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 2012;129:434-40.)

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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 denaturating gradient gel electrophoresis,[9,10] infants with sensitization[10] 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-pyrosequencing[13] 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.

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

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

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).

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.

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 GGGNGGCWGCAG 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 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 *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