

Low diversity of the gut microbiota in infants with atopic eczema

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Background: It is debated whether a low total diversity of the gut microbiota in early childhood is more important than an altered prevalence of particular bacterial species for the increasing incidence of allergic disease. The advent of powerful, cultivation-free molecular methods makes it possible to characterize the total microbiome down to the genus level in large cohorts.

Objective: We sought to assess microbial diversity and characterize the dominant bacteria in stool during the first year of life in relation to atopic eczema development.

Methods: Microbial diversity and composition were analyzed with barcoded 16S rDNA 454-pyrosequencing in stool samples at 1 week, 1 month, and 12 months of age in 20 infants with IgE-associated eczema and 20 infants without any allergic manifestation until 2 years of age (ClinicalTrials.gov ID NCT01285830).

Results: Infants with IgE-associated eczema had a lower diversity of the total microbiota at 1 month ($P = .004$) and a lower diversity of the bacterial phylum Bacteroidetes and the genus *Bacteroides* at 1 month ($P = .02$ and $P = .01$) and the phylum Proteobacteria at 12 months of age ($P = .02$). The microbiota was less uniform at 1 month than at 12 months of age, with a high interindividual variability. At 12 months, when the microbiota had stabilized, Proteobacteria, comprising gram-negative organisms, were more abundant in infants without allergic manifestation (Empirical Analysis of Digital Gene Expression in R [edgeR] test: $P = .008$, $q = 0.02$).

Conclusion: Low intestinal microbial diversity during the first month of life was associated with subsequent atopic eczema. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Allergic disease, *Bacteroides* species, diversity, eczema, hygiene hypothesis, infant, microbiota, molecular microbiology, pyrosequencing, *Sutterella* species

It is debated whether low diversity of the gut microbiota in infancy is more important than the prevalence of specific bacterial taxa when trying to explain why the prevalence of allergic disease is increasing in affluent countries. Initially, several studies using conventional cultivation or fluorescent *in situ* hybridization reported differences in the intestinal microbiota at a species level between allergic and nonallergic children.¹⁻³ Allergic infants were colonized less often with *Bacteroides* species and bifidobacteria^{1,2} and more often with *Staphylococcus aureus*,² and they had a lower ratio of bifidobacteria to clostridia.³ However, there have been contradictory results in more recent studies. Two large European prospective studies did not confirm any relationship with any particular bacterial group.^{4,5}

As an alternative explanation, it has been suggested that low diversity of the intestinal microbiota would explain the increase in allergic disease in affluent societies.^{6,7} The underlying rationale is that the gut immune system reacts to exposure to new bacterial antigens and repeated exposure would enhance the development of immune regulation. Although this theory emerged more than a decade ago,⁸ there are still only a few studies relating this diversity with allergy, likely because of methodological limitations. In 3 studies using molecular techniques, terminal RFLP,⁶ and denaturing gradient gel electrophoresis,^{9,10} infants with sensitization¹⁰ or eczema^{6,9} were reported to have fewer peaks/bands than healthy infants. Yet no specific microbes were identified with these molecular methods. Furthermore, the sensitivity of the methods appears to be low because the median number of peaks/bands was much lower than the expected number of bacterial species.^{6,9,10}

A new generation of powerful noncultivation-based microbiologic methods has now made it possible to analyze the total microbiota down to the genus level, even in large cohorts.^{11,12} Previously uncultivated bacteria can now be detected, and there is no need to decide what bacteria to analyze in advance. Thus the assessment can be made without prejudice. This will allow for more comprehensive knowledge of the intestinal microbiota and its effects on the immune system. We have used barcoded 16S rRNA 454-pyrosequencing¹³ to assess the microbial diversity and characterize the dominant bacteria in stool during the first year of life in infants who either had atopic eczema or did not have any allergic manifestation up to 2 years of age.

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Abbreviations used

edgeR: Empirical Analysis of Digital Gene Expression in R
 OTU: Operational taxonomic unit
 RDP: Ribosomal Database Project

METHODS**Study design**

The infants included in this study were part of a larger study in southeastern Sweden between 2001 and 2005 evaluating allergy prevention with the probiotic *Lactobacillus reuteri* ATCC 55730.¹⁴ In this study the infants received probiotics or placebo daily from day 1 to 3 until 12 months of age. Clinical follow-ups were done at 1, 3, 6, 12, and 24 months of age, and telephone interviews were done at 2, 4, 5, 8, 10, and 18 months of age. A questionnaire was completed on each occasion. Stool samples were collected from the infants at 5 to 7 days and 1 and 12 months of age. The samples were immediately frozen at -20°C after collection and later stored at -70°C . Among the 188 infants completing the original study and from whom stool samples were available from all 3 sampling occasions, 20 infants with atopic eczema and 20 without any allergic manifestation were randomly selected for this study.

There were no differences regarding potential confounders, such as sex, birth order, cesarean section, family history of allergic disease, breast-feeding, antibiotics, and probiotic supplementation, between the infants with and without atopic eczema (Table I). Children admitted to the neonatal ward during the first week of life were excluded from the original study. All infants were breast-fed for at least 1 month, and no infant received antibiotics before 1 month of age. Informed consent was obtained from both parents before inclusion. The Regional Ethics Committee for Human Research at Linköping University approved the study. The study is registered at ClinicalTrials.gov (ID NCT01285830).

Diagnostic criteria of atopic eczema

Eczema was defined as a pruritic, chronic, or chronically relapsing noninfectious dermatitis with typical features and distribution.¹⁴ The diagnosis of atopic eczema required that the infant with eczema was sensitized also.¹⁵ Infants were regarded as sensitized if they had at least 1 positive skin prick test response, detectable circulating allergen-specific IgE antibodies, or both. Skin prick tests were done on the volar aspects of the forearm with egg white, fresh skimmed cow's milk (lipid concentration, 0.5%), and standardized cat, birch, and timothy extracts (Soluprick; ALK-Abelló, Hørsholm, Denmark) at 6, 12, and 24 months of age. Histamine hydrochloride (10 mg/mL) was used as a positive control, and albumin diluents were used as a negative control. The test result was regarded as positive if the mean diameter of the wheal was greater than 3 mm. Circulating IgE antibodies to egg white and cow's milk were analyzed at 6, 12, and 24 months of age in venous blood (UniCap Pharmacia CAP System; Pharmacia Diagnostics, Uppsala, Sweden). The cutoff level was 0.35 kU/L, according to the protocol of the manufacturer. In addition, circulating IgE to a mixture of food allergens, including egg white, cow's milk, cod, wheat, peanut, and soybean, was analyzed at 6, 12, and 24 months of age (UniCap Pharmacia CAP System, fx5, Pharmacia Diagnostics).

DNA extraction, 16S rRNA gene amplification, and sequencing

Extraction of bacterial DNA from the fecal samples and 16S rRNA gene amplification were done according to the methods described in a previous publication,¹³ with the following modifications: the primer pair used, targeting the variable regions 3 and 4 of the 16S rRNA gene, was 341f 5'-CCTAC GGGGGCCWGCAG with adaptor B and 805r 5'-GACTACHVGGGTAT CTAATCC with adaptor A,¹⁶ and sample-specific sequence barcodes consisting of 5 nucleotides were used. The barcodes contained no homopolymers, and a pair of barcodes differed in at least 2 positions. A negative PCR reaction without template was also included for all primer pairs in each run. The PCR was run for 25 cycles. The PCR products with proximal lengths of 450 bp

TABLE I. Descriptive data of children included in the study

	Atopic eczema, % (no.)	Healthy, % (no.)	P value*
Boys	60 (12)	50 (10)	.53
Firstborn	45 (9)	50 (10)	.75
Cesarean delivery	15 (3)	0 (0)	.23
Furred pets	0 (0)	5 (1)	1.00
Maternal atopy	85 (17)	90 (18)	1.00
Paternal atopy	70 (14)	60 (12)	.51
Breast-feeding			
1 mo	100 (20)	100 (20)	1.00
12 mo	25 (5)	35 (7)	.49
Antibiotics			
1-12 mo	15 (3)	30 (6)	.45
12-24 mo	50 (10)	30 (6)	.20
Day care			
0-12 mo	0 (0)	5 (1)	1.00
12-24 mo	70 (14)	85 (17)	.45
Probiotic group	30 (6)	55 (11)	.11

*The χ^2 test was used except when the expected frequency for any cell was less than 5, when the Fisher exact test was used.

were purified with AMPure beads (Becton Dickinson, Franklin, NJ) by using a Magnet Particle Separator (Invitrogen, Carlsbad, Calif). The concentrations were measured with a Qubit fluorometer (Invitrogen), the quality was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, Calif), and the samples were pooled together and amplified in PCR mixture in oil emulsions and sequenced on different lanes of a 2-lane PicoTiterPlate on a Genome Sequencer FLX system (Roche, Basel, Switzerland) at the Royal Institute of Technology in Stockholm.

Sequence processing and taxonomic classification

Sequence processing was carried out with the AmpliconNoise software package,¹⁷ correcting for errors introduced in PCR and pyrosequencing, as well as removing chimeric sequences. Also, reads lacking a correct primer, having less than 360 successful pyrosequencing flows, or both were removed.¹⁷ Denoised sequences were trimmed to 198 bp after primer and barcode removal and clustered by means of complete linkage clustering into operational taxonomic units (OTUs) at the 97% similarity level with AmpliconNoise.¹⁷ Each denoised sequence, as well as the most abundant sequence for each OTU, was Basic Local Alignment Search Tool searched with default parameters against a local Basic Local Alignment Search Tool database comprising 836,814 near full-length bacterial 16S rRNA gene sequences from the Ribosomal Database Project (RDP), version 10.10.¹⁸ The sequences inherited the taxonomic annotation (down to the genus level) of the best-scoring RDP hit fulfilling the criteria of 95% or greater identity over an alignment of length of 180 bp or greater. If no such hit was found, the sequence was classified as "no match." If multiple best hits were found and these had conflicting taxonomies, the most detailed level of consensus taxonomy was assigned to the OTU.

After removal of pyrosequencing noise and chimeric sequences, 271,355 high-quality, typically 198-bp-long sequence reads remained, with 1,137 to 12,909 reads per sample (mean, 2,261). These corresponded to 3,597 unique sequences and 1,818 OTUs clustered at the 97% similarity level by using complete linkage clustering. The majority (98%) of reads were of clear bacterial origin and had an RDP relative within 95% sequence similarity. Statistics on numbers of sequences and OTUs are presented in Table E1 in this article's Online Repository at www.jacionline.org.

Statistical analysis

Statistical significance testing overrepresentation and underrepresentation of the bacterial lineages was made at the phylum, class, genus, and OTU (3% dissimilarity) levels. Comparisons were made with the Bioconductor R package Empirical Analysis of Digital Gene Expression in R (edgeR),¹⁹ and

TABLE II. Shannon diversity index of the total microbiota, dominant phyla, and significant genera in stool samples obtained at various ages from infants who did or did not have atopic eczema during the first 2 years of life

	Atopic eczema (n = 20), median	IQR	Healthy (n = 20), median	IQR	P value*
1 wk					
Total microbiota	1.59	1.33–1.77	1.58	1.42–1.83	.78
Firmicutes	0.81	0.48–1.27	0.86	0.51–1.10	.53
Proteobacteria	0.15	0.03–0.30	0.32	0.05–0.37	.19
Actinobacteria	0.29	0.07–0.41	0.27	0.10–0.37	.58
Bacteroidetes	0.02	0.00–0.51	0.20	0.00–0.39	.60
1 mo					
Total microbiota	1.47	1.16–1.66	1.69	1.53–2.15	.004
Firmicutes	0.55	0.34–1.11	0.61	0.44–0.92	.72
Proteobacteria	0.15	0.06–0.35	0.27	0.12–0.33	.29
Actinobacteria	0.36	0.12–0.46	0.42	0.20–0.67	.26
Bacteroidetes	0.05	0.00–0.36	0.48	0.08–0.60	.02
<i>Bacteroides</i> species	0.01	0.00–0.28	0.44	0.08–0.49	.01
12 mo					
Total microbiota	2.90	2.25–3.30	2.62	2.22–3.27	.65
Firmicutes	2.31	1.71–2.58	1.89	1.49–2.39	.12
Proteobacteria	0.04	0.01–0.07	0.07	0.04–0.13	.02
Actinobacteria	0.21	0.11–0.41	0.17	0.02–0.38	.43
Bacteroidetes	0.16	0.03–0.36	0.50	0.12–0.65	.08

IQR, Interquartile range.

*Mann-Whitney *U* test.

P values were converted to false discovery rate values (*q* values) to correct for multiple testing.¹⁹ The edgeR test is a statistical test that is designed for the analysis of replicated count-based expression data. The Shannon diversity index was used to measure the biodiversity in samples. Briefly, it is a test that takes into account the number of species and the evenness of the species, typically with a value between 1.5 and 3.5.²⁰ It was calculated as $-\sum \log(p_i)p_i$, where p_i denotes the frequency of OTU i ,²¹ and differences in this index were tested with the Mann-Whitney *U* test by using R software (<http://www.r-project.org/>). Clustering of OTUs was analyzed with Fast Unifrac (<http://bmf2.colorado.edu/fastunifrac/>)²² by calculating weighted sample distances.

Repeated-measures ANOVA was used in analyses of multiple longitudinal measures of a specific phylum or genus in subjects in 2 different groups. The χ^2 test was used for categorical data, unless the expected frequency for any cell was less than 5, when the Fisher exact test was used (SPSS version 16.0; SPSS, Inc, Chicago, Ill).

RESULTS

Infants with atopic eczema (ie, IgE-associated eczema) had a lower diversity of the total microbiota and the bacterial phylum Bacteroidetes and its genus *Bacteroides* at 1 month of age than infants who did not have any allergic manifestation during the 2 first years of life (Table II). The diversity of the phylum Proteobacteria, comprising gram-negative bacteria, was also reduced in the atopic infants, significantly so at 12 months of age (Table II). Furthermore, these phyla and genera differed significantly between atopic and nonatopic infants with repeated-measures ANOVA including all sampling time points during the first year of life (1 month, 1 week, and 12 months: *P* = .049 for the total microbiota, *P* = .04 for Bacteroidetes, *P* = .02 for *Bacteroides* species, and *P* = .02 for Proteobacteria). Probiotic supplementation was a potential confounder. Even after exclusion of the probiotic-treated infants, however, several significant differences and some statistical tendencies were still observed (*P* = .03 for the total microbiota, *P* = .06 for Proteobacteria, *P* = .096 for

Bacteroidetes, and *P* = .03 for *Bacteroides* species at 1 month and *P* = .06 for Proteobacteria and *P* = .01 for Bacteroidetes and *Bacteroides* species at 12 months; data not shown). Nine infants received antibiotics at between 2 and 12 months of age. Excluding them did not affect the result at 12 months (*P* = .02 for Proteobacteria).

The relative abundance of the dominant bacterial phyla at various ages is shown in Fig 1. During the first month of life, there was a high intersubject variability (see Fig E1 in this article's Online Repository at www.jacionline.org) and no significant differences at the phylum level between infants who did and did not have atopic eczema. The relative abundance of Bacteroidetes, Proteobacteria, and Actinobacteria, the latter a phylum comprising bifidobacteria, which are associated with breast-feeding, was high in both groups. At 12 months, however, levels of these phyla had decreased, and Firmicutes, comprising gram-positive aerobe and anaerobe bacteria, had become dominant, resembling an adult microbiota pattern. At this age, the relative abundance of Proteobacteria was lower (edgeR test: *P* = .008, *q* = 0.02) and the relative abundance of Firmicutes tended to be higher (edgeR test: *P* = .06, *q* = 0.10) in atopic than nonatopic infants (Table III). Infants who have received antibiotics or probiotics did not differ significantly in terms of relative abundance from those who have not (data not shown). Despite this, the differences in relative abundance between healthy and atopic infants were more significant if infants receiving antibiotics were excluded (edgeR test: *P* = .01 and *q* = 0.02 for Firmicutes, *P* = .005 and *q* = 0.02 for Proteobacteria, and *P* = .03 and *q* = 0.05 for Bacteroidetes at 12 months; data not shown). Excluding infants receiving probiotics did not affect the relative abundance significantly.

The relative abundance of the dominant bacterial classes and genera is presented in Table III to compare our findings with those of previous reports, which often relate allergic disease with bacterial classes and genera rather than phyla. Because 144 genera were identified, *P* values were converted to false discovery rate

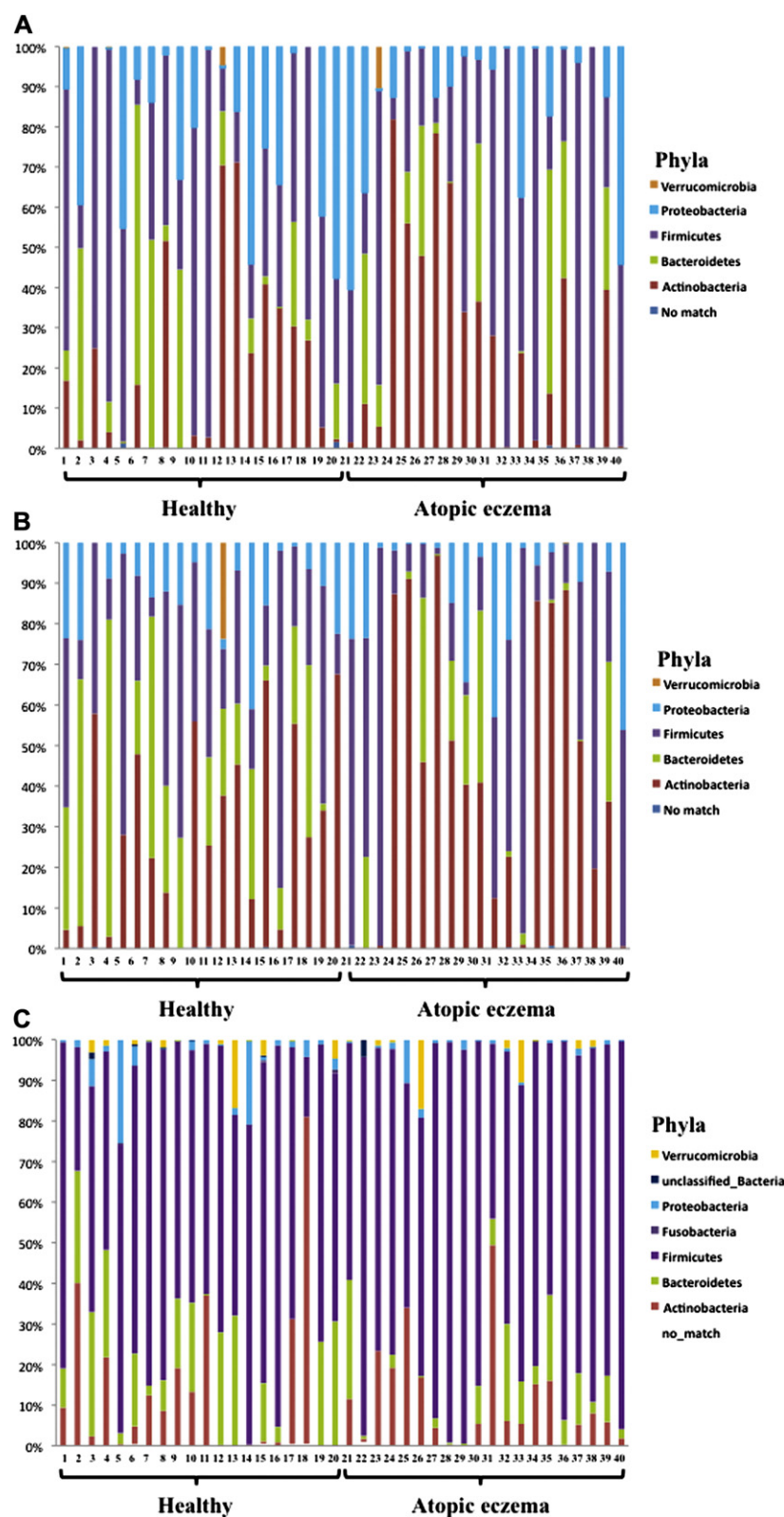


FIG 1. Relative abundance of dominant bacterial phyla in stool samples in each subject at 1 week (**A**) and 1 (**B**) and 12 (**C**) months of age in 20 infants with atopic eczema and 20 infants without any allergic manifestations.

TABLE III. Mean of the relative abundance of dominant phyla (boldface), classes, families, and genera (relative abundance >1% at any age) in stool samples obtained at various ages from infants who did or did not have AE during the first 2 years of life

	1 wk		1 mo		12 mo	
	Healthy (n = 20), mean % (SD)	AE (n = 20), mean % (SD)	Healthy (n = 20), mean % (SD)	AE (n = 20), mean % (SD)	Healthy (n = 20), mean % (SD)	AE (n = 20), mean % (SD)
Actinobacteria	21 (23)	28 (27)	31 (22)	43 (35)	14 (20)	11 (12)
<i>Bifidobacterium</i> species	21 (23)	28 (27)	29 (22)	41 (35)	14 (20)	10 (11)
<i>Collinsella</i> species	<1	<1	1 (3)	<1	<1	<1
Proteobacteria	20 (20)	14 (18)	12 (10)	12 (15)	4 (7)*	1 (2)*
Gammaproteobacteria class	20 (21)	13 (24)	12 (29)	12 (22)	3 (5)	1 (3)
Enterobacteriaceae (unclassified)	18 (21)	8 (15)	7 (11)	5 (10)	2 (4)	<1
Bacteroidetes	15 (21)	12 (18)	24 (22)	9 (15)	15 (12)	7 (9)
<i>Bacteroides</i> species	14 (21)	11 (16)	21 (22)	7 (13)	13 (12)	6 (6)
<i>Parabacteroides</i> species	1 (3)	2 (4)	2 (4)	<1	<1	<1
<i>Prevotella</i> species	<1	<1	<1	<1	<1	1 (5)
Firmicutes	43 (28)	45 (33)	32 (22)	35 (32)	65 (19)	74 (16)
Bacilli class	25 (25)	29 (31)	14 (20)	16 (26)	5 (20)	6 (27)
<i>Streptococcus</i> species	12 (10)	10 (17)	9 (9)	11 (14)	5 (9)	2 (5)
<i>Enterococcus</i> species	5 (11)	9 (16)	1 (3)	3 (6)	<1‡	4 (14)‡
<i>Lactobacillus</i> species	<1	2 (4)	2 (4)	<1	<1	<1
Clostridia class	18 (25)	15 (24)	16 (26)	18 (30)	55 (20)	65 (18)
<i>Veillonella</i> species	3 (8)	2 (4)	2 (2)	3 (6)	2 (3)	1 (2)
Lachnospiraceae Incertae Sedis	1 (3)	<1	<1	1 (6)	4 (5)	7 (6)
Peptostreptococcaceae Incertae Sedis	1 (2)	1 (4)	<1	<1	3 (3)†	5 (4)†
Erysipelotrichaceae Incertae Sedis	<1	<1	<1	2 (6)	4 (4)	4 (6)
<i>Clostridium</i> species	<1	<1	2 (8)	2 (6)	1 (4)	<1
Lachnospiraceae	<1	<1	<1	<1	7 (7)	6 (6)
<i>Faecalibacterium</i> species	<1	<1	<1	<1	3 (4)	2 (4)
<i>Ruminococcus</i> species	<1	<1	<1	<1	1 (2)	3 (3)
<i>Anaerostipes</i> species	<1	<1	<1	<1	1 (4)	1 (3)
Erysipelotrichi class	<1	<1	3 (19)	2 (26)	4 (7)	9 (3)
Verrucomicrobia	<0.1	<0.1	1 (2)	<0.1	2 (4)	1 (4)
<i>Akkermansia</i> species	<1	<1	1 (5)	<1	2 (4)	2 (4)

AE, Atopic eczema.

*edgeR: $P = .01$, $q = 0.03$.†edgeR: $P = .02$, $q = 0.04$.‡edgeR: $P = .002$, $q = 0.005$.

values (q values) to correct for multiple testing. *Bifidobacterium* species, *Bacteroides* species, *Streptococcus* species, *Enterococcus* species, and sequences collectively classified to unclassified Enterobacteriaceae were the most abundant genera, especially during the first month of life. There was no significant difference between atopic and nonatopic subjects for any of the dominant bacterial genera, except for *Enterococcus* species and Peptostreptococcaceae Incertae Sedis, which were more abundant in atopic infants at 12 months of age. Among less abundant genera (relative abundance, <1%), only a few differed significantly between atopic and nonatopic infants after correcting for multiple testing. The microaerophilic gram-negative *Sutterella* species, belonging to the phylum Proteobacteria, was more abundant in the nonatopic infants both at 1 and 12 months of age (healthy vs atopic, mean [SD]: 0.2% [0.4%] vs 0.006% [0.02%], $P = .008$ and $q = 0.02$ at 1 month; 0.3% [0.5%] vs 0.2% [0.5%], $P = .006$ and $q = 0.02$ at 12 months). The gram-negative anaerobic *Fusobacterium* species, belonging to the phylum Fusobacteria, was also more abundant in this group at 12 months of age (healthy vs atopic, mean [SD]: 0.01% [0.02%] vs 0.002% [0.009%], $P = .006$ and $q = 0.02$). On the other hand, the gram-positive anaerobes *Eggerthella* species, belonging to the phylum Actinobacteria, and *Coprobacillus* species, belonging to the phylum Firmicutes, were more abundant in the atopic infants at 12 months (healthy vs

atopic, mean [SD]: 0.1% [0.2%] vs 0.8% [1.0%], $P < .001$ and $q = 0.002$ and 0.01% [0.04%] vs 0.4% [0.09%], $P < .001$ and $q < 0.001$, respectively). The gram-positive anaerobic *Peptoniphilus* species, belonging to the phylum Firmicutes, was more abundant at 1 month of age in the atopic infants (healthy vs atopic, mean [SD]: 0% [0%] vs 0.002% [0.006%], $P = .01$ and $q = 0.03$).

DISCUSSION

Using the new high-throughput 16S-based molecular microbiology, we could confirm and extend previous findings that low intestinal diversity during the first month of life is associated with an increased risk of subsequent atopic disease.^{6,9,10} In contrast to previous studies, we could also show that the differences in diversity and relative abundance were attributed to specific bacterial phyla and genera, possibly because the sensitivity of our analyses was higher than in previous diversity studies.^{6,9,10} At 12 months, mean OTUs per sample were 69 in our study compared with 8.5 bands/sample (in denaturing gradient gel electrophoresis) in a recent the study by Bisgaard et al.¹⁰ It is noteworthy that the most important differences appeared in the first months of life, supporting the theory that factors influencing the early maturation of the immune system might be especially important for subsequent allergy development.²³ However, the study did not clarify

the debate over whether a low total diversity of the gut microbiota in early childhood is more important than the altered prevalence of particular bacterial species in allergy development. Total diversity was important, but the differences in diversity and relative abundance seemed to be defined to specific bacteria.

The low diversity of the phylum Bacteroidetes and its genus *Bacteroides* in infants with atopic eczema is consistent with the results of previous studies reporting low levels of these bacteria to be associated both with allergic disease² and factors associated with allergic disease, such as a Western lifestyle^{11,12} and cesarean section.²⁴ *Bacteroides* species have also been demonstrated to have anti-inflammatory properties. Thus *Bacteroides fragilis* prevented the induction of colitis through suppression of the proinflammatory cytokines TNF and IL-23 in an experimental colitis model²⁵ and also mediated a conversion from CD4⁺ T cells into IL-10-producing forkhead box protein 3 regulatory T cells during commensal colonization, eliciting mucosal tolerance in another murine model.²⁶ Furthermore, *Bacteroides thetaiotaomicron* modulates the expression of a large quantity of genes involved in mucosal barrier reinforcement.^{27,28}

Although our results indicate that the microbial diversity is more important than colonization with any particular bacteria, 1 bacterial phylum, Proteobacteria, appeared to be less abundant in the atopic infants. This phylum comprises gram-negative bacteria, typically with endotoxin (LPS) incorporated into the cell wall. Endotoxin elicits a T_H1 response through the innate immune system by enhancing IL-12 production from monocytes and dendritic cells,²⁹ and low exposure to endotoxin has been associated with an increased risk of atopic eczema.³⁰ Also, the low allergy prevalence among children growing up on farms and in less affluent countries has been attributed to high endotoxin exposure.^{31,32} Thus a strong endotoxin exposure might downregulate atopy-promoting T_H2 responses, possibly causing the negative association between atopic eczema and the high abundance and diversity of Proteobacteria in the present study.

Previously, bifidobacteria and clostridia, especially *Clostridium difficile*, have been associated with allergic disease.^{3,33} None of these bacteria were related to allergic disease in this study; nor was *Clostridium* a dominant bacterial genus. However, there were other genera within the phylum Firmicutes that were more abundant in the atopic than the nonatopic infants. Interestingly, Firmicutes have been associated with other conditions related to a westernized lifestyle, such as obesity.^{12,34}

Importantly, assessments of stool samples merely reflect luminal colonic microbiota and not necessarily the colonization of the small intestine, in which the major part of the gut immune system is situated. The higher oxygen content in the upper gut favors facultative bacteria, such as streptococci and lactobacilli,³⁵ which therefore might be more important than our results indicate.

In conclusion, the results support the hypothesis that low microbial diversity early in life is associated with an increased risk for allergic disease. The importance of bacteria belonging to the phyla Bacteroidetes and Proteobacteria was corroborated, whereas the importance of other bacteria previously associated with allergic disease, such as bifidobacteria and clostridia, could not be confirmed.

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Key message

- Low microbial diversity early in life is associated with increased risk for allergic disease.

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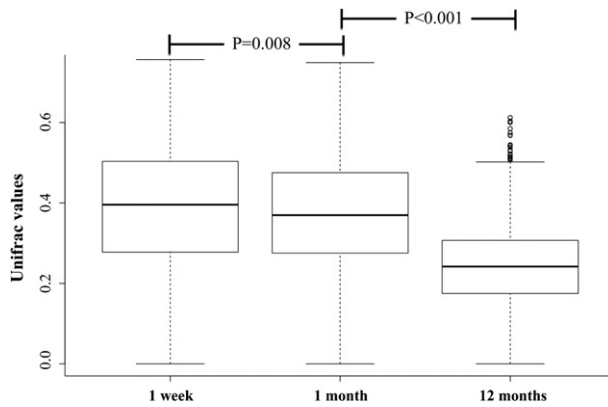


FIG E1. Pairwise community differences. Distributions of Unifrac distance levels at 1 week, 1 month, and 12 months for all 40 infants, regardless of atopy status. The decrease in Unifrac levels indicated that the microbial communities became more uniform across subjects over time. The differences in Unifrac values were analyzed with the Wilcoxon paired test.

TABLE E1. The mean of sequences per infant, total OTUs per infant, and correctly taxonomically classified OTUs (bacterial genus) per infant in stool samples obtained at various ages from infants who did or did not have atopic eczema during the first 2 years of life

	1 wk		1 mo		12 mo	
	Healthy (n = 20), mean (SD)	Atopic eczema, (n = 20), mean (SD)	Healthy (n = 20), mean (SD)	Atopic eczema (n = 20), mean (SD)	Healthy (n = 20), mean (SD)	Atopic eczema (n = 20), mean (SD)
Sequences	2231 (458)	1992 (268)	2047 (414)	2090 (324)	2578 (2459)	2630 (2129)
OTUs	32 (38)	21 (13)	25 (11)	21 (6)	69 (45)	67 (31)
Classified OTUs	24 (26)	17 (10)	20 (10)	18 (6)	51 (31)	51 (22)

No analyses were significant with the Student *t* test.