

Original article

Reduced diversity in the early fecal microbiota of infants with atopic eczema

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Background: It might be that early intestinal colonization by bacteria in westernized infants fails to give rise to sufficient immune stimulation to support maturation of regulatory immune mechanisms.

Objective: The purpose of the present study was to characterize the very early infantile microbiota by using a culture-independent approach and to relate the colonization pattern to development of atopic eczema in the first 18 months of life.

Methods: Fecal samples were collected from 35 infants at 1 week of age. Twenty infants were healthy, and 15 infants were given diagnoses of atopic eczema at the age of 18 months. The fecal microbiota of the infants was compared by means of terminal restriction fragment length polymorphism (T-RFLP) and temporal temperature gradient gel electrophoresis (TTGE) analysis of amplified 16S rRNA genes.

Results: By means of T-RFLP analysis, the median number of peaks, Shannon-Wiener index, and Simpson index of diversity were significantly less for infants with atopic eczema than for infants remaining healthy in the whole group and for the Swedish infants when *AluI* was used for digestion. The same was found when TTGE patterns were compared. In addition, TTGE analysis showed significantly less bands and lower diversity indices for the British atopic infants compared with those of the control subjects.

Conclusion: There is a reduced diversity in the early fecal microbiota of infants with atopic eczema during the first 18 months of life.

Clinical implications: This finding, if confirmed in larger patients and control populations, might inspire future strategies for primary prevention of IgE-mediated atopic eczema. (J Allergy Clin Immunol 2007;119:1001-1008.)

Key words: Atopic eczema, intestinal microbiota, diversity, terminal restriction fragment length polymorphism, temporal temperature gradient gel electrophoresis

Atopic eczema is often the first manifestation of atopic disease in infants,¹ and children with severe cutaneous disease are at high risk of later having sensitization to inhaled allergens and persistent respiratory allergic disease. In Western countries there has been a constant increase in the incidence of allergic disease over the last decades.

Lack of microbial exposure during infancy has been suggested as one factor responsible for the allergy epidemic in westernized populations.^{2,3} Experimental evidence demonstrates the importance of microbiota in shaping the development of the immune system.^{4,5} An increase in the number of T cells has been observed on colonization of germ-free mice by bacteria and in germ-free animals in which insufficient numbers or absence of T cells in the Peyer's patches was related to failure of induction of oral tolerance.^{5,6}

Infections with gastrointestinal pathogens, rather than with airborne viruses, seem to protect against allergy.⁷ However, most microbes encountered by the immune system are nonpathogenic and do not give rise to symptomatic infections, although they provide positive stimuli for the immune system. Infants in developing countries are colonized earlier by fecal bacteria and have a faster turnover of bacterial strains in the microbiota than infants in developed countries.^{8,9} This has led to the hypothesis that the commensal intestinal microbiota of Western infants fails to support the development of tolerance to allergens.¹⁰ Key groups of microorganisms could either be lacking or present in excess, or an overall low diversity in the microbiota could be responsible. The timeframe during which maturation of regulatory immune mechanisms occurs is not known, and thus it is uncertain at what age microbial stimulation would be of most importance.

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Abbreviations used

Cy5:	Indodicarbocyanine
T-RF:	Terminal restriction fragment
T-RFLP:	Terminal restriction fragment length polymorphism
TTGE:	Temporal temperature gradient gel electrophoresis

Studies of the intestinal microbiota in children have revealed differences, although inconsistent, in early colonization patterns between those with and without allergies.¹¹⁻¹⁴ In the ALLERGYFLORA project, a comprehensive study of 318 infants from Sweden, England, and Italy, no highly significant ($P < .01$) differences in colonization by various groups of culturable intestinal bacteria were found during the first year of life between infants having or not having atopic eczema in the first 18 months of life.¹⁵

The 16S rRNA gene has previously been the target for analysis of the human intestinal microbiota,^{16,17} and techniques based on the study of ribosomal genes, using universal primers and generating fingerprints, have been used as powerful tools for the study of microbial diversity in complex samples.¹⁸⁻²⁰ One such method, single-strand conformation polymorphism, was used by Ott et al²¹ to show that diversity of colonic mucosa-associated bacterial microflora was reduced in patients with active inflammatory bowel disease.

Terminal restriction fragment length polymorphism (T-RFLP) analysis has been shown to be a suitable method for the study of the development of the fecal microbiota of infants,²² and methods based on denaturing electrophoresis, such as denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis, have frequently been used to characterize human microbiota.^{23,24} The purpose of the present study was to characterize the very early infantile microbiota by using T-RFLP and temporal temperature gradient gel electrophoresis (TTGE) and to relate the colonization pattern to development of atopic eczema in the first 18 months of life.

METHODS

Subjects and sample collection

The ALLERGYFLORA project consisted of 3 cohorts of approximately 100 infants each from Göteborg, Sweden; London, Great Britain; and Rome, Italy.¹⁵ The aim was to investigate whether colonization by culturable fecal bacteria was related to the development of atopic eczema and sensitization by 18 months, taking into account the possible influence of lifestyle and dietary factors. From the 318 participants, 35 infants were included in the present fecal diversity study. Cases were those children with atopic eczema, defined as those either fulfilling Williams' UK diagnostic criteria for atopic dermatitis²⁵ or the International Study of Asthma and Allergy in Childhood criteria of an itching rash that has come and gone for at least 6 months and has affected typical locations.²⁶ A SCORAD value was obtained by using validated software (SCORAD-Card; TPS, Rome, Italy).²⁷ At

18 months of age, 5 mL of blood was drawn by means of venipuncture, and the serum was frozen at -70°C . Serum total IgE (IgE-FEIA) and specific IgE levels against a mix of common food allergens (Fv5: egg white, cows' milk, codfish, wheat, peanut, and soya bean) and an inhalant allergen mix (Phadiatop: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, horse and dog dander, timothy grass, *Cladosporium* species, olive, mugwort, and nettle) were measured (all from Pharmacia Diagnostics, Uppsala, Sweden). The analysis was done in one laboratory to minimize method variability. Infants who were clearly atopic (ie, who also had increased total IgE levels, defined as greater than the mean plus 1 SD for the whole cohort [18 kU/L]) and had positive specific IgE serology (positive for either Fv5 or Phadiatop) were preferentially included as cases. Control infants were from the same cohorts and had neither any allergic manifestations (eczema, rhinitis, or asthma) by 18 months of age nor increased total or specific IgE levels. Control infants were also selected to ensure that an equal proportion of infants had been delivered by means of cesarean section because delivery mode is known to significantly influence microbiota composition.^{28,29} All the infants were breast-fed. The characteristics of the patients and control subjects are summarized in Table I.

Fresh fecal samples were collected from the infants when they were 1 week old. The samples were collected at home by the parents and placed in a gas-proof plastic bag in which an anaerobic atmosphere was generated (AnaeroGen Compact; Oxoid Ltd, Basingstoke, Hampshire, England). The samples were stored at 4°C until delivery to the laboratory, where they were frozen to -80°C and stored until processing. Informed consent was obtained, and the study was approved by local ethics committees.

DNA extraction

DNA from feces was isolated and purified by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in combination with glass-bead beating and use of the BioRobot EZ1 (tissue kit and card; Qiagen). Briefly, 120 mg (wet weight) of fecal sample was homogenized and lysed in 1.4 mL of ASL-buffer (DNA Stool Mini Kit, Qiagen) at 95°C for 5 minutes. Fifteen glass beads (2 mm in diameter) were added to the tubes containing the sample, and the tubes were shaken for 30 minutes at 4°C in an Eppendorf Mixer (Model 5432; Eppendorf, Hamburg, Germany). After centrifugation at $20,800g$ for 1 minute, 1.2 mL of supernatant was collected in a 2.0-mL tube and treated with 1 InhibitEX tablet (Qiagen) to remove the DNA-damaging substances and PCR inhibitors. After 3 minutes of centrifugation at $20,800g$, 200 μL of supernatant was treated with proteinase K and buffer AL, according to the manufacturer's instructions. One hundred microliters of suspension was diluted with 100 μL of PBS (pH 7.3; Basingstoke), and DNA in the sample was extracted with the BioRobot EZ1 in accordance with the manufacturer's instructions. Buffer ASL without sample was treated in parallel to serve as a negative control of sample preparation.

T-RFLP analysis

For T-RFLP, 16S rRNA genes were amplified by using the forward primer indodicarbocyanine (Cy5)-ENV1 (5'-AGA GTT TGA TII TGG CTC AG-3') and the reverse primer ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3'). The forward primer was fluorescently labeled with Cy5 at the 5' end. PCR was performed as previously described, except using 4 μL of template DNA in the reaction mixture.²¹ The size (approximately 1504 bp) of PCR products was verified on a 1% agarose gel in $1\times$ TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, and 2.5 mmol/L EDTA, pH 8.3) after staining with ethidium bromide. PCR products from 3 separate reactions were pooled and further purified by using the MinElute PCR purification Kit (Qiagen). The amount of DNA was

TABLE I. Population, risk/protective factors, and allergic outcomes

	Swedish infants		British infants		Italian infants	
	Eczema* (n = 8)	Control (n = 8)	Eczema* (n = 4)	Control (n = 7)	Eczema* (n = 3)	Control (n = 5)
Cesarean delivery	2	2	1	3	2	2
Asthma	1	0	2	0	0	0
Rhinitis	1	0	3	0	1	0
Specific IgE positive†	5	0	4	0	1	0
Total IgE (kU/L), median	43	6.5	150	4	70	14
Total IgE (kU/L), range	16-260	2-10	120-640	2.1-9	23-210	4.5-23
SCORAD, range‡	0-10.4	0	0-39.4	0-7.25§	0-10.6	0
Antibiotics during pregnancy	1	1	2	2	3	2
Antibiotics at 6 mo	2	1	3	0	0	1
Hereditry	6	5	3	3	2	1

*Atopic eczema defined as either fulfilling Williams' UK diagnostic criteria for atopic dermatitis²⁵ or the International Study of Asthma and Allergy in Childhood criteria of an itching rash that has come and gone for at least 6 months and has affected typical locations.²⁶

†Phadiatop or Fx5 positive.

‡Calculated by using validated software (SCORAD-Card, TPS).²⁷

§One infant with nonpruritic dermatitis on examination at 18 months but not fulfilling either William's UK or International Study of Asthma and Allergy in Childhood criteria for eczema.

estimated by running 1 μ L of purified PCR product on a 0.8% agarose gel in TB buffer in parallel with known concentrations of λ phage DNA (Roche Diagnostics, Mannheim, Germany).

Aliquots (approximately 200 ng) of purified PCR products were digested for 5 hours at 37°C with either 15 U of *MspI* or *AluI* (Roche Diagnostics GmbH) in a total volume of 10 μ L, after which the enzymes were inactivated by heating at 65°C for 15 minutes, as recommended by manufacturer. Four microliters of digests were mixed with 4 μ L of formamide loading dye (3.3 μ L of deionized formamide and 0.7 μ L of 25 mmol/L EDTA with 5% wt/vol dextran blue) and 1 μ L of internal size standard, and the mixture was denatured at 94°C for 3 minutes. The internal size standards contained ALFexpress Sizer 50 (Amersham Biosciences, Piscataway, NJ) and 697 bp of PCR product amplified from *Escherichia coli* ATCC 11775 by using primer 685r (5'-TCT ACG CAT TTC ACC GCT AC-3'; *E. coli* numbering 705-685) and Cy5-ENV1. External size standards, consisting of ALFexpress Sizer 50-500 (Amersham Biosciences) and the Cy5-labeled 697-bp PCR product, were also loaded on the sample-containing gels to estimate the lengths of the terminal restriction fragments (T-RFs). The fluorescently labeled fragments were separated and detected with an ALFexpress II DNA sequencer, as previously described.²² The peak areas of fluorescently labeled T-RFs were estimated by using the ALFwin Fragment Analyser 1.03 program (Amersham Biosciences). From each sample, PCR digestions and electrophoresis were done twice to assess the variability of the method.

TTGE analysis

For TTGE, the V3, V4, and V9 regions of bacterial 16S rRNA genes were amplified by means of PCR with primers p5-gc (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3')³⁰ and p6 (5'-CGG TGT GTA CAA GGG CCG GGA ACG -3').³¹ The reaction mixture contained 2 μ L of template DNA, 12.5 pmol of each primer, and 12.5 μ L of HotStarTaq Master Mix (Qiagen) in a final volume of 25 μ L. The PCR was run in a Mastercycler (Eppendorf) by using the following program: 95°C for 15 minutes; 32 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 72°C for 10 minutes. The sizes and amounts of the amplicons were checked on a 1% agarose gel containing ethidium bromide.

TTGE analysis of the amplicons was performed with a DCode Universal Mutation Detection System (Bio-Rad Laboratories,

Sundbyberg, Sweden). Gels were made of 8% (wt/vol) polyacrylamide (Acrylamide/Bis, 37.5:1), 8 mol/L urea, and 1.25 $\times$ TAE buffer prepared from 50 $\times$ TAE buffer (Bio-Rad Laboratories). The gels were run at a constant voltage of 75 V for 18 hours with a temperature gradient from 61.2°C to 68.4°C at a ramp rate of 0.4°C/h. For better resolution, the voltage was fixed at 20 V for 20 minutes at the beginning of the electrophoresis. Aliquots of 3 to 8 μ L of amplified DNA together with 5 μ L of loading dye (2 g of Ficoll, 0.02 g of bromophenol blue, and 8 mL of H₂O) were loaded into each well. Amplified 16S rDNA fragments of representative operative taxonomic units, derived from previously analyzed infantile fecal samples,²² were used as reference. The gels were stained with SYBR Green I Nucleic Acid Gel Stain (Roche Diagnostics) for 30 minutes in the dark and photographed on a UV transillumination table (302 nm) with a Canon PowerShot G5 digital camera (Canon, Tokyo, Japan).

Statistical analysis

For T-RFLP analysis, the numbers of peak in each community were counted, and the relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak area of the respective T-RF divided by the total peak area of all T-RFs detected within a fragment length range of between 30 and 697 bp. Only the T-RFs that had relative abundance of 1% or greater in both duplicates were considered in the analysis. TTGE profiles were analyzed with BioNumeric (Applied Maths, Sint-Martens-Latem, Belgium). The number of bands was counted, and a densitometric curve was obtained for each gel lane. The relative intensity of each band in a sample was calculated as the intensity of the respective band divided by the sum of all band intensities in the densitometric curve. Shannon-Wiener (Shannon; H') and Simpson indices (D) were calculated by using the following equations: $H' = -\sum p_i \ln p_i$ and $D = \sum p_i^2$, where p_i is the relative abundance/intensity of the i th peak/band in the community.³² The use of $1 - D$ (Simpson index of diversity) instead of the original formulation of the Simpson index ensures that the value of the index will increase with increasing diversity.

The differences in bacterial diversity between the atopic and nonatopic infants were tested nonparametrically by using Mann-Whitney rank sum tests and parametrically by using logistic regression modeling, both with Stata version 8.2 software (StataCorp, College Station, Tex).³³ The logistic regression models, including each diversity measure in turn as a continuously distributed

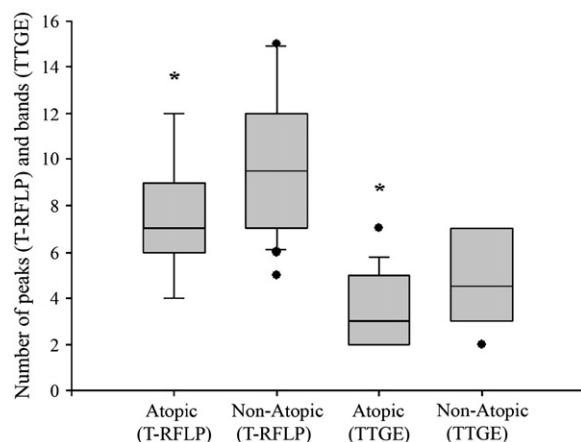


FIG 1. Median number of peaks and bands after T-RFLP of 16S rDNA with *AluI* for cutting and TTGE, respectively, generated from the fecal microbiota of 1-week-old infants that at 18 months had atopic eczema or stayed healthy. For each group, median and 10th, 25th, 75th, and 90th percentiles are shown. * $P < .05$.

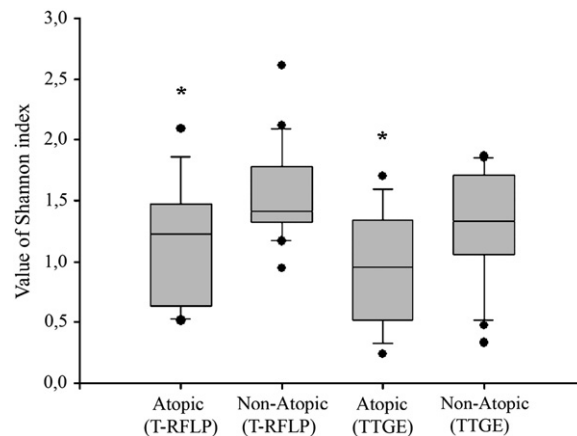


FIG 2. Shannon-Wiener index after T-RFLP of 16S rDNA with *AluI* for cutting and TTGE, respectively, generated from the fecal microbiota of 1-week-old infants that at 18 months had atopic eczema or stayed healthy. For each group, median and 10th, 25th, 75th, and 90th percentiles are shown. *For T-RFLP, $P < .01$ and for TTGE, $P < .05$.

explanatory variable, were further elaborated to include sex and study center. Because the number of observations is small, potential confounding effects of mode of delivery, siblings, antibiotics during pregnancy, and maternal history of allergy were explored by including each of these singly in the logistic regression model, along with sex and study center. For graphic presentation, we show the median values of each diversity index, with 10th, 25th, 75th, and 90th percentiles.

RESULTS

Microbiota complexity determined by means of T-RFLP in relation to development of atopic disease

Bacterial DNA was extracted from fecal samples collected by 1 week of age from infants who later had atopic eczema and increased total and specific IgE levels ($n = 15$), as well as from infants who were healthy without increased IgE levels for their first 18 months ($n = 20$). The infants were derived from Sweden ($n = 8 + 8$), Great Britain ($n = 4 + 7$), and Italy ($n = 3 + 5$; Table I). The median number of peaks after cutting with *AluI* was significantly less in those with atopic eczema (7.0) than in infants who remained healthy (9.5; $P = .03$; Fig 1). The same trend was found when *MspI* was used for cutting, resulting in a median number of peaks of 8.0 for atopic infants and 10.0 for nonatopic infants.

In the Swedish infants the median number of peaks for those who later had atopic eczema was 7.0 by using *AluI* for cutting. This was significantly different compared with the number of peaks in 1-week-old infants who remained healthy at 18 months (10.0, $P = .05$). When *MspI* was used, a similar trend was seen, but the difference was not significant (8.0 vs 10.0 peaks). In the British and Italian groups no significant differences were found between atopic and nonatopic infants either with *AluI* or *MspI*. The microbiota of Italian atopic versus nonatopic

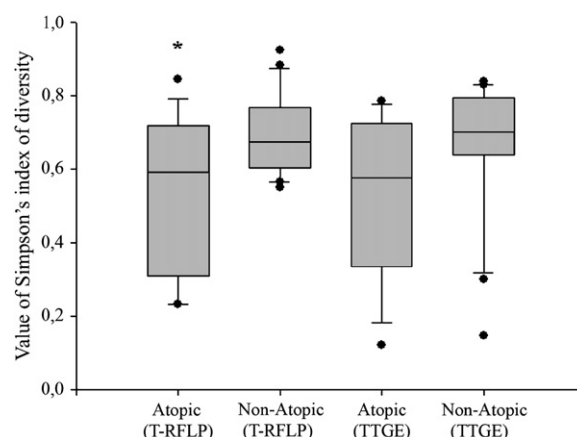


FIG 3. Simpson index after T-RFLP of 16S rDNA with *AluI* for cutting and TTGE, respectively, generated from the fecal microbiota of 1-week-old infants that at 18 months had atopic eczema or stayed healthy. For each group, median and 10th, 25th, 75th, and 90th percentiles are shown. * $P < .05$.

infants showed 5.0 versus 7.0 peaks with *AluI* and 7.0 versus 12.0 peaks with *MspI*, respectively. For the British infants, the results were 10.5 versus 11.0 for *AluI* and 12.0 versus 13.0 for *MspI*.

For the entire group, when *AluI* was used for cutting, the Shannon index was significantly lower for atopic than for healthy infants ($P = .01$, Fig 2), and this was also the case for the Swedish group ($P = .007$). Simpson index of diversity was significantly lower for infants with eczema compared with those staying healthy ($P = .05$, Fig 3). The same was true for the Swedish group ($P = .05$). No significant differences were found within the British or Italian groups, but the trends were the same (data not shown). When *MspI* was used, the trends were the same, but no statistically significant differences were obtained (data not shown).

TABLE II. Associations of atopy with each measure of fecal diversity by means of nonparametric tests and logistic regression modeling

	Median values		Mann-Whitney <i>U</i> test*	Logistic regression*	
	Nonatopic	Atopic		Unadjusted	Adjusted†
T-RFLP peaks	9.5	7.0	.03	0.02	0.008
T-RFLP Shannon	1.41	1.22	.01	0.006	0.002
T-RFLP Simpson	0.67	0.59	.05	0.007	0.007
TTGE bands	4.5	3.0	.05	0.04	0.008
TTGE Shannon	1.33	0.95	.03	0.03	0.01
TTGE Simpson	0.70	0.58	.05	0.05	0.03

*Stata *P* values.

†For sex and center.

Microbiota complexity determined by means of TTGE in relation to development of atopic disease

TTGE was used to evaluate the band pattern of the microbiota in fecal samples from 1-week-old infants in relation to later development of atopy. The TTGE patterns of all infants were scanned, and the number of bands, as well as the Shannon index and Simpson index of diversity, were calculated (Figs 1-3). The number of bands were lower in the infants with atopy than in infants remaining healthy (3.0 vs 4.5, $P = .05$; Fig 1). The Shannon index was lower in infants with atopy ($P = .03$, Fig 2), whereas the Simpson index of diversity was not significantly different among the groups ($P = .053$, Fig 3). When the 3 groups were examined separately, the microbiota of British infants subsequently having atopy gave rise to less bands (2.0 vs 5.0 bands, $P = .04$), a lower Shannon index ($P = .02$), and a lower Simpson index of diversity ($P = .04$) than the microbiota of those who remained healthy (data not shown). The microbiota of the Swedish infants who later became atopic was characterized by fewer (but not statistically significant) bands (4.0 vs 6.5), a lower Shannon index ($P = .03$), and a lower Simpson index of diversity ($P = .04$) compared with the microbiota of Swedish nonatopic infants.

Logistic regression modeling

Table II shows the *P* values for each diversity index tested by using nonparametric tests and using logistic regression with and without adjustment for sex and study center. The direction of the associations was unaltered by adjustment, but the significance of the findings was generally enhanced. Additional adjustment for mode of delivery, siblings, antibiotics during pregnancy, and maternal history of allergy (singly) did not change the pattern of results greatly (data not shown). There were no consistent associations of these potential confounding factors with the measures of fecal diversity at 1 week of age (data not shown).

The odds ratios of atopy per additional band or peak for the TTGE and RFLP data, adjusting for sex and center by using logistic regression, as modeled in Table II, were as follows: 0.50 (0.28-0.89) per additional TTGE band and 0.65 (0.44-0.96) per additional RFLP peak.

Interpretation of these odds ratios should bear in mind that the number detected with TTGE (range, 2-7 bands) is about half that detected with RFLP (range, 4-15 peaks). Thus across the range of the data, each measure of diversity had a similar effect on atopy risk. We do not present odds ratios relating to the Shannon and Simpson indices because these do not have intuitive units.

Among the 15 atopic infants, the rank correlation between SCORAD score and number of peaks was not substantial or significant for either TTGE bands (rank correlation, -0.03 ; $P = .93$) or RFLP peaks (rank correlation, -0.10 ; $P = .71$). Similarly, total IgE levels among atopic infants were not correlated substantially or significantly with fecal diversity, as measured by using TTGE bands (rank correlation, -0.17 ; $P = .54$) or RFLP peaks (rank correlation, $+0.18$; $P = .51$). However, when log-transformed total IgE was modeled as an outcome in all infants studied, adjusting for sex and center, it was inversely related to the number of TTGE bands (proportionate reduction per additional band, 0.59; 95% CI, 0.45-0.78; $P = .001$) and less strongly to the number of RFLP peaks (proportionate reduction per additional peak, 0.83; 95% CI, 0.70-0.99; $P = .04$).

DISCUSSION

A culture-dependent study on the fecal microbiota at different ages from 1 week to 1 year of age in 318 Swedish, British, and Italian infants was conducted previously.¹⁵ Of this cohort, 15 clearly atopic infants at the age of 18 months and 20 nonatopic infants were chosen. Although no significant differences in colonization by different bacterial groups were found by using cultivation-dependent techniques,¹⁵ we detected, by means of T-RFLP and TTGE, a significantly lower diversity in the fecal microbiota of 1-week-old infants who later had atopy than in infants who remained healthy during their first 18 months of life. Culture-dependent methods have provided a lot of information about the intestinal microflora but are biased by the fact that a vast majority of the bacteria residing in this niche have not yet been cultivated.^{34,35} On the other hand, PCR-based analysis can introduce

different types of biases and insufficient cell lysis before PCR could distort the view of the intestinal community composition.³⁶ However, all samples included in this study were equally treated.

With T-RFLP, the difference in diversity was shown for the total cohort, as well as for the Swedish group of infants. With TTGE, it was possible to measure a lower diversity for children having eczema within the Swedish and British groups and within the whole cohort compared with those who remained healthy. The number of individuals included in the Italian group was too low to render significance, but the same trend was found. T-RFLP and TTGE are both methods that, by means of PCR amplification of 16S rRNA genes, can visualize the bacterial composition in a sample. The outcome depends on several methodological parameters. T-RFLP has a higher sensitivity than TTGE, detecting bacterial groups that are present as 0.1% in a sample compared with 1% for TTGE.^{19,37,38} Thus bacterial populations of 10⁹/g for T-RFLP and 10¹⁰/g for TTGE could be detected. On the other hand, TTGE has a more fine-tuned discriminatory power than T-RFLP. For example, different genera and sometimes species of the family Enterobacteriaceae give rise to different band position in TTGE,³⁹ whereas in T-RFLP most of the genera of this family end up in the same peak.²²

No significant differences were obtained between atopic and healthy infants when the restriction endonuclease *MspI* was used. This might relate to the microbiota of 1-week-old infants having a low complexity and that the dominating bacterial groups were more readily differentiated by the restriction endonuclease *AluI*. The microflora of breast-fed infants remains at low complexity until weaning.^{22,40} However, for how long the microbial diversity will remain at a lower level for infants with eczema at 18 months than for healthy infants is still to be studied.

Although regular calculations of diversity indices are not frequently done in studies of the intestinal microbiota, Wang et al¹⁷ found both the Shannon index and the reciprocal Simpson index to be less in the jejunum than in the distal ileum, ascending colon, and rectum in a healthy volunteer. Moreover, a lower Shannon index was found in the intestinal microbiota of subjects with Crohn's disease and ulcerative colitis compared with in noninflammatory control subjects by using single-strand conformation polymorphism.²¹

In this study we found a relation between the diversity of 1-week-old infants' microbiota and their health status at 18 months of age with regard to atopic eczema. During the neonatal period, several factors have been shown to influence microbiota development. These include antibiotic intake, infant nutrition (breast as opposed to formula milk), and the presence of siblings.^{15,41} Nevertheless, our results indicate, for the first time, that sufficient diversity in the fecal microbiota at this early age might be an important factor for the prevention of the development of atopic eczema. This is certainly along the lines of the hygiene hypothesis revised, as suggested by Wold.¹⁰

REFERENCES

- Eichenfield LF, Hanifin JM, Beck LA, Lemanske RF Jr, Sampson HA, Weiss ST, et al. Atopic dermatitis and asthma: parallels in the evolution of treatment. *Pediatrics* 2003;111:608-16.
- Strachan DP. Hay fever, hygiene and household size. *BMJ* 1989;289:1259-60.
- Bach JF. The effects on infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.
- Bauer H, Horowitz RE, Levenson SM, Popper H. The response of the lymphatic tissue to microbial flora. Studies on germfree mice. *Am J Pathol* 1963;42:471-83.
- Maeda Y, Noda S, Tanaka K, Sawamura SA, Alba Y, Ishikawa H, et al. The failure of oral tolerance induction is functionally coupled to the absence of T-cells in Peyer's patches under germfree conditions. *Immunobiology* 2001;204:442-57.
- Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1977;159:1739-45.
- Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, et al. Exposure to foodborne and orofaecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* 2000;320:412-7.
- Adlerberth I, Carlsson B, deMan P, Jalil F, Khan SR, Larsson P, et al. Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand* 1991;80:602-10.
- Adlerberth I, Jalil F, Hansson LÅ, Carlsson B, Mellander L, Larsson P, et al. High turn-over rate of *Escherichia coli* strains in the intestinal flora of infants in Pakistan. *Epidemiol Infect* 1998;12:587-98.
- Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 1998;53(suppl):20-5.
- Kalliomäki M, Kirjavainen P, Eerola E, Pentti K, Salmimäki S, Isolari E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001;107:129-34.
- Kirjavainen PV, Arvola T, Salminen SJ, Isolari E. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning. *Gut* 2002;51:51-5.
- Watanabe S, Narisawa Y, Arase S. Differences in faecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* 2003;111:587-91.
- Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, et al. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA birth cohort study. *Gut* 2007;56:661-7.
- Adlerberth I, Strachan DP, Matricardi PM, Ahrné S, Orfei L, Åberg N, et al. Gut microbiota and development of atopic eczema in three European birth cohorts. *J Allergy Clin Immunol* 2007;120:343-50.
- Wang X, Heazlewood SP, Krause DO, Florin TH. Molecular characterization of microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *J Appl Microbiol* 2003;95:508-20.
- Wang M, Ahrné S, Jeppsson B, Molin G. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS Microbiol Ecol* 2005;54:219-31.
- Schwieger F, Tebbe CT. A new approach to utilize PCR-single-strand conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microbiol* 1998;64:4870-6.
- Marsh TL. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* 1999;2:323-7.
- Valinski L, Della Vedova G, Jiang T, Borneman J. Oligonucleotide fingerprinting of rRNA genes for analysis of fungal community composition. *Appl Environ Microbiol* 2002;68:5999-6004.
- Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory disease. *Gut* 2004;53:685-93.
- Wang M, Ahrné S, Antonsson M, Molin G. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of faecal microbiota in infants of different ages. *J Microbiol Methods* 2004;59:53-69.
- Zoetendal EG, Akkermans ADL, de Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human faecal samples reveals

- stable and host-specific communities of active bacteria. *Appl Environ Microbiol* 1998;64:3854-9.
24. Murray CS, Tannock GW, Simon MA, Harmsen HJM, Wellin GW, Custovic A, et al. Faecal microbiota in sensitized wheezy and non-sensitized non-wheezy children: a nested case control study. *Clin Exp Allergy* 2005;35:741-5.
 25. Williams HC. Diagnostic criteria for atopic dermatitis. *Lancet* 1996;348:1391-2.
 26. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995;8:483-91.
 27. Tripodi S, Panetta V, Pelosi S, Pelosi U, Boner AL. Measurement of body surface area in atopic dermatitis using specific PC software (ScoradCard). *Pediatr Allergy Immunol* 2004;15:96-101.
 28. Bennet R, Nord CE. Development of the faecal anaerobic microflora after caesarean section and treatment with antibiotics in newborn infants. *Infection* 1987;15:332-6.
 29. Bezirtoglou E. The intestinal microflora during the first weeks of life. *Anaerobe* 1997;3:173-7.
 30. Nübel U, Engelen B, Felske A, Snaidr J, Weishuber A, Amann RI, et al. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* 1996;178:5636-43.
 31. Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH. Analysis of Actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* 1997;63:3233-41.
 32. Magurran AE. An index of diversity. In: *Measuring biological diversity*. Oxford: Blackwell Science Ltd; 2004. p. 100-33.
 33. StataCorp. Stata Statistical Software: release 8.2. College Station (Tex): Stata Corp; 2005.
 34. Langendijk PS, Schaf F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH, et al. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. With genus-specific 16S rRNA-targeted probes and its application in faecal samples. *Appl Environ Microbiol* 1995;61:3069-75.
 35. Suau A, Bonnet R, Sutren M. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* 1999;65:4799-807.
 36. Wintzingerode FV, Hoebel UB, Stackebrandt E. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 1997;21:213-29.
 37. Dunbar J, Ticknor LO, Kuske CR. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl Environ Microbiol* 2000;66:2943-50.
 38. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR. Molecular ecological analysis of the gastrointestinal microbiota: a review. *J Nutr* 2004;134:465-72.
 39. Olsson C, Ahm  S, Pettersson B, Molin G. DNA based classification of food associated Enterobacteriaceae previously identified by Biolog GN Microplates. *System Appl Microbiol* 2004;27:219-28.
 40. Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *J Med Microbiol* 1982;15:189-203.
 41. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 2006;118:511-21.