

Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age

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Background: Changes in the human microbiome have been suggested as a risk factor for a number of lifestyle-related disorders, such as atopic diseases, possibly through a modifying influence on immune maturation in infancy.

Objectives: We aimed to explore the association between neonatal fecal flora and the development of atopic disorders until age 6 years, hypothesizing that the diversity of the intestinal microbiota influences disease development.

Methods: We studied the intestinal microbiota in infants in the Copenhagen Prospective Study on Asthma in Childhood, a clinical study of a birth cohort of 411 high-risk children followed for 6 years by clinical assessments at 6-month intervals, as well as at acute symptom exacerbations. Bacterial flora was analyzed at 1 and 12 months of age by using molecular techniques based on 16S rRNA PCR combined with denaturing gradient gel electrophoresis, as well as conventional culturing. The main outcome measures were the development of allergic sensitization (skin test and specific serum IgE), allergic rhinitis, peripheral blood eosinophil counts, asthma, and atopic dermatitis during the first 6 years of life.

Results: We found that bacterial diversity in the early intestinal flora 1 and 12 months after birth was inversely associated with the risk of allergic sensitization (serum specific IgE $P = .003$; skin prick test $P = .017$), peripheral blood eosinophils ($P = .034$), and allergic rhinitis ($P = .007$). There was no association with the development of asthma or atopic dermatitis.

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Conclusions: Reduced bacterial diversity of the infant's intestinal flora was associated with increased risk of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia, but not asthma or atopic dermatitis, in the first 6 years of life. These results support the general hypothesis that an imbalance in the intestinal microbiome is influencing the development of lifestyle-related disorders, such as allergic disease. (*J Allergy Clin Immunol* 2011;128:646-52.)

Key words: Allergic sensitization, allergic rhinitis, peripheral blood eosinophils, atopic dermatitis, asthma, denaturing gradient gel electrophoresis, infants, gastrointestinal, microbiota, fecal microflora, human microbiome

Symbiotic interactions of microorganisms are widespread in nature and support fundamentally important processes linking health and disease to the bacterial ecology. Changes in the human microbiome have been associated with a number of lifestyle-related disorders, such as inflammatory bowel disease,¹ obesity,^{2,3} diabetes,⁴ rheumatoid arthritis,⁵ and atopic dermatitis and allergy.⁶⁻⁸

The gastrointestinal tract provides a vast and continuous source for bacterial stimulation of the immune system from infancy. We have prospectively studied the possible association between the composition of the bacterial community of the intestine in infancy and the development of atopic disorders, including allergic sensitization, allergic rhinitis, peripheral blood eosinophilia, asthma, and atopic dermatitis, during the first 6 years of life in a high-risk birth cohort of 411 infants. The composition of the fecal bacteria was identified by using a molecular technique examining 16S rRNA PCR coupled with denaturing gradient gel electrophoresis (DGGE) and conventional culturing. Molecular techniques provide a more sensitive measure of the microbiota than conventional culturing but have rarely been applied on a large scale because of resource requirements.

We hypothesized that the diversity of the intestinal microbiota influences the development of asthma, atopic dermatitis, and allergy.

METHODS

The study is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines for reporting observational epidemiologic studies.⁹

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Copenhagen and Frederiksberg Ethics Committee

Abbreviations used

COPSAC: Copenhagen Prospective Study on Asthma in Childhood
DGGE: Denaturing gradient gel electrophoresis
GEE: Generalized estimating equation
PCA: Principal component analysis

(KF 01-289/96 and KF 11-107/02) and the Danish Data Protection Agency (2008-41-1754).

Subjects and study design

The Copenhagen Prospective Study on Asthma in Childhood (COPSAC) is a prospective clinical birth cohort study. During 1998-2001, the study enrolled 411 infants born to mothers with a history of asthma, excluding children born before 36 weeks' gestation and anyone suspected of chronic diseases or lung symptoms before inclusion, as previously described in detail.¹⁰⁻¹² Children attended the clinical research unit 1 month after birth and at scheduled visits every 6 months, as well as for any acute symptoms from the airways or skin during the first 6 years of life. Doctors working in the clinical research unit evaluated symptoms of atopic disease from clinical examinations, with support from parents' daily diaries. Diagnosis and treatment were controlled by the doctors at the research clinic, who acted as general practitioners for the cohort.

End point definitions

Specific IgE levels assessed at ½, 1½, 4, and 6 years of age were determined by means of ImmunoCAP (Phadia AB, Uppsala, Sweden)¹³ against cat, dog, horse, birch, timothy grass, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, mugwort, molds (*Paspalum notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, and *Alternaria alternata*), egg, milk, peanut, cod, wheat, and soya bean allergens. Values of 0.35 kU/L or greater were considered indicative of sensitization and were analyzed as the dichotomized index of any sensitization.

Skin prick test was performed at ½, 1½, 4, and 6 years of age with cat, dog, horse, birch, timothy grass, *D pteronyssinus*, *D farinae*, mugwort, *Alternaria alternata*, *C herbarum*, egg, milk, peanut, cod, wheat, rye, beef, pork, and soya bean allergen extracts (ALK-Abelló, Copenhagen, Denmark), as well as raw egg and milk. A mean wheal diameter of 2 mm or more larger than that elicited by the negative control at ½ or 1½ year, and of 3 mm or larger at 4 or 6 years, was considered indicative of sensitization and was analyzed as the dichotomized index of any sensitization.

The peripheral blood eosinophil count ($10^9/L$) was assessed at ½, 1½, 4, and 6 years and analyzed as a continuous variable.

Allergic rhinitis in the seventh year of life was diagnosed by the doctors at the research clinic based on parental interviews regarding the child's history of symptoms. Rhinitis was defined as troublesome sneezing or blocked or runny nose in the past 12 months in periods without accompanying cold or flu.^{14,15}

Asthma at 6 years was diagnosed by the doctor at the research unit according to international guidelines, with an emphasis on a history of recurrent troublesome lung symptoms recorded in diaries and with a need for short-acting β_2 -agonists, as previously described in detail.^{11,12,16} The diagnosis required symptom improvement during a 3-month trial of inhaled corticosteroids and relapse when this medication was stopped.

Atopic dermatitis was diagnosed based on the criteria of Hanifin and Rajka, as previously detailed.¹⁷

Fecal samples

Fecal samples were collected in sterile plastic containers at the ages of 1 and 12 months and stored at 4°C until they were transported (within 24 hours) to Statens Serum Institute (Copenhagen, Denmark). Each sample was mixed on arrival with 1 mL of 10% vol/vol glycerol broth (SSI, Copenhagen,

Denmark) and frozen at -80°C until further processing. Both 1 and 12 months samples were available for 253 children.

DNA extraction

Two hundred milligrams of the fecal sample was added to a 2-mL vial containing 1.4 mL of ASL buffer and 0.3 g of zirconium beads (diameter, 0.1 mm; Biospec Product, Inc, Bartlesville, Okla) and homogenized at 30 Hz for 6 minutes by using the TissueLyser system (Qiagen Retsch GmbH, Haan, Germany).¹⁸

DNA was extracted by using the QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in a final volume of 100 μ L.

PCR

Primers PRBA338-GCf and PRUN518r^{18,19} were used to amplify the V3 region of bacterial 16S rRNA. All primers were purchased from MWG Biotech (Ebersberg, Germany). A 40-bp GC clamp was attached to the 5' end of PRBA338-GCf. The PCR reaction mixture contained 1 \times PCR buffer (Applied Biosystems, Foster City, Calif), 1 μ L of genomic DNA, 0.1 μ mol/L of both primers, 200 μ mol/L of each deoxynucleoside triphosphate (Roche Applied Science, Penzberg, Germany), 1.5 mmol/L $MgCl_2$ (Applied Biosystems), 0.5 μ L of BSA (10 mg/mL), and 1.25 U of Taq DNA polymerase (Applied Biosystems) in a final volume of 50 μ L. PCR was performed by using the following parameters: 94°C for 5 minutes followed by 30 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, with a final extension of 7 minutes at 72°C.

DGGE

PCR fragments were separated by means of DGGE, as described by Muyzer and Smalla,²⁰ with a Bio-Rad DCode System (Bio-Rad Laboratories, Hercules, Calif). A 30% to 65% gradient was used to separate PCR products on 8% polyacrylamide gels (wt/vol; acrylamide/bisacrylamide, 37.5:1) in 0.5 \times Tris-acetate-ETDA. A 100% denaturant is defined as 7 mol/L urea and 40% (vol/vol) deionized formamide. Twenty microliters of PCR amplicon was loaded in each lane and electrophoresed at 70 V for 16 hours at 60°C. Gels were stained with SYBR-Gold (Molecular Probes, Invitrogen, Carlsbad, Calif) for 20 minutes and photographed under UV transillumination by using a Gel Doc system (Bio-Rad Laboratories).

Band matching on DGGE fingerprint profiles was performed by using BioNumerics software 4.50 (Applied Maths, St-Martens-Latem, Belgium). The number of band classes distinguished depended on the optimization and position tolerance, which was set at 0.05%. For each child, the final DGGE data were reported as a profile consisting of 1s or 0s, indicating the presence of a certain band or not, respectively.

Neonatal intestinal bacterial flora identification by means of culturing

From each fecal sample, approximately 200 mg was transferred to 1 mL of sterile ultrapure water and mixed thoroughly. Samples were then plated on 6 different selective and nonselective media (media all produced by SSI Diagnostica, Hillerød, Copenhagen, Denmark, www.ssi.dk). Blood agar (5%) and chocolate agar plates were used as culture media for *Streptococcus* and other bacterial genera; a blue agar plate was used for gram-negative bacilli, especially enterobacteriaceae; and a Sabouraud plate was used for yeast and fungi. These 4 media were incubated aerobically at 37°C for 48 hours. The cycloserine cefoxitin fructose agar plate was selective the medium for *Clostridium difficile* and was incubated anaerobically (5% CO_2 , 3% H_2 , 5% O_2 , and 87% N_2 at 37°C) for 48 hours. A specific developed anaerobic plate for strict anaerobes was incubated in anaerobic jars (GasPAK; BD, Franklin Lakes, NJ) at 37°C for 5 to 6 days and checked every day. All anaerobic microorganisms were tested for the absence of growth under aerobic conditions (for 2 days), and antimicrobial susceptibility was tested by using a disc of 5 μ g of metronidazole and 1000 μ g of kanamycin (Oxoid Limited, Hampshire,

United Kingdom). Subsequently, microbial identification was performed according to growth on selective media, characteristics of colonies, and cellular morphology and confirmed by using the automated identification system VITEK 2 (Bio Mérieux, Nürtingen, Germany). Isolates from anaerobic plates were preserved at -80°C for future identification. No quantitative culture method was used. The microorganisms were identified primarily at the species level.

Environmental risk factors

Information on the type of delivery, mother's use of antibiotics, breast-feeding of the baby, and cat or dog in the home during the first year of life was obtained at the clinic visits at 1 month, 6 months, and 1 year of life.

Statistical analysis

Initially, all DGGE bands were considered and included in the statistical analyses to look for a pattern (ie, a combination of specific DGGE bands) that could be descriptive of a certain outcome. However, this approach was unsuccessful because no patterns were found to be consistently present in children with the same reported outcome or outcomes. Although each sample had relatively few bands (6-20 bands), the positioning was virtually distinct for each sample. Instead, we chose to use the number of bands in each DGGE profile for each child (so-called band richness) as a measure of bacterial diversity. This was simply done by summarizing the profile of each child (consisting of 1s and 0s). Band classes were analyzed by means of principal component analysis (PCA), and the resultant principal component scores were plotted in 2-dimensional scatter plots. The plots were colored according to the end point, exploring for patterns in these variables. The PCA models were developed in LatentX version 2.00 (Latent5, Copenhagen, Denmark).

The end point variables of asthma and allergic rhinitis were dichotomized variables, and any association with colonization of bacterial groups and band richness was tested by means of logistic regression. Specific IgE levels, skin prick test results, and peripheral blood eosinophil counts measured at 4 time points were assessed by using a generalized estimating equation (GEE) analysis (repeated-measures analysis). Specific IgE levels and skin prick test results were analyzed as dichotomized variables in log-linear models. Peripheral blood eosinophil counts were analyzed as a continuous variable in a linear model. Peripheral blood eosinophil counts were log transformed to obtain normal distributed data before analysis. Atopic dermatitis was assessed by time to onset, and Cox regression analysis was used to test differences in risk by band richness and bacterial culture.

Band richness at 1 and 12 months was analyzed for independent effects on outcome in the same model and as a combined measure (average band richness at 1 and 12 months).

A significance level of .05 was used in all types of analyses. All analyses were made in SAS version 9.2 software for Windows (SAS Institute, Inc, Cary, NC).

Missing data were treated as missing observations. Loss to follow-up was treated as censored subjects in Cox regression analysis.

RESULTS

Four hundred eleven infants were enrolled at 1 month of age. Cultures from fecal samples were successful in 346 neonates at 1 month and 325 infants at 12 months. DGGE PCR was completed in 300 children at 1 month and 301 children at 12 months, and 253 were completed at both time points. Baseline characteristics for the main cohort, the 253-member subgroup with complete data on DGGE PCR data, and the excluded population are compared in Table E1 (available in this article's Online Repository at www.jacionline.org), including information on sex; ethnicity; *ORMDL3* risk alleles; atopic heredity;

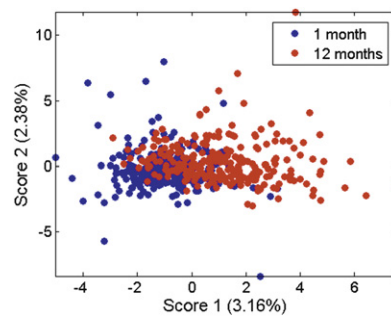


FIG 1. PCA score plot colored for the DGGE at the 1- or 12-month sample. The scores from the 2 primary principal components are plotted against each other, visualizing the main systematic variation in the DGGE data. The first principal component is caused by the infant's age at sampling and explains only 3% of the total variation in the DGGE data, indicating that these data represent very little systematic variation (patterns).

sociodemographics; anthropometrics; mode of delivery; the mother's exposure to tobacco, antibiotics, and paracetamol; and the infant's exposures to breast-feeding, day care, furred pets, older siblings, and nicotine in hair. The selected cohort was biased toward significantly higher household income and a slightly older father and mother.

A range of 1 to a maximum of 10 bacterial species was isolated per specimen, whereas we identified up to 20 bands by using the DGGE method (see Table E2 in this article's Online Repository at www.jacionline.org). Because the diversity was so big within samples, we chose to present the data at the family level.

Analysis of PCR combined with DGGE

The 16S rRNA DGGE profiles showed a significantly greater number of bands at 12 months compared with 1 month (mean of 8.5 bands [range, 2-20 bands] vs mean of 6.0 bands [range, 1-14 bands], $P < .0001$).

Initially, we calculated the correlation coefficient between all bands and all outcome variables. This resulted in very low correlation coefficients (results not shown), indicating either that no individual bands are linked to any outcome or that such a link depends on a combination of several bands. Fig 1 shows a PCA score plot colored for the DGGE at 1- or 12-month samples, confirming the above-reported difference between the 1- and 12-month profiles. The first principal component explains only 3% of the total variation in the DGGE data, indicating that these data represent very little systematic variation (patterns). Any other systematic variation would account for less than 3%, and indeed, no other pattern could be linked to the clinical outcomes (results not shown). This shows that no single DGGE band or a combination of DGGE bands (ie, patterns) is responsible for the clinical outcomes.

Subsequently, we analyzed the band richness and found that the band richness at 1 and 12 months was not significantly correlated ($n = 253$, $P > .1$, Pearson correlation coefficient $r = -0.051$); that is, high band richness at 1 month did not associate with higher band richness at 12 months.

We then analyzed for independent effects of band richness at 1 and 12 months on allergic sensitization in a multivariate model finding separate significant effects from 1- and 12-month band richness (Table I). Therefore we analyzed the average of 1- and 12-month band richness, which strengthened all estimates

TABLE I. Risk of atopic disease from DGGE band richness in the infant's intestinal flora

End point	Age (y)	No.	Band richness at:	Estimate (95% CL)	P value
Repeated assessments					
Specific IgE	½, 1½, 4, and 6 y	910	1 mo	-0.106 (-0.202 to -0.098)	.031
			12 mo	-0.093 (-0.179 to -0.070)	.034
			Average	-0.196 (-0.324 to -0.069)	.0027
Skin prick test	½, 1½, 4, and 6 y	914	1 mo	-0.054 (-0.155 to 0.047)	>.1
			12 mo	-0.114 (-0.224 to -0.005)	.040
			Average	-0.179 (-0.326 to -0.032)	.017
Peripheral blood eosinophils	½, 1½, 4, and 6 y	836	1 mo	-0.018 (-0.042 to 0.007)	>.1
			12 mo	-0.017 (-0.038 to 0.003)	.10
			Average	-0.035 (-0.067 to -0.003)	.034
Current disease					
Allergic rhinitis	7 y	28/162	1 mo	0.78 (0.64 to 0.96)	.016
			12 mo	0.88 (0.76 to 1.02)	.08
			Average	0.71 (0.56 to 0.91)	.007
Current asthma	6 y	27/229	1 mo	1.01 (0.86 to 1.19)	>.1
			12 mo	0.98 (0.85 to 1.12)	>.1
			Average	0.98 (0.79 to 1.21)	>.1
Time to onset					
Atopic dermatitis	0-6 y	127/253	1 mo	0.96 (0.89 to 1.03)	>.1
			12 mo	1.00 (0.95 to 1.06)	>.1
			Average	0.97 (0.89 to 1.06)	>.1

The independent effects of band richness at 1 and 12 months were analyzed together as explanatory variables in the same model. The average band richness was analyzed separately (in boldface).
CL, Confidence limit.

(Table I). The average infant band richness was inversely associated with the development of sensitization assessed both by specific IgE levels in serum ($P = .0027$) and skin prick test results ($P = .017$), as well as allergic rhinitis ($P = .007$), in logistic regression analysis models (Table I). The average infant's band richness was also inversely associated with peripheral blood eosinophilia in a GEE model ($P = .034$). There were no associations between band richness and development of atopic dermatitis ($P > .1$) neither during the first 6 years of life nor when the Cox regression analyses were restricted to the first 2 years of life ($P > .1$). Band richness showed no association with current asthma at the age of 6 years in a logistic regression analysis ($P > .1$). There was no association with atopic dermatitis in combination with sensitization either (data not shown).

The average band richness was not significantly associated with cesarean section or mother's use of antibiotics in the third trimester, solely breast-feeding of the baby, or having a dog or cat at home at birth (data not shown). As expected from this, adjusting for these covariates did not materially affect the associations between band richness and outcomes (see Table E3 in this article's Online Repository at www.jacionline.org).

There was a trend suggesting that band richness was reduced (14.8 vs 13.8) in children with culture positive for staphylococaceae ($P = .06$).

Culturing of neonatal intestinal bacterial flora by 1 and 12 months

The same PCA procedure as explained above was applied for the culturing data, but again, no patterns were found in data related to any of the investigated outcomes.

The prevalence of the most common bacterial genera and species detected in fecal culture in 1- and 12-month samples were

divided into 5 common genera groups (see Table E4 in this article's Online Repository at www.jacionline.org). Bacterial groups detected in fewer than 3% of infants were not analyzed further and are therefore not shown in Table E4.

The average number of bacteria increased from 1 to 12 months of age. Particularly, the prevalence of enterobacteriaceae, enterococcaceae, yeast, and fungi increased, whereas the prevalence of staphylococcaceae decreased from 1 to 12 months (see Table E5 in this article's Online Repository at www.jacionline.org).

Sensitization (specific IgE) was associated with cultures of staphylococcaceae at 1 month ($P = .035$) but not at 12 months (Table E4). Otherwise, sensitization, peripheral blood eosinophil counts, allergic rhinitis, or asthma was not associated with any of the cultured groups of microflora at 1 and 12 months of age ($P > .1$ in all tests) nor was the risk rate for atopic dermatitis during the first 2 or 6 years of life increased from any of the cultured bacteria ($P > .1$ in all tests). There was no association detected with the combined end point of atopic dermatitis and sensitization either (data not shown).

DISCUSSION

Principal findings

Reduced bacterial diversity in the infant's intestinal flora increased the risk of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia but was not associated with the risk of asthma or atopic dermatitis during the first 6 years of life. Although the causal direction of these associations between the bacterial diversity of the intestinal flora and the development of atopic disease cannot be determined, the findings support the general hypothesis that imbalance of the human microbiome in infancy modifies the development of lifestyle-associated disorders, such as atopic disease.

Limitations of the study

The main limitation of the study is the general difficulty identifying all bacterial species in the gut flora. Cultures are mainly limited by the unknown number of nonculturable bacteria and the expected loss of anaerobic bacteria. Transportation of fecal specimens might reduce the number of bacteria cultured.

The number of bacteria identified by means of both culture and the DGGE method was lower than seen in studies applying single-strand polymorphisms, finding a much higher diversity even within the same species.²¹ We used the conserved region V3 to identify species in the community. Recently, this region has been compared with other 16S regions and was found to be suitable for detecting species in complex communities.²² Furthermore, our DDGE gels were analyzed by using a computer program set to eliminate background bands.

We used summation scores of the bacterial diversity, which ignores possible effects from specific species and does not account for cross-correlation patterns.

The external validity of the study is limited by the selection of children born of mothers with a history of asthma. The association reported in this study therefore requires replication in unselected populations.

Strengths of the study

A major strength of this study is the long-term, closely monitored nature of a birth cohort with comprehensive assessments of clinical symptom presentations together with objective assessments. COPSAC is a long-term clinical birth cohort study with deep phenotyping and objective exposure assessments and has provided 6-year clinical surveillance of a birth cohort attending this central clinical research unit instead of other health care facilities. Recall bias of symptoms and exposures was minimized with close prospective follow-up at 6-month visits at the research unit in addition to symptom review by doctors, with support from daily diary cards. Risk of misclassification was low because the families only saw the doctors employed at the clinical research unit for diagnosis and treatment of any atopy-related symptom rather than their family practitioners; that is, they were not prone to the misclassification common for these diseases in the general medical community. Clinical diagnosis and treatments were based on clinical interviews (not questionnaires) at the research unit by experienced study physicians in accordance with standard operating procedures and predefined algorithms.

Another strength of this study is the comprehensive microbiological characterization, analyzing samples of the intestinal flora by using both culture-independent methods and conventional cultures in more than 300 infants at both 1 and 12 months of age. This is the largest study of bacterial diversity measured by DNA techniques and atopic diseases reported to date. Conventional bacteriologic culture methods are vulnerable to sampling, transportation, and storage, as well as laboratory praxis, quality of media, and bacterial growth conditions in studying the composition of the intestinal microbiota.^{23,24} In addition, a large number of anaerobic bacteria cannot be cultivated on available media. Molecular techniques based on 16S rRNA PCR combined with DGGE are an efficient method to investigate the bacterial biodiversity in fecal samples, and DGGE is a sensitive culture-independent method widely accepted for bacterial biodiversity studies.^{20,23,25} The DGGE method only provides a relative assessment of the main bacterial strains without identifying the actual

number and identity of the species. The 16S rRNA PCR DGGE approach can also lead to some distortions. Because 16S rRNA PCR DGGE bands correspond to the G+C content of the amplified V3 region of 16S rDNA, bacterial species with similar G+C content in the amplified region might migrate to the same position and appear as a single band, resulting in fewer bands. The presence of intragenomic 16S rDNA heterogeneity in bacterial strains having more than 1 copy of the 16S gene might lead to an overestimation of the number of bacteria.¹⁹

Bacterial diversity at 1 and 12 months was not correlated but was similarly and independently associated with allergic sensitization. These 2 independent tests therefore provide internal replication of the bacterial diversity-sensitization association, reducing the probability that this is a chance finding.

Interpretation

The human intestinal microbiome is important for maintaining normal homeostasis in the host. These bacterial functions include fermentation of nondigestible dietary residue, production of short-chain fatty acids, vitamin synthesis, control of intestinal epithelial cell growth and differentiation, gut hormone production, protection against pathogens, and maturation and homeostasis of the immune system.^{3,4,26} The neonatal period is particularly critical in terms of mucosal defense and immunologic priming, and it has been hypothesized that the maturation of the immune system is dependent on the bacterial milieu, with a potential skewing from an unfavorable ecology. Distortion of some of those functions by reduced bacterial diversity of the gut has been proposed in patients with inflammatory bowel disease,¹ obesity,^{2,3} diabetes,⁴ rheumatoid arthritis,⁵ and atopic diseases, such as atopic dermatitis and allergy.⁶⁻⁸

We observed that a reduced bacterial diversity during the first year of life assessed by DNA-based identification was significantly associated with allergic sensitization both assessed by skin test and serum specific IgE measurement. Furthermore, the relevance of the allergic sensitization was strengthened by the observation of an association between bacterial diversity and allergic rhinitis (ie, clinically meaningful symptom manifestation). Finally, bacterial diversity in the infant was associated with peripheral blood eosinophilia, which is a systemic atopic disease marker.

Both 1- and 12-month bacterial diversity were independently associated with allergic sensitization in a multivariate model. This indicates that both measures are involved in the same underlying mechanism and that diversity in the whole first year of life rather than at a single time point is important. To provide an optimal measure of diversity in the first year of life, we therefore combined information on 1- and 12-month diversity. The combined measure (mean band richness) generally demonstrated improved estimates for the associated outcomes.

Our findings are in line with reports on the role of the balance of the intestinal ecosystem for other lifestyle-related disorders,^{1-5,21} whereas the published evidence on the particular association between intestinal flora and the development of atopic disease is ambiguous. The hypothesis of an association between bacterial diversity and the development of atopic disease has been around for almost 2 decades since it was first proposed by Holt²⁷ and Bjorksten.²⁸ However, the available evidence remains ambiguous and without general acceptance of the idea in the scientific community. This is partly because of indiscriminate use of study end

points, such as recurrent wheeze, asthma, atopic dermatitis, sensitization, and their combinations. Even though these are common comorbidities, the extrapolation of risk factors between these disorders is probably not justified.²⁹

Our finding of an inverse association between early bacterial diversity and the development of allergic sensitization is consistent with the other 2 available reports,^{30,31} but not 3 others.³²⁻³⁴ In the latter studies, sensitization was assessed in infancy, when very few children are sensitized, reducing the power for such association analyses.³²⁻³⁴

Our finding of no association between bacterial diversity in the infant and the development of atopic dermatitis is in agreement with some³² and at variance with other^{35,36} previous studies. *Escherichia coli* and *Clostridium difficile* in the intestinal flora at 1 month were both associated with atopic dermatitis by 2 years of age in the KOALA birth cohort by using quantitative PCR to identify the 5 main bacterial species.³³ Combining the phenotypes of atopic dermatitis and sensitization in 2 studies also suggested differences in the composition of intestinal flora preceding such a phenotype.^{37,38} A number of case-control studies of intestinal flora in infants^{39,40} and older children^{41,42} suggested associations between atopic dermatitis and a variety of bacterial strains but with no replication of any particular bacteria and without adjusting for multiple comparisons.

The aim of our project was an unbiased assessment of any association between the human microbiome and the development of atopy. It was not the study hypothesis that any particular bacteria would be associated with atopy and hence the choice of PCR-DGGE as the primary method. Neither PCAs of DGGE band positions nor conventional cultures showed a pattern of any particular bacteria associating with any of the end points. In addition, it was the interesting observation that the associations with allergic sensitization, peripheral blood eosinophilia, and allergic rhinitis were largely similar when comparing bacterial diversity by 1 and 12 months of age, and the estimates were generally strengthened when using the average bacterial diversity at these 2 time points. Together, this supports the interpretation that bacterial diversity rather than particular bacterial strains is the important link to atopic disease. This is in line with a previous study using a similar fingerprint DNA-based method (terminal RFLP) finding meaningful significant associations between lifestyle and a measure of bacterial diversity but no significant clustering in the PCAs.⁴³ Therefore we have not had reason to sequence the DGGE for any particular culprit. Still, our findings are not incompatible with a role for certain pathogenic bacteria. It is likely that pathogenic bacteria displace beneficial bacteria by overgrowing and thereby reducing the diversity. Indeed, we observed a significant inverse association between culture of *Staphylococcus* species and the development of sensitization, and we also observed a trend ($P = .06$) suggesting an inverse association between cultures of *Staphylococcus* species measured at 1 month and the general diversity of the intestinal microbiota, which is consistent with an interpretation of a role for certain pathogenic bacteria through suppression of the general biodiversity. This interpretation finds support in the report on reduced bacterial diversity and increased growth of *Staphylococcus aureus* in stool samples from infants becoming overweight at age 7 years.²

Colonization of the airways has only been studied recently, probably because of the common misconception that the lower airways are sterile, when in fact the bronchial tree contains a

characteristic microbiota that is disturbed in asthmatic airways.⁴⁴ COPSAC showed a strong association between colonization of the airways with common pathogenic bacteria and development of asthma by age 5 years.¹² These findings are also compatible with the interpretation of a role for pathogenic bacteria, disturbing the local bacterial ecology by overgrowing beneficial bacteria. Together, these observations lend support to a general hypothesis of an association between the human microbiome and the development of atopic diseases. Still, the evidence remains fragmented based on associations between bacteria in different organs associated with different atopic end points.

We found no association between the bacterial diversity of the gut and the development of asthma or atopic dermatitis. This might align with the different genetics of sensitization, asthma, and eczema, as recently demonstrated by genomic analyses.⁴⁵

Conclusions

This large-scale study of the diversity of fecal microbiota composition in infancy performed by DNA-based identification and conventional culture suggests an inverse association between the diversity of the intestinal microbiota in infancy and the development of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia. This supports the general hypothesis that the diversity of the human microbiome influences the long-term development of lifestyle-dependent immune disease manifestations, such as atopic disease.

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Key messages

- **Reduced bacterial diversity of intestinal flora in the infant was associated with increased risk of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia but not asthma or atopic dermatitis in the first 6 years of life.**
- **This suggests that an imbalance in the intestinal microbiome is influencing the development of allergic disease.**

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TABLE E1. Baseline characteristics in the full cohort, study group, and dropout group

	Full cohort	Study group	Dropout	P value
No.	411	253	158	—
Genetics				
Male sex (%)	49	49	50	NS*
White (%)	97	97	96	NS*
<i>ORMDL3</i>				NS*
CT (%)	48	47	49	
TT (%)	29	33	24	
Atopic heredity				
Maternal asthma (%)	100	100	100	—
Maternal allergic rhinitis (%)	76	74	78	NS*
Maternal eczema (%)	48	51	44	NS*
Paternal asthma (%)	17	19	14	NS*
Paternal allergic rhinitis (%)	33	36	27	.07*
Paternal eczema (%)	13	14	11	NS*
Sociodemographics				
Household income, mean (SD), 100,000 DKK	494 (207)	516 (205)	452 (204)	.003†
Mother's education				NS*
Low (%)	60	59	62	
Medium (%)	27	29	23	
High (%)	14	13	16	
Mother's occupation				NS*
Student (%)	11	9	15	
Unemployed (%)	10	10	10	
Nonprofessional (%)	34	32	36	
Professional (%)	46	49	38	
Urban living (%)	93	93	94	NS*
Father's age (y), mean (SD)	32.0 (5.2)	32.6 (5.2)	31.0 (5.0)	.002†
Mother's age (y), mean (SD)	30.0 (4.5)	30.7 (4.4)	29.0 (4.6)	.0004†
Birth				
Birth weight, mean (SD), z score	0.43 (1.07)	0.42 (1.16)	0.44 (0.92)	NS†
Birth length, mean (SD), z score	1.48 (1.22)	1.45 (1.31)	1.54 (1.08)	NS†
Birth BMI, mean (SD), z score	-0.53 (1.11)	-0.51 (1.19)	-0.54 (0.98)	NS†
Gestational age (wk), mean (SD)	39.9 (1.57)	39.8 (1.60)	40.0 (1.51)	NS†
Cesarean section (%)	21	25	13	NS*
Apgar score >9 (%)	97	96	97	NS*
Prenatal exposures				
Maternal smoking in third trimester (%)	15	15	16	NS*
Antibiotic use in third trimester (%)	14	14	15	NS
Paracetamol use in third trimester (%)	14	14	15	NS
Postnatal exposures				
Solely breast-fed >4 wk (%)	82	83	79	NS*
Duration of solely breast-feeding (d), mean (SD)	113 (62)	117 (62)	105 (62)	.09†
Day care, age at start, median (IQR)	346 (246-433)	345 (243-422)	352 (256-475)	NS‡
Cat in household first year (%)	14	13	15	NS*
Dog in household first year (%)	13	12	16	NS*
Older siblings (%)				NS*
0	61	57	67	
1	28	32	20	
≥2	11	11	13	
Nicotine in hair at 1 y, median (IQR)	0.74 (0.3-2.5)	0.64 (0.3-2.3)	0.95 (0.4-3.2)	NS‡

IQR, Interquartile range; NS, not significant ($P > .05$).

* χ^2 Test.

†Unpaired t test.

‡Wilcoxon rank sum test.

TABLE E2. Comparison of bacterial diversity assessed by using DGGE bands and bacterial cultures at 1 and 12 months

	Mean	Minimum	Maximum
DGGE bands			
1 mo	6.0	1	12
12 mo	8.5	2	20
Species no.			
1 mo	1.8	0	5
12 mo	2.2	0	6

TABLE E3. Risk of atopic disease from DGGE band-richness in infant intestinal flora in models adjusting for multiple environmental risk factors

End-Point	Age	No.	Band richness at	Estimate [95% CL]	P value
Repeated assessments		No.		GEE-estimate[95% CL]	
Specific IgE	½, 1½, 4 & 6 y	884	1 mo	-0.127 [-0.227; -0.027]	.013
			12 mo	-0.092 [-0.184; 0.000]	.050
			Average	-0.211 [-0.348; -0.075]	.0023
Skin prick test		889	1 mo	-0.052 [-0.156; 0.053]	>.1
			12 mo	-0.101 [-0.211; -0.010]	.075
			Average	-0.161 [-0.308; -0.014]	.032
Peripheral blood eosinophil		812	1 mo	-0.018 [-0.042; 0.006]	>.1
			12 mo	-0.016 [-0.038; 0.006]	>.1
			Average	-0.034 [-0.066; -0.001]	.044
Current disease		Npos/No		Odds ratio [95% CI]	
Allergic rhinitis	7 y	27/160	1 mo	0.78 [0.64-0.95]	.014
			12 mo	0.88 [0.75-1.03]	.107
			Average	0.71 [0.55-0.91]	.007
Current asthma	6 y	27/225	1 mo	0.98 [0.83-1.16]	>.1
			12 mo	0.97 [0.84-1.12]	>.1
			Average	0.95 [0.77-1.18]	>.1
Time to onset		Npos/No.		Hazard ratio [95% CI]	
Atopic dermatitis	0-6 y	123/242	1 mo	0.97 [0.90-1.04]	>.1
			12 mo	1.02 [0.96-1.08]	>.1
			Average	1.00 [0.91-1.09]	.1

The independent effects of band richness at 1 month and 12 month were analyzed together as explanatory variables in the same model. The average band richness was analyzed separately (in boldface). Environmental risk factors included in the models were cesarean section, mother's use of antibiotics in third trimester; solely breast feeding of the baby, or having a dog or cat at home at birth.

TABLE E4. Risk of atopic disease from conventional fecal cultures at 1 and 12 months

	Enterobacteriaceae	Enterococaceae	Staphylococaceae	Anaerobes	Yeasts and fungi
1 mo					
Repeated assessments of end point by age ½, 1½, 4, and 6 years analyzed in a GEE model					
Specific IgE (1129 end point assessments)	0.062 (−0.372 to 0.496); <i>P</i> > .1	−0.107 (−0.586 to 0.372); <i>P</i> > .1	0.508 (0.059 to 0.957); <i>P</i> = .035	−0.113 (−0.662 to 0.437); <i>P</i> > .1	0.107 (−0.558 to 0.771); <i>P</i> > .1
Skin prick test (1131 assessments)	−0.091 (−0.647 to 0.465); <i>P</i> > .1	0.084 (−0.531 to 0.698); <i>P</i> > .1	0.183 (−0.382 to 0.748); <i>P</i> > .1	0.002 (−0.672 to 0.677); <i>P</i> > .1	−0.098 (−1.140 to 0.944); <i>P</i> > .1
Peripheral blood eosinophil counts (10 ⁹ /L; 1025 end point assessments)	−0.052 (−0.177 to 0.074); <i>P</i> > .1	−0.008 (−0.142 to 0.127); <i>P</i> > .1	0.004 (−0.123 to 0.131); <i>P</i> > .1	0.016 (−0.140 to 0.171); <i>P</i> > .1	0.037 (−0.161 to 0.234); <i>P</i> > .1
Current disease analyzed by using a logistic regression model					
Allergic rhinitis, age 7 y (n = 192)	0.69 (0.32 to 1.50); <i>P</i> > .1	0.50 (0.18 to 1.39); <i>P</i> > .1	0.89 (0.39 to 2.02); <i>P</i> > .1	0.86 (0.33 to 2.25); <i>P</i> > .1	0.77 (0.21 to 2.77); <i>P</i> > .1
Asthma, age 6 y (n = 282)	0.94 (0.46 to 1.92); <i>P</i> > .1	1.17 (0.55 to 2.51); <i>P</i> > .1	1.69 (0.83 to 3.46); <i>P</i> > .1	0.94 (0.39 to 2.26); <i>P</i> > .1	1.17 (0.38 to 3.60); <i>P</i> > .1
Time to disease onset analyzed by using Cox regression analyses					
Atopic dermatitis ever (n = 346)	0.91 (0.66 to 1.27); <i>P</i> > .1	1.30 (0.92 to 1.85); <i>P</i> > .1	1.26 (0.90 to 1.77); <i>P</i> > .1	0.84 (0.56 to 1.27); <i>P</i> > .1	1.03 (0.61 to 1.76); <i>P</i> > .1
12 mo					
Repeated assessments of end point by age ½, 1½, 4, and 6 years analyzed in a GEE model					
Specific IgE (1129 end point assessments)	−0.248 (−0.844 to 0.347); <i>P</i> > .1	0.211 (−0.222 to 0.645); <i>P</i> > .1	0.146 (−0.513 to 0.805); <i>P</i> > .1	0.432 (−0.093 to 0.957); <i>P</i> > .1	−0.036 (−0.506 to 0.433); <i>P</i> > .1
Skin prick test (1129 end point assessments)	0.058 (−0.752 to 0.867); <i>P</i> > .1	−0.066 (−0.616 to 0.484); <i>P</i> > .1	0.044 (−1.004 to 1.092); <i>P</i> > .1	0.496 (−0.112 to 1.104); <i>P</i> > .1	0.167 (−0.414 to 0.747); <i>P</i> > .1
Peripheral blood eosinophil counts (10 ⁹ /L; 993 end point assessments)	0.050 (−0.103 to 0.202); <i>P</i> > .1	0.049 (−0.069 to 0.167); <i>P</i> > .1	−0.116 (−0.361 to 0.130); <i>P</i> > .1	−0.149 (−0.303 to 0.005); <i>P</i> > .1	0.067 (−0.057 to 0.191); <i>P</i> > .1
Current disease analyzed by using a logistic regression model					
Allergic rhinitis, age 7 y (n = 304)	0.57 (0.22 to 1.47); <i>P</i> > .1	1.20 (0.57 to 2.53); <i>P</i> > .1	1.07 (0.29 to 3.96); <i>P</i> > .1	1.80 (0.75 to 4.30); <i>P</i> > .1	0.57 (0.23 to 1.39); <i>P</i> > .1
Asthma, age 6 y (n = 282)	1.20 (0.40 to 3.64); <i>P</i> > .1	1.30 (0.61 to 2.75); <i>P</i> > .1	0.72 (0.16 to 3.21); <i>P</i> > .1	1.30 (0.55 to 3.07); <i>P</i> > .1	0.76 (0.33 to 1.77); <i>P</i> > .1
Time to disease onset analyzed by using a Cox regression analyses					
Atopic dermatitis ever (n = 346)	0.91 (0.59 to 1.41); <i>P</i> > .1	0.93 (0.68 to 1.28); <i>P</i> > .1	1.09 (0.62 to 1.93); <i>P</i> > .1	0.84 (0.56 to 1.25); <i>P</i> > .1	0.91 (0.65 to 1.28); <i>P</i> > .1

(1) Enterobacteriaceae include: *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter cowanii*, *Enterobacter aerogenes*, *Enterobacter sakazakii*, *Escherichia hermanii*, and *Escherichia fergusonii*; *Citrobacter freundii*, *farmeri*, *koseri*, *amalonaticus*, *sedlakii*, and *braakii*; *Serratia marcescens*, *fonticola*, *plymuthica*, and *liquefaciens*; *Klebsiella pneumoniae* and *oxytoca*; *Proteus mirabilis*, *penneri*, and *vulgaris*; *Morganella morganii*; *Pantoea agglomerans*; and *Raoultella ornithinolytica*.

(2) Enterococaceae include: *Enterococcus faecalis*, *avium*, *gallinarum*, *faecium*, *raffinosis*, *casseliflavus*, and *durans*.

(3) Staphylococaceae include: *Staphylococcus aureus*, *epidermidis*, *warneri*, *lugdunensis*, *hominis*, *haemolyticus*, *lentus*, *auricularis*, *simulans*, *pasteuri*, *sciuri*, and *vitulinus*.

(4) Anaerobes include: *Clostridium difficile* and other anaerobic species.

(5) Yeasts and fungi include: *Candida albicans*, *Candida tropicalis*, *Candida inconspicua* and other fungi.

TABLE E5. Prevalence of the most common bacterial families detected in fecal culture in 1- and 12-month samples

	1 mo (n = 346)	12 mo (n = 325)
Enterobacteriaceae (1)	64% (221)	85% (275)
Enterococcaceae (2)	26% (90)	43% (141)
Staphylococcaceae (3)	32% (109)	8% (27)
Anaerobes (4)	22% (75)	21% (68)
Yeasts and fungi (5)	11% (38)	32% (103)

Bacteria detected in fewer than 10 infants were not analyzed further.

List of genera and species isolated from the fecal samples—(1) Enterobacteriaceae include: *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter cowanii*, *Enterobacter aerogenes*, *Enterobacter sakazakii*, *Escherichia hermannii*, and *Escherichia fergusonii*; *Citrobacter freundii*, *farmeri*, *koseri*, *amalonaticus*, *amalonaticus*, *sedlakii*, and *braakii*; *Serratia marcescens*, *fonticola*, *plymuthica*, and *liquefaciens*; *Klebsiella pneumoniae* and *oxytoca*; *Proteus mirabilis*, *penneri* and *vulgaris*; *Morganella morganii*; *Pantoea agglomerans*; and *Raoultella ornithinolytica*.

(2) Enterococcaceae include: *Enterococcus faecalis*, *avium*, *gallinarum*, *faecium*, *raffinosis*, *casseliflavus*, and *durans*.

(3) Staphylococcaceae include: *Staphylococcus aureus*, *epidermidis*, *warneri*, *lugdunensis*, *hominis*, *haemolyticus*, *lentus*, *auricularis*, *simulans*, *pasteuri*, *sciuri*, and *vitulinus*.

(4) Anaerobes include: *Clostridium difficile* and other anaerobic species.

(5) Yeasts and fungi include: *Candida albicans*, *Candida tropicalis*, *Candida inconspicua* and other fungi.