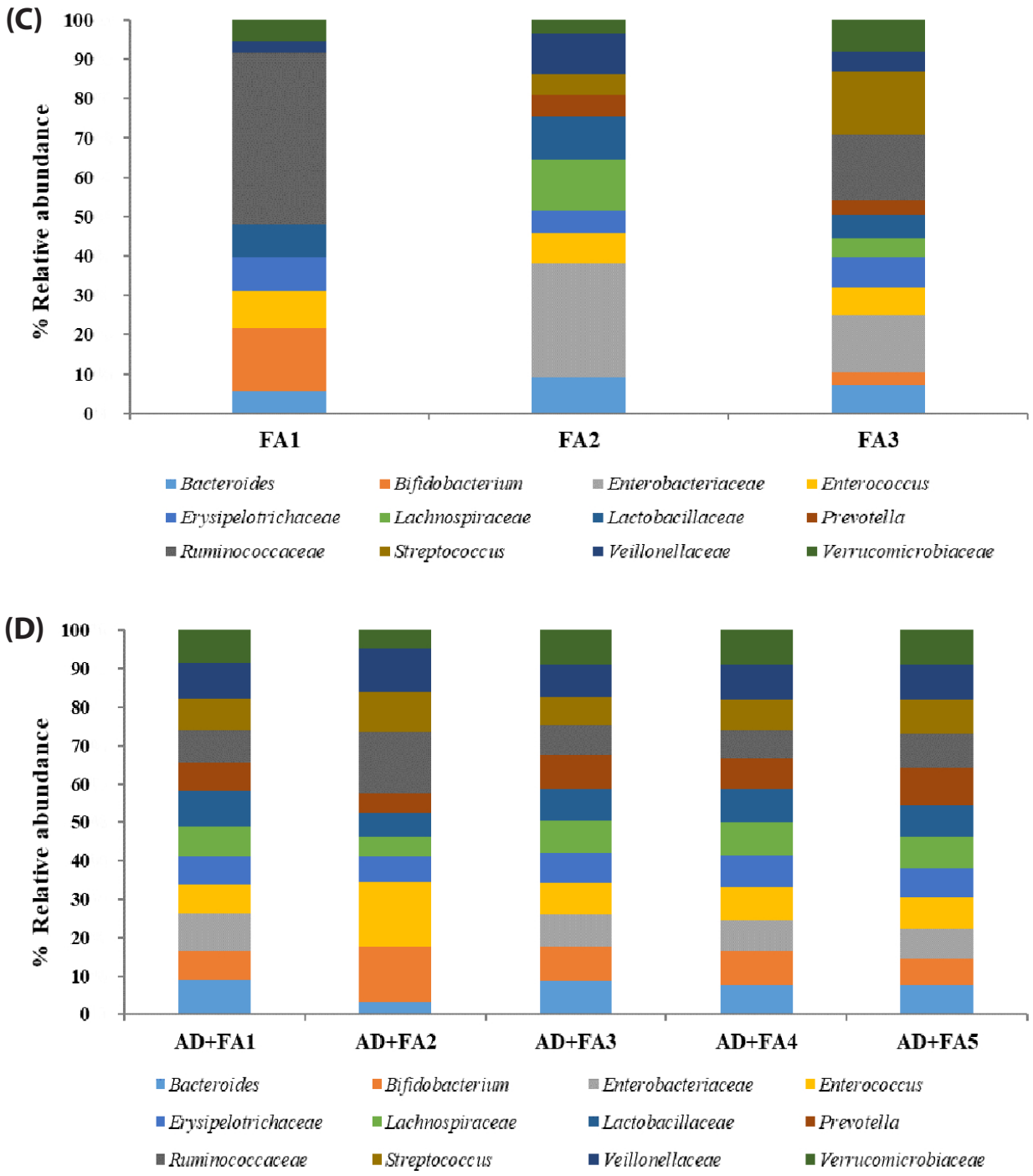

Figure S1. (Continued)

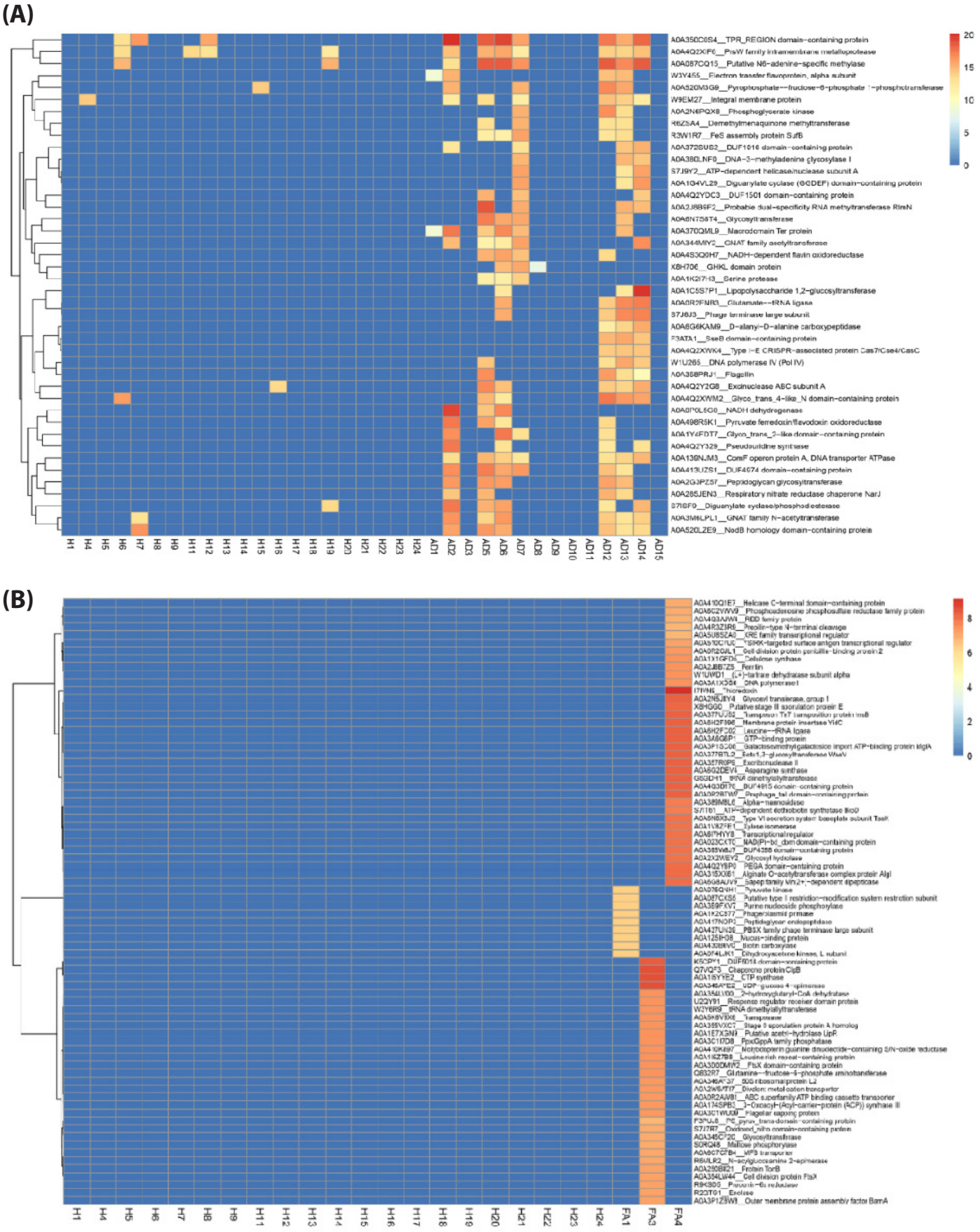

Figure S2. Relative protein expression of 12 bacterial familes compared between (A) controls and AD, (B) controls and FA, (C) controls and AD+FA. H, control, AD, atopic dermatitis, FA, food allergy.

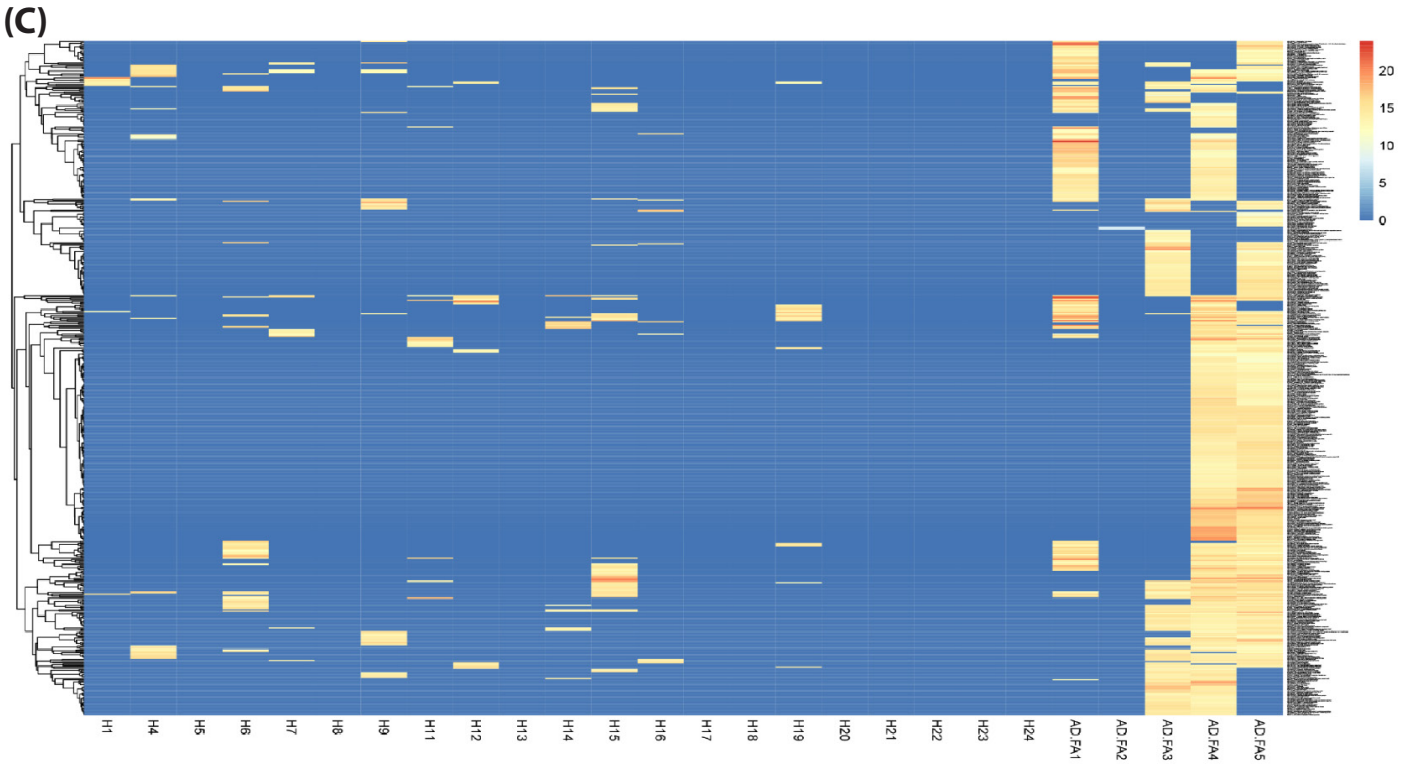

Figure S2. (Continued)

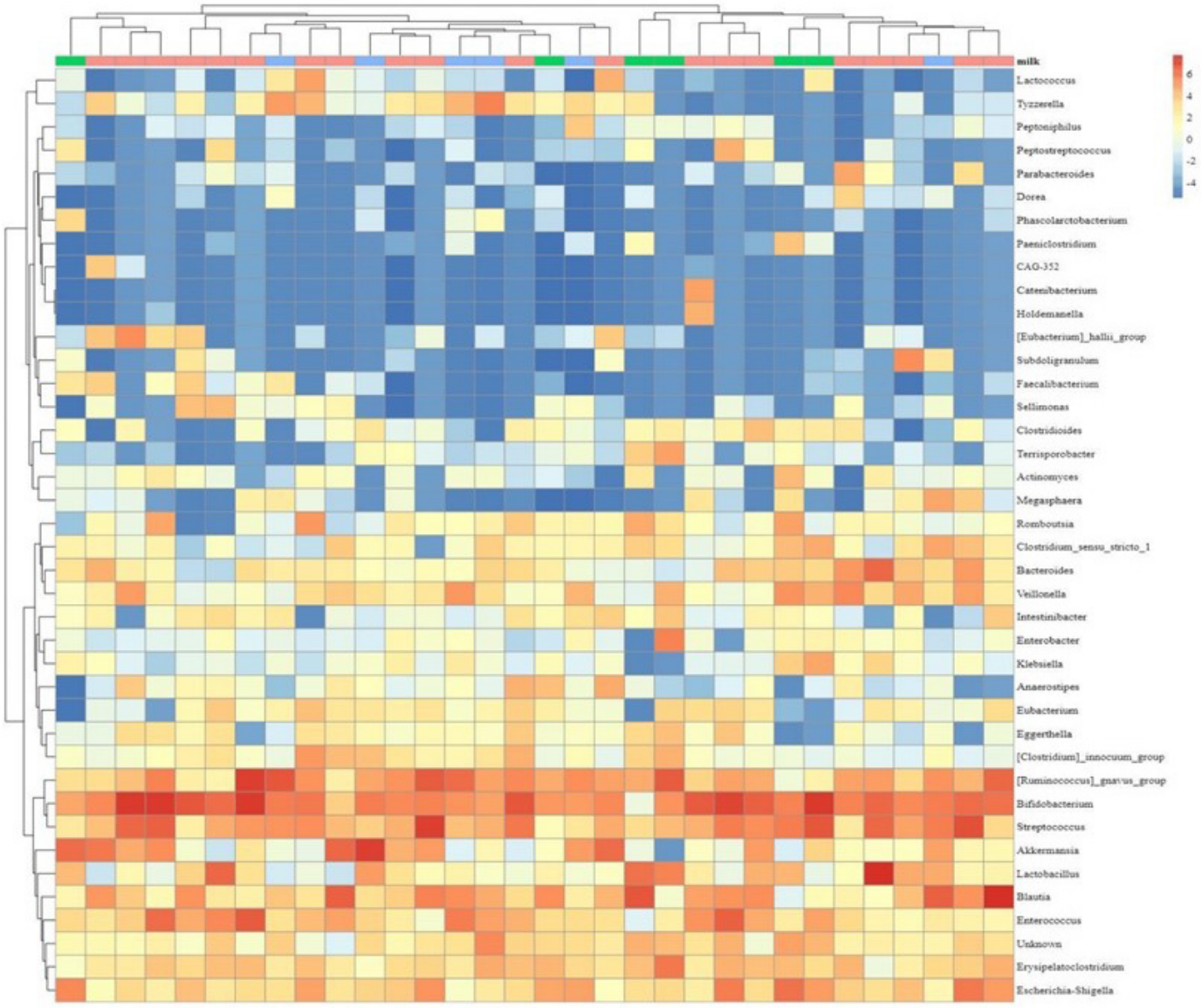

Figure S3. Hierarchical clustering dendrogram of gut microbiota. Dendrograms of gut microbiota in participants who received infant formula without probiotics or prebiotics (green), infant formula containing prebiotics (pink), and infant formula containing probiotics (blue) for the top 40 families, based on Bray-Curtis distance metrics. The heat map depicts the relative abundance of each family in each sample. The color scale for the heat map is displayed in the upper right corner of the figure.

References

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2. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. Journal of applied microbiology. 2004 Dec 1;97(6):1166-77.
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