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Temporal and long-term gut microbiota variation in allergic disease: A prospective study from infancy to school age

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Abstract

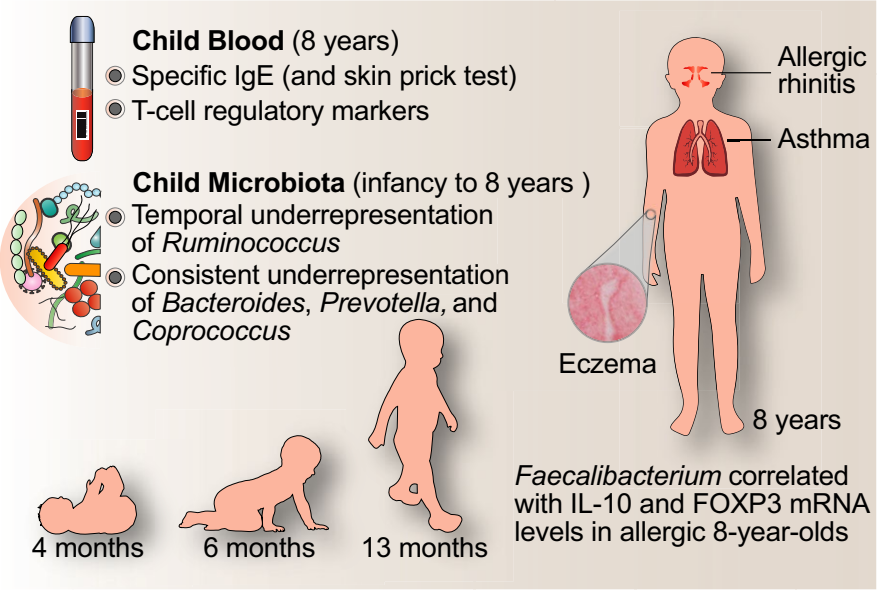
Background: Compositional changes in the early-life gut microbiota have been implicated in IgE-associated allergic diseases, but there is lack of longitudinal studies. We examined gut microbiota development from infancy to school age in relation to onset of IgE-associated allergic diseases. At 8 years of age, we also examined the relationship between gut microbiota and T-cell regulation, estimated as responses to polyclonal T-cell activation.

Methods: Stool samples were collected from 93 children at 4, 6, 13 months, and 8 years of age. The gut microbiota was profiled using 16S rRNA gene sequencing. Peripheral blood was drawn from all children, and mononuclear cells were polyclonally activated. Levels of IL-10 and FOXP3 mRNA copies were determined using real-time quantitative reverse transcriptase-PCR.

Results: At 8 years of age, 21 children were diagnosed with IgE-associated allergic disease and 90% displayed allergic comorbidity. Seventy-two children were nonallergic and nonsensitized. Statistical tests with multiple testing corrections demonstrated temporal underrepresentation of *Ruminococcus* and consistent underrepresentation of *Bacteroides*, *Prevotella*, and *Coprococcus* in allergic compared to nonallergic children from infancy to school age. The gut microbiota of the allergic 8-year-olds was enriched in *Bifidobacterium* and depleted of *Lactobacillus*, *Enterococcus*, and *Lachnospira*. In allergic 8-year-olds, *Faecalibacterium* correlated with IL-10 mRNA levels ($r_s = 0.49$, $P_{adj} = 0.02$) with the same trend for FOXP3 ($r_s = 0.39$, $P_{adj} = 0.08$).

Conclusions: We identified both temporal and long-term variation in the differential abundance of specific bacterial genera in children developing IgE-associated allergic disease. Improved dietary interventions aiming at expanding immune-modulatory taxa could be studied for prevention of allergic disease.

KEYWORDS
allergy, diversity, intestinal colonization, microbiome, T-cell response



GRAPHICAL ABSTRACT

Gut microbiota development was studied prospectively from infancy to school age in relation to onset of IgE-associated allergic diseases. We also examined correlations between gut microbiota and T-cell regulatory markers. We identified temporal underrepresentation of *Ruminococcus* and consistent underrepresentation of *Bacteroides*, *Prevotella*, and *Coprococcus* in allergic compared to nonallergic children from infancy to school age. In allergic 8-year-olds, *Faecalibacterium* correlated with polyclonally activated IL-10 mRNA levels of peripheral blood mononuclear cells, with the same trend for FOXP3.

1 | INTRODUCTION

Microbial exposure of the mucosal surfaces is critical for immune development and regulation.[1] In the postnatal period, the progressive colonization of the gastrointestinal tract (GIT) parallels maturation of both the innate and adaptive immune system.[2,3] It is increasingly recognized that this colonization process is critical to drive immune maturation, but it still remains to define what constitute a GIT microbiota that promote tolerance establishment.[1,2]

Although the prevalence of eczema, respiratory allergic disease, and food allergy varies with age, allergic comorbidity is common,[4] and a shared risk factor could be bacterial depletion in the GIT. Underrepresentation of specific bacterial taxa has been implicated in the development of eczema and asthma (reviewed in Ref.[2]) and more recently in food sensitization[5] and food allergy.[6,7] These previous studies focused on the early establishment of the GIT microbiota, and there is still lack of longitudinal studies. Using massive parallel sequencing, we conducted a longitudinal prospective study to examine the development, composition, and diversity of the gut microbiota from infancy to school age and related that to onset of allergic disease in the first 8 years of life. We also examined gut microbial composition and its relationship with responses to polyclonal T-cell activation as indicators of T-cell regulation.

2 | METHODS

2.1 | Study design

We included 93 children from a randomized placebo-controlled trial investigating the effects of probiotics for primary prevention of allergic disease (NCT 00894816), with stool samples collected at 4, 6, 13 months, and 8 years of age that were either diagnosed with IgE-associated allergic disease at 8 years or nonallergic. As described,[8,9] 179 infants with no prior allergic manifestations were randomized to daily intake of infant cereals with (n = 89) or without (n = 90) the probiotic *Lactobacillus paracasei* F19 (*Lactobacillus* F19) 1×10^8 colony-forming units per serving from 4 to 13 months of age. Parents reported signs and symptoms of eczema, wheeze, asthma, food allergy, and medications using diary cards. Study staff reviewed the diary cards and interviewed parents monthly using a structured questionnaire on signs and symptoms of allergic disease. Written and oral information was provided before enrollment and parents signed written consent. In all, 171 completed the intervention phase that was conducted in 2001-2003. A clinical follow-up was conducted in 2009-2011 at 8-9 years of age.[10] Approval of the trial was obtained from the ethics committee in Umeå, Sweden (00-028 and 2008-214-31M).

2.2 | Diagnosis of allergic disease at 8 years of age

All children were examined by one of two trained study pediatricians (Dr C. E. West or Dr M. Borgström). Parents completed an adapted questionnaire used in the Obstructive Lung disease in northern Sweden pediatric cohort study, based on the ISAAC core questions[11] with additional questions on symptoms of wheeze, eczema, and other allergic conditions.[12] All children underwent a standardized spirometry reversibility test, a skin prick test to relevant environmental allergens, and specific IgE tests to cow's milk, egg white, wheat, codfish, and peanut,[10] see Data S1.

As previously described,[10] clinical diagnoses were established based on information from clinical examinations, questionnaires, and review of medical records. The diagnosis of eczema included typical skin lesions responsive to topical steroids. The severity was examined using the SCORing Atopic Dermatitis (SCORAD) index.[13] Asthma included a physician's diagnosis of asthma with wheeze that responded to bronchodilator therapy and/or a clinical history of wheeze and an increase in forced expiratory volume in 1 second >12% from baseline following terbutaline inhalation at the study visit. Allergic rhinitis included a clinical history of symptoms suggestive of allergic rhinitis plus evidence of sensitization to inhalant allergens at the study visit. The diagnosis of IgE-mediated food allergy was made if there was a clinical history of immediate (<60 minutes) symptoms after contact with and/or ingestion of food and specific IgE levels >0.35 kU/L to the offending food. A diagnosis of allergic disease was made if there was a diagnosis of eczema, asthma, allergic rhinitis, and/or food allergy. A diagnosis of IgE-associated allergic disease included the former criteria plus evidence of an IgE association.

2.3 | Stool sampling and DNA isolation

Stool samples were collected at 4, 6, 13 months, and 8 years of age, see Data S1.

2.4 | 16S rRNA Metagenomic Sequencing Library Preparation and 16S rRNA gene sequencing

The library was prepared according to Illumina's Protocol (*Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*) with few modifications: The initial amplification was carried out for 20 cycles with the forward primer construct 5′-TCGTCGGCAGCGTCA GATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG**-3′, comprising the 341F universal bacterial primer sequence (in bold); and an Illumina specific adapter overhang sequence and the reverse primer construct 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC**TACHVG GGTATCTAATCC**-3′, comprising the universal bacterial primer 805R (in bold) and an Illumina specific adapter overhang sequence. After cleaning, a 5 μL aliquot of each of the 2-step samples was submitted to an Indexing reaction using the Nextera XT Index Kits v2 (Illumina Inc, San Diego, CA, USA), in a 13-cycle PCR. Purification of PCR products was carried out using Agencourt AmPure XP Beads (Beckman Coulter AB, Stockholm, Sweden) on a KingFisher Flex System (Thermo Fisher, Waltham, MA, USA). Finished libraries were quantified using Invitrogen Quant-iT fluorometric assay (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Samples were then pooled to equimolar amounts, all controls from the extraction phase, as well as a negative (blank) PCR control, were submitted to PCR and sequenced with the respective samples. 16S rRNA gene sequencing (region V3-V4) was used to profile the gut microbiome using Illumina MiSeq (v3 kit, 600 cycles; Illumina Inc, San Diego, CA, USA) at the Clinical Genomics Facility, Science for Life Laboratory, Solna, Sweden.

2.5 | Microbiota analyses

Initially read-pairs were demultiplexed using a wrapper script around QIIME (https://github.com/druvus/16S-demultiplexing) and stitched together using FLASH.[14] The composition and diversity of the gut microbiota were assessed using Quantitative Insights into Microbial Ecology (QIIME);[15] chimeric sequences were handled using UCHIME.[16] Of 288 samples, the total sequencing yield was 7 036 846 reads, with a mean of 21 370 reads per sample. Reads were assigned to operational taxonomic units (OTUs) using a closed-reference OTU picking protocol, where uclust[17] was applied to search sequences against a subset of the Greengenes (GG) version 13.8 database.[18] Reads were assigned to OTUs based on their best hit to this database at the 97% identity threshold. Reads that did not match a reference sequence were discarded. Taxonomy was assigned to each read by accepting the GG taxonomy string of the best matching GG sequence. OTUs that did not pass a filter of 0.005% of the total number of sequences were excluded resulting in 886 unique OTUs that were further included in the analyses. α-diversity was calculated using Faith's phylogenetic diversity.

2.6 | Blood sampling and isolation of peripheral blood mononuclear cells (PBMCs)

Venous blood was drawn in Vacutainer® tubes (Becton Dickinson, Plymouth, UK) supplied with heparin (10 IU/mL) without added preservative, and PBMCs were isolated by Ficoll-Paque gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden).

2.7 | PBMC stimulation, RNA extraction, and real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Peripheral blood mononuclear cells (10^6/mL) were incubated with a mixture of anti-CD3 monoclonal antibody (mAb) (clone OKT3, IgG_{2b}, 50 ng/mL, American Tissue Culture Collection, Rockville, MD) and anti-CD28 mAb (clone CD28.1, IgG_1, 1 μg/mL, DakoCytomation, Glostrup, Denmark) in HEPES-buffered RPMI 1640 supplemented with 0.4% human serum albumin and antibiotics in humid air with 5% CO_2 for 6 hours as described.[19] Parallel cultures of PBMCs in medium alone served as controls. Following incubation, cells were collected by centrifugation and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in RNase-free

water containing 1 kU/mL RNasin ribonuclease inhibitor (Promega, Madison, WI) and stored at −80°C until qRT-PCR analysis.

Concentrations of interleukin-10 (IL-10) and FOXP3 mRNA copies were determined using real-time qRT-PCR assays with gene-specific reverse transcription from total RNA in each qRT-PCR run using the 3′-primer as template and recombinant thermostable *Thermus thermophilus* (tTh) DNA polymerase (Applied Biosystems, Foster City, CA) or tTh (LightCycler 480 RNA master hydrolysis probes, Cat. No.04991885001; Roche, Mannheim, Germany) as reverse transcriptase. Reverse transcription was followed by qPCR with the same enzyme as polymerase, primers placed in different exons and a reporter dye marked probe placed over the exon boundary in the amplicon. Parallel qRT-PCR analysis of serial dilutions of an RNA copy standard was included in each qRT-PCR run. For sequences of primers and probes see Forsberg et al[20] for the IL-10 assay and Morgan et al[21] for the FOXP3 assay. The concentration of 18S rRNA was determined in each sample using a commercial real-time qRT-PCR assay (Applied Biosystems, Foster City, CA). The samples were compared to serial dilutions of a pool of total RNA extracted from polyclonally stimulated PBMCs.[22] One unit was defined as the amount of 18S rRNA in 10 pg of the RNA standard, which corresponds to approximately 100 lymphocytes. Samples were analyzed in triplicate in qRT-PCR for mRNAs and 18S rRNA. Emission from released reporter dye was monitored by the ABI prism 7700 sequence detection system (Applied Biosystems). Results were expressed as mRNA copies per 18S rRNA unit. All samples included in the study contained >20 units 18S rRNA per reaction mixture.

TABLE 1 Description of the study cohort

	Nonallergic (n = 72) Mean ± SD	Allergic (n = 21) Mean ± SD	*P*-value
Age, years	8.8 ± 0.2	8.8 ± 0.2	
Gestational age, weeks	40.2 ± 1.4	40.2 ± 1.3	
Birth weight, g	3722 ± 487	3553 ± 421	.011[a]
Birth length, cm	51.1 ± 2.2	50.5 ± 1.5	
Duration of breastfeeding, months	8.1 ± 4	6.9 ± 3.6	.08[a]
Weight (at 8 y), kg	32.8 ± 6.9	30.9 ± 5.6	
Length (at 8 y), cm	137.3 ± 6.4	136.8 ± 6	
BMI Z-score (at 8 y)	0.5 ± 1.2	0.1 ± 0.8	.071[a]
	%	%	
Girls	41.6	57.1	
First child	57.0	38.1	
Maternal allergy	33.3	61.9	.036[b]
Paternal allergy	40.2	57.1	
Both parents with allergy	16.6	47.6	.005[b]
Sibling(s) with allergy	36.1	57.1	.025[b]
Furry pets (at 8 y)	62.5	33.3	.025[b]
Never furry pets	18.1	52.4	.0005[b]
Lactobacillus F19 intervention (at 4-13 mo)	48.6	42.8	
Probiotics in the diet (at 8 y)	26.4	23.8	
Antibiotics ever during 13 mo	33.3	76.2	.027[b]
Antibiotics ever 13 mo-8 y	80.6	90.5	
Maternal University education	66.7	76.2	
Paternal University education	48.6	42.9	
Smoking mother	4.2	9.5	
Smoking father	2.8	9.5	

[a]For Wilcoxon rank-sum test.
[b]For the test of equal or given proportions.

2.8 | Statistics

The unpaired Wilcoxon rank-sum test or Test of Equal or Given Proportions were used to assess differences in demographic characteristics. To investigate relative changes in gut microbiota between allergic and nonallergic children over time the DESeq2 package[23] was used. This method tests the null hypothesis that the logarithmic fold change (LFC) between tested groups at a certain time point is zero. For differential analysis of count data, DESeq2 applies GLM (Generalized linear model), Bayes approach for estimation of shrinkage, maximum likelihood test for dispersions, and the Wald test; here, *P* values from the subset of OTUs that pass the independent filtering step ($\alpha = 0.1$) are adjusted for multiple testing using the Benjamini and Hochberg procedure ($\alpha = 0.05$).[23] LefSe pipeline (linear discriminant analysis [LDA] effect size)[24] was used for discovery of putative time point and allergy-related bacterial biomarkers. The LefSe algorithm uses nonparametric factorial Kruskal-Wallis sum rank test, unpaired Wilcoxon rank-sum test, and LDA, to estimate the effect size of each differentially abundant OTU; to pass significance, α parameter for tests was set to 0.05 and the threshold on the logarithmic score of LDA analysis was set to 2.0. Spearman's rho (r_s) was used to test associations between IL-10, FOXP3 mRNA expression levels, and OTUs, and reported *P* values were adjusted for multiple comparison using false discovery rate (FDR). Differences in α-diversity were estimated using unpaired Wilcoxon rank-sum test; differences in relative abundance of bacterial species between nonallergic and allergic groups at certain time points were estimated using Kruskal-Wallis test. Statistical analyses were performed using R 3.2.3[25] and SPSS version 23 (SPSS Inc. Chicago, IL, USA).

3 | RESULTS

3.1 | Participants

A description of the study cohort is presented in Table 1. At 8 years of age, 21 children were diagnosed with IgE-associated allergic disease while 72 remained free of any allergic manifestation and had no evidence of sensitization. In the former group, 20 children were sensitized to airborne allergens and 13 were sensitized to food allergens. Fourteen children had atopic eczema, 14 had allergic rhinitis, 4 had asthma, and 5 had food allergy. The majority was diagnosed with two or more IgE-associated

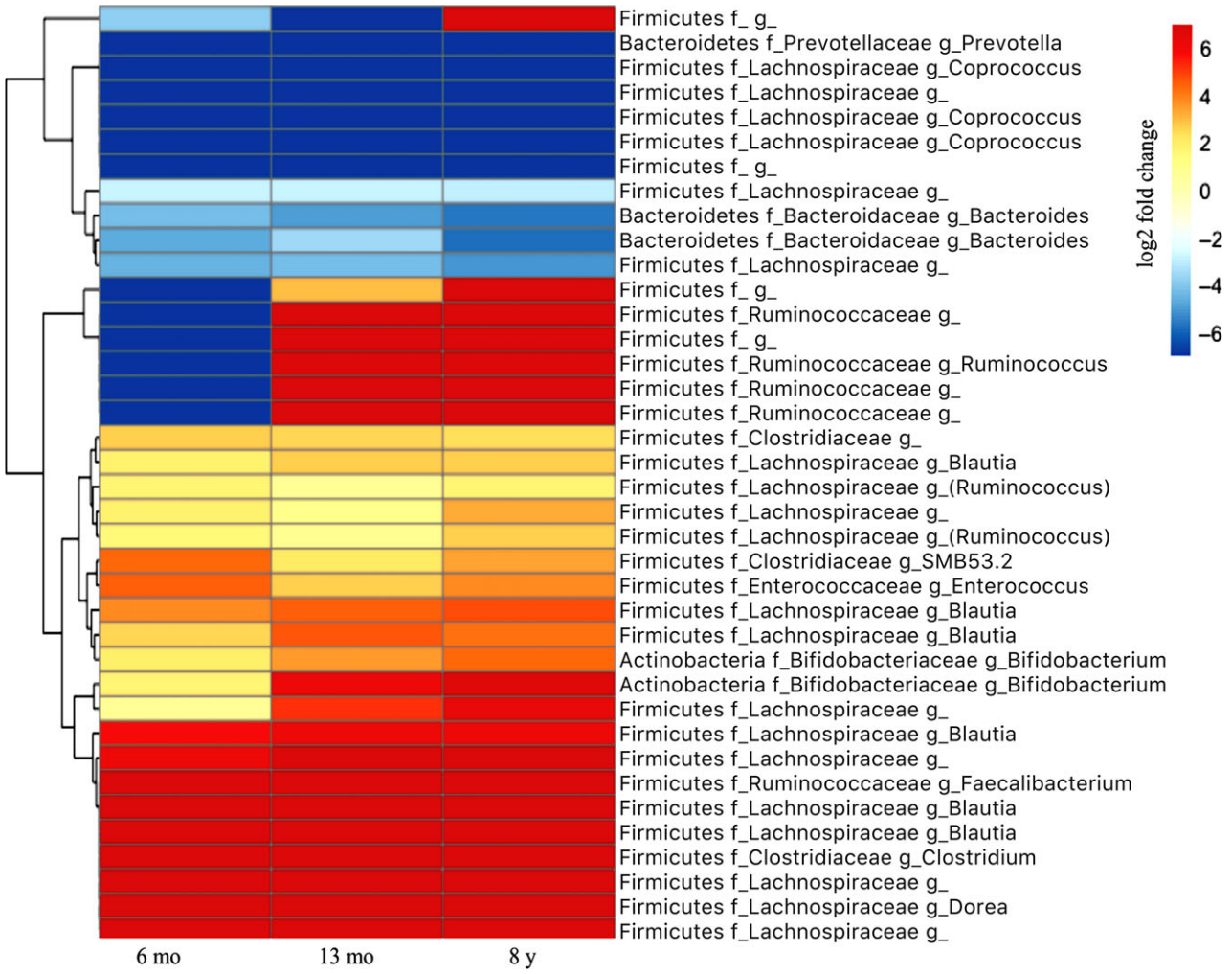


FIGURE 1 The 38 most significantly different OTUs, as a ratio, between allergic and nonallergic children over time; each cell presents a log2 fold change of relative abundance of a certain OTU between allergic and nonallergic children at a certain time point, normalized for the relative abundance of that OTU at baseline, that is 4 mo. N = 63 for the longitudinal analysis

allergic diseases at 8 years or had one diagnosed IgE-associated allergic disease plus a history of outgrown allergic manifestations upon allergen exposure (eczema, urticaria, food allergy, and/or wheeze/asthma). Only 2 children had a single IgE-associated allergic disease (Table S1).

3.2 | Gut microbiota development in allergic disease

The longitudinal design made it possible to study compositional changes in gut microbiota from infancy to school age in relation to development of IgE-associated allergic disease. DESeq2 revealed an interaction effect, that is differences in gut microbiota composition between allergic and nonallergic children over time. The heat map (Figure 1) depicts the 38 most significantly different OTUs over time; each cell presents a log2 fold change of relative abundance of a certain OTU between allergic and nonallergic children at a certain time point, normalized for the relative abundance of that OTU at baseline, that is 4 months. At that age, none had allergic disease nor had they been treated with probiotics. All presented results were statistically significant after correction for multiple testing ($P < .05$); *Prevotella* and *Coprococcus* were underrepresented in allergic compared to nonallergic children at all time points, whereas *Blautia*, *Faecalibacterium*, *Clostridium*, and *Dorea* were consistently overrepresented. OTUs assigned to the *Ruminococcaceae* family were underrepresented at age 6 months and then increased in allergic compared to nonallergic children in a time-dependent manner. *Bacteroides* was underrepresented at the early samplings and then decreased even further in allergic children, whereas the abundance of *Bifidobacterium* increased in allergic children with time. Next, we examined the effect of potential confounders by including breastfeeding duration, exposure to antibiotics, and furry pets in the DeSeq2 model. This model was even stronger ($P < .01$), and the main findings of both temporal and consistent over- and underrepresentation of signature OTUs remained robust; however, additional OTUs assigned to *Roseburia* were consistently overrepresented in allergic children (Figure S1).

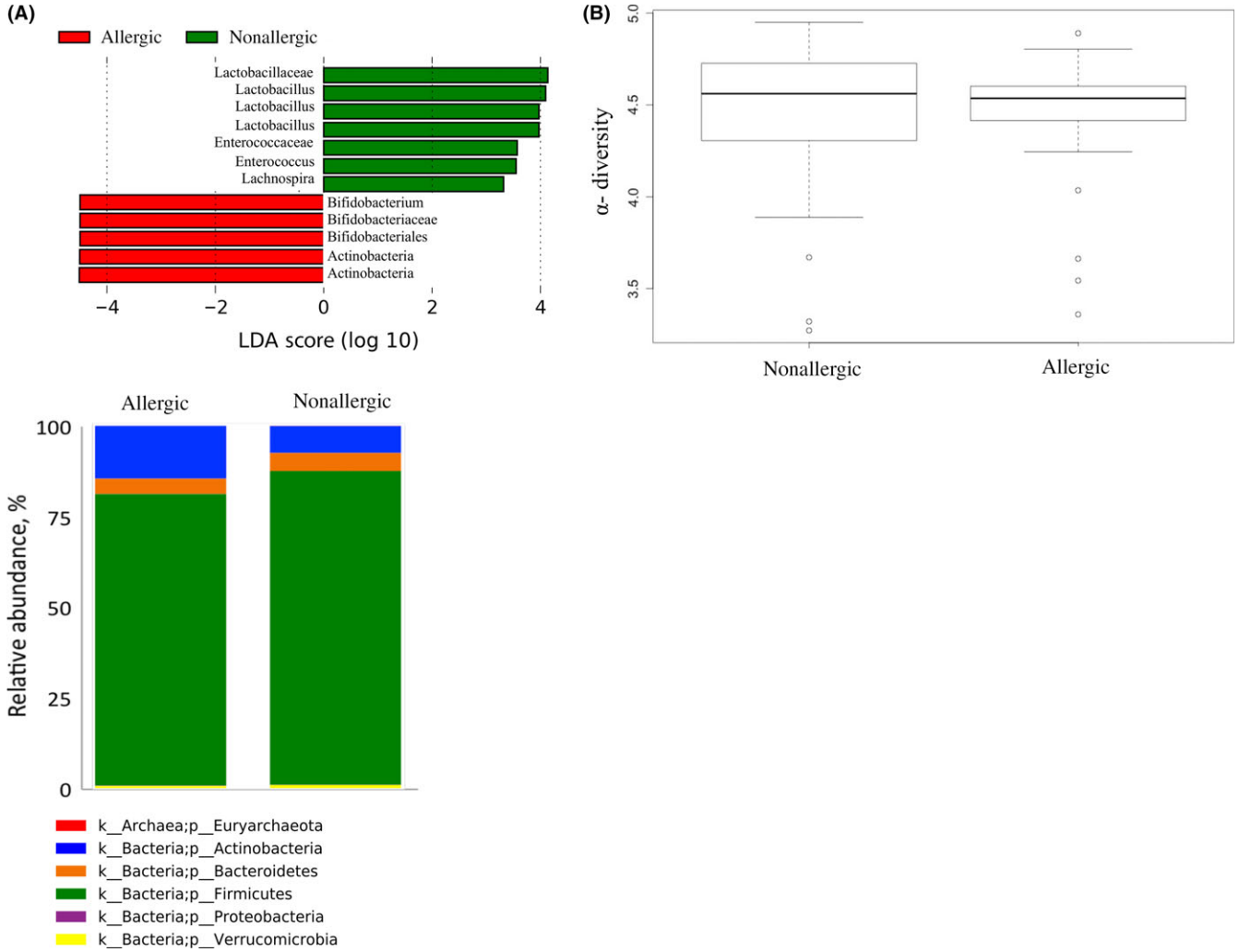


FIGURE 2 A, Relative abundance of bacterial taxa at 8 y of age (N = 93) with enrichment of *Bifidobacterium* in allergic children, and *Lactobacillus* and *Enterococcus* in nonallergic children. B, There was no difference in α-diversity between the groups at 8 y of age

3.3 | Taxa differences in allergic 8-year-olds

LefSe analyses revealed enrichment of *Bifidobacterium* in allergic 8-year-olds (Figure 2A), and OTUs assigned to *Bifidobacterium adolescentis* were most abundant (P = .009), while there was depletion of *Lactobacillus*, *Enterococcus*, and *Lachnospira* (Figure 2A). Within-group microbial diversity (α-diversity) was not affected by allergy diagnosis at that age (P = .38, Figure 2B) nor at the earlier samplings (data not shown).

3.4 | Correlations between regulatory markers and *Faecalibacterium*

We examined associations between IL-10 and FOXP3 mRNA levels in activated PBMCs stimulated with polyclonal T-cell activators and the 10 most abundant OTUs in allergic and nonallergic children at 8 years of age. *Faecalibacterium* correlated with IL-10 and FOXP3 mRNA levels (r_s = 0.49 P_{adj} = 0.02; and r_s = 0.38 P_{adj} = 0.08, respectively) in allergic children, although the latter fell just short of the significance level after correction for multiple testing (Figure 3). Additionally, there was a trend for OTUs belonging mainly to *Ruminococcaceae*, to correlate with both IL-10 and FOXP3, whereas OTUs from *Lachnospiraceae* correlated inversely with these markers in allergic children (Table S2). Unstimulated IL-10 and FOXP3 mRNA levels were low, and no correlations were observed (data not shown).

3.5 | Minor effects of probiotics

We previously reported higher counts of *Lactobacillus* and the supplemented strain *Lactobacillus* F19 in the probiotic group during the intervention.[8] Using the Illumina sequencing platform, *Lactobacillus* and *Enterococcus* appeared overrepresented in the placebo group at baseline, whereas *Phascolarctobacterium* and *Ruminococcus* appeared overrepresented in the *Lactobacillus* F19 group at that age, although these differences were not statistically significant. We could confirm higher relative abundance of *Lactobacillus* in the probiotic group at 6 months (P_{adj} < 0.05), but not at 13 months (Figure 4). There were

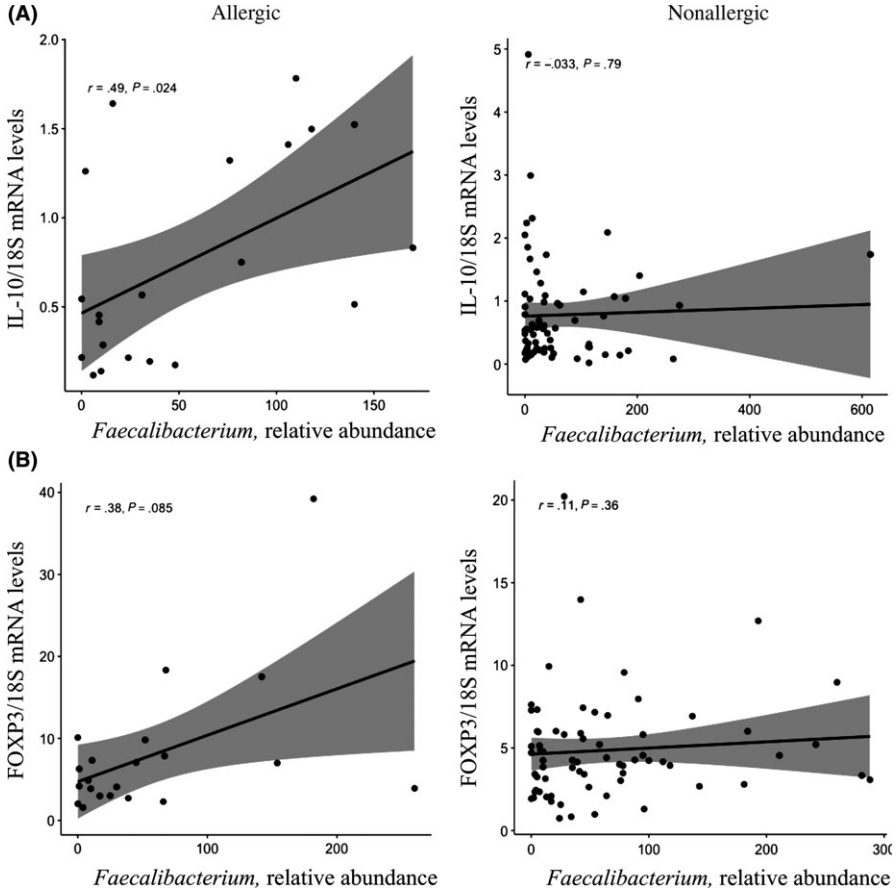


FIGURE 3 Correlation between (A) IL-10 and (B) FOXP3 mRNA expression levels in peripheral blood mononuclear cells after polyclonal T-cell stimulation and *Faecalibacterium* at 8 y of age (N = 93): *Faecalibacterium* correlated with IL-10 and FOXP3 mRNA levels in allergic but not in nonallergic children

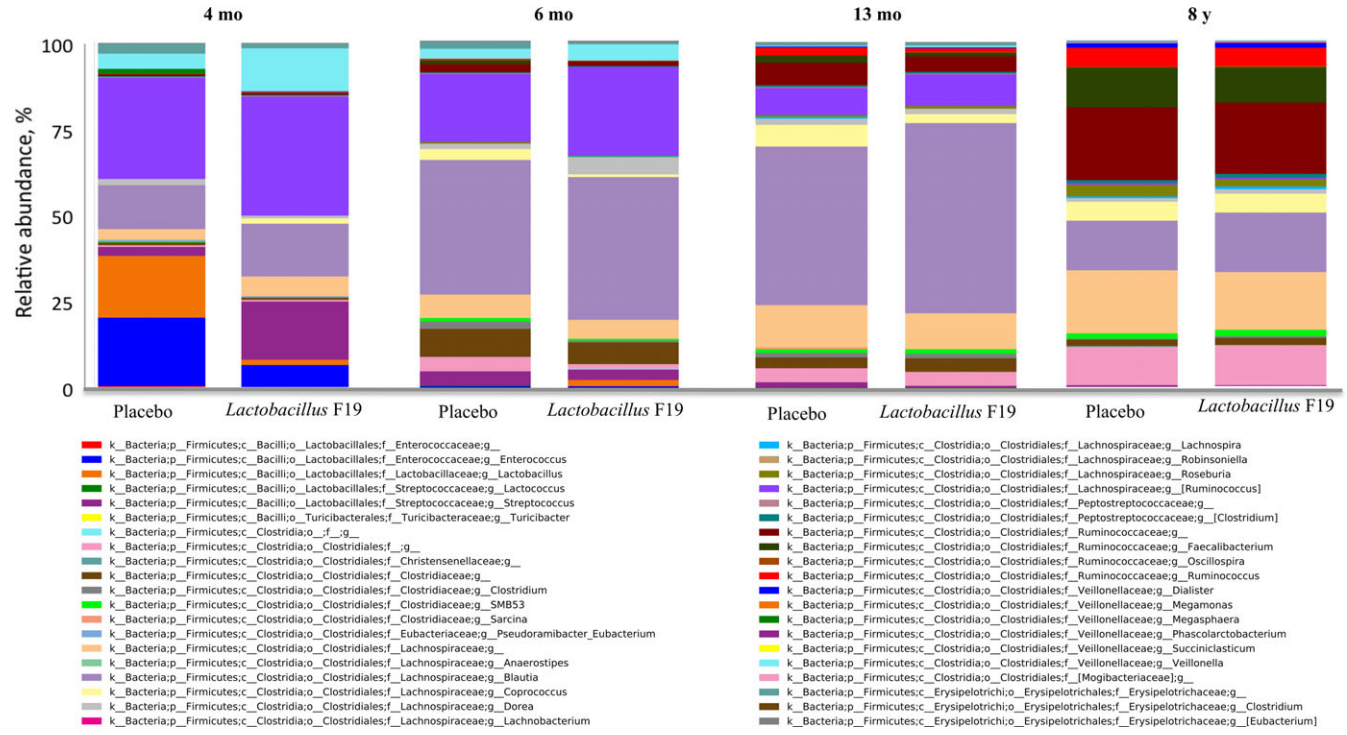


FIGURE 4 Relative abundance of bacterial taxa within the Firmicutes phylum according to intake of the probiotic *Lactobacillus* F19 in infancy at 4, 6, 13 mo, and 8 y of age. There was a higher relative abundance of *Lactobacillus* in the probiotic group at 6 mo ($P_{adj} < 0.05$), but not at the other samplings

no differences in global microbial composition according to probiotic supplementation (Figure S2).

4 | DISCUSSION

With its prospective long-term follow-up, this study extends previous findings of a role of gut microbial depletion in the development of IgE-associated allergic disease by demonstrating not only temporal but also long-term differences in the differential abundance of specific bacterial taxa until 8 years of age. After correction for multiple comparisons, we identified three bacterial genera, *Coprococcus*, *Prevotella*, and *Bacteroides*, which were consistently underrepresented in allergic children, whereas *Ruminococcus* was underrepresented at the 6-month sampling, then increases with age, and becomes overrepresented, compared with nonallergic children. We also identified *Faecalibacterium* to be associated with a tolerogenic response in allergic 8-year-olds.

Commensal Clostridia of the phylum Firmicutes are abundant in the gut microbiota and colonize a specific region of the mucosa, in close proximity to intestinal cells.[26] As Clostridia colonize regions nearby the mucosa, they are theorized to be key players in the cross talk with intestinal cells and they are potent producers of butyrate,[26] which has anti-inflammatory effects.[27] *Coprocccus*, a member of the Clostridia class, was consistently underrepresented in children that developed IgE-associated allergic disease. *Coprocuccus* is capable of fermenting carbohydrates to produce butyrate as the metabolic end product.[28] In addition to its anti-inflammatory effects, butyrate is also the preferred energy substrate in the colon with a role in maintaining gut barrier integrity.[26] Clinical studies have demonstrated reduced gut integrity not only in food allergy,[29] but also in eczema and asthma.[30] It is conceivable that lack of butyrate-producing bacteria has implications for gut integrity and that underrepresentation of genera, for example *Coprococcus*, may deteriorate gut barrier function. Further support for a role of *Coprococcus* is corroborated by a study reporting alleviation of infant eczema to be associated with increases in *Coprococcus eutactus*.[31] *Ruminococcus*, another member of Clostridia and a butyrate-producer, was underrepresented in the gut microbiota of 6-month-old infants that developed IgE-associated allergic disease in our study, but then increased with age to become overrepresented compared with children that remained nonallergic. In the Canadian CHILD cohort, underrepresentation of the *Ruminococcaceae* family was a feature in food-sensitized 1-year-olds, independent of delivery method, antibiotics, and breastfeeding.[5] In our previous study in Australian infants,[3] we identified that underrepresentation of *Ruminococcaceae* preceded the development of IgE-associated eczema. In addition, low abundance of *Ruminococcus* was associated with exaggerated Toll-like receptor-2–induced inflammatory innate cytokine responses at 6 months of age in that study.[3] Dysfunctional innate inflammatory responses have been reported to underlie deviant immune maturation of adaptive T-cell responses in allergic children,[32] and it is likely that the patterns and trajectory of both innate and adaptive T-cell responses are modulated by microbial exposure.

Here, we took the opportunity to examine the relationship between gut microbiota and markers of T-cell regulation. In allergic 8-year-olds, *Faecalibacterium* correlated to the expression levels of regulatory IL-10. *Faecalibacterium* is an important commensal bacterium, and thus far, *Faecalibacterium prausnitzii* is the sole identified species. *F. prausnitzii* has previously been demonstrated to induce a tolerogenic response in experimental colitis models and in adult PBMCs.[33,34] *Faecalibacterium* is considered a bacterial biomarker for intestinal health, but in our cohort, *Faecalibacterium* was consistently overrepresented in children developing IgE-associated allergic disease. Overrepresentation of specific subspecies of *F. prausnitzii* was reported in a metagenomic study including four patients with atopic eczema.[35] Analysis at the subspecies level was not the scope of this study, and the tolerogenic capacity of *F. prausnitzii* should be further explored in allergic disease.

Lachnospiraceae, another family within the Clostridia class, were inversely associated with IL-10 and FOXP3 mRNA expression in the allergic 8-year-olds, although these associations were not statistically significant after correction for multiple testing. At that age, *Lachnospira*, *Enterococcus*, and *Lactobacillus* were underrepresented in allergic compared with nonallergic children; however, this was not seen at the earlier samplings. In contrast, *Bacteroides* was consistently underrepresented in children developing IgE-associated allergic disease. Genera within the Bacteroidetes phylum are gut symbionts that stimulate epithelial mucin production and degradation, with importance for gut barrier function.[36] In another Swedish cohort, there was reduced α-diversity of Bacteroidetes and abundance of *Bacteroides* in infants subsequently developing atopic eczema;[37] however, long-term data on gut microbiota were not reported.

Genera that were overrepresented in children developing IgE-associated allergic disease included *Clostridium*. Specific *Clostridium* species have been associated with increased allergy risk.[2] As assessed by qPCR, colonization with *C. difficile* in infancy was associated with eczema, wheeze, and asthma in a large Dutch prospective cohort.[38] *Bifidobacterium*, particularly OTUs assigned to *B. adolescentis*, was also overrepresented in allergic 8-year-olds in our cohort. For further certainty at the species level, confirmation by qPCR or a metagenomics approach would be needed; however, our finding is consistent with a previous study using denaturing gradient gel electrophoresis that reported *B. adolescentis* to be the prevailing bifidobacterial species in allergic 5-year-olds.[39]

As the included children were part of a probiotic allergy prevention study in infancy,[9] we also assessed the impact of probiotics, but the effects were minor. *Lactobacillus* was increased at 6 months in infants that received the probiotic *Lactobacillus* F19, but no differences in global microbiota composition or diversity according to probiotic intake were observed at any sampling. Consistent with our results, one previous probiotic allergy prevention study using massive parallel sequencing has reported long-term data on gut microbiota composition, with only minor and transient differences according to probiotic intake.[40]

The main strengths of our study are the prospective design, repeated stool samplings for analysis with culture-independent

massive parallel 16S rRNA gene sequencing technology, and extensive and careful clinical characterization of the allergic phenotype. The effects of delivery mode and early feeding[41] that have major influences on gut microbiota establishment could be disregarded as all included infants were vaginally delivered and breastfed. When controlling for breastfeeding duration, antibiotics, and exposure to furry pets[42] in the longitudinal analysis, our main findings remained robust. Although commonly used in clinical studies both for practical and ethical reasons, it remains a limitation that we used fecal samples and not mucosal biopsies, as the mucosa-associated microbiota may interact more directly with the immune system.[43] A possible limitation is that we did not study Treg cells; however, as it can be difficult to define Treg cells,[44] we chose to analyze IL-10 and FOXP3 as regulatory markers. Additionally, the resolution was not at the species level and metagenomics studies are warranted.

In summary, we identified three bacterial genera, *Coprococcus*, *Prevotella*, and *Bacteroides*, which were consistently underrepresented in allergic children, whereas *Ruminococcus* was temporally underrepresented. We also identified *Faecalibacterium* to be associated with a tolerogenic response in allergic 8-year-olds. These observations suggest directions for the development of improved dietary interventions aiming at expanding immune-modulatory taxa for prevention of allergic disease.

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CONFLICTS OF INTEREST

Dr CE West has received research support from Arla Foods; fees for lectures from Arla Foods, Semper AB, and Nutricia; and royalties from UptoDate. Dr O Hernell is a member of Scientific Advisory Boards of Semper AB, Hero, and Arla; and received research grants/honoraria from Arla, Hero, Mead Johnson Nutrition, and Semper. Dr L Engstrand received research support from Ferring Pharmaceuticals and fees for lectures from BioGaia AB. Dr K Simonyté Sjödin, Dr M-L Hammarström, Dr P Rydén, and Dr A Sjödin have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

CE West, ML Hammarström, O Hernell, and L Engstrand designed the study. K Simonyté Sjödin did the bioinformatics and statistical analysis with support from P Rydén and A Sjödin. K Simonyté Sjödin, P Rydén, and CE West interpreted data. ML Hammarström, O Hernell, A Sjödin, and L Engstrand provided intellectual input and contributed to the discussions. K Simonyté Sjödin and CE West drafted the first version of the manuscript. All authors contributed to the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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