Low gut microbiota diversity in early infancy precedes asthma at school age

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Low gut microbiota diversity in early infancy precedes asthma at school age

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Running title: Early gut microbiota diversity and asthma at school age

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ABSTRACT

Background: Low total diversity of the gut microbiota during the first year of life is associated with allergic diseases in infancy, but little is known how early microbial diversity is related to allergic disease later in school age.

Objective: To assess microbial diversity and characterize the dominant bacteria in stool during the first year of life in relation to the prevalence of different allergic diseases in school age, such as asthma, allergic rhinoconjunctivitis and eczema.

Methods: The microbial diversity and composition was analyzed with barcoded 16S rDNA 454 pyrosequencing in stool samples at one week, one month and 12 months of age in 47 infants which were subsequently assessed for allergic disease and skin prick test reactivity at seven years of age (ClinicalTrials.gov ID NCT01285830).

Results: Children developing asthma (n=8) had a lower diversity of the total microbiota than non-asthmatic children at one week (p=0.04) and one month (p=0.003) of age, whereas allergic rhinoconjuctivitis (n=13), eczema (n=12) and positive skin prick reactivity (n=14) at seven years of age did not associate with the gut microbiota diversity. Neither was asthma associated with the microbiota composition later in infancy (at 12 months). Children having IgE-associated eczema in infancy and subsequently developing asthma had lower microbial diversity than those that did not. There were no significant differences, however, in relative abundance of bacterial phyla and genera between children with or without allergic disease.

Conclusion and Clinical relevance: Low total diversity of the gut microbiota during the first month of life was associated with asthma but not allergic rhinoconjunctivitis in children at seven years of age. Measures affecting microbial colonisation of the infant during the first month of life may impact asthma development in childhood.

Key words

Asthma; allergic rhinoconjunctivitis; birth; children; diversity; hygiene hypothesis; microbiota; molecular microbiology

Introduction

A limited microbial exposure may underlie the increase of allergic diseases in affluent countries [1]. Recent reports indicate that a high diversity of the gut microbiota in infancy may be more important than the prevalence of specific bacterial taxa [2-4]. The suggested 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 sharing several common features, the phenotype and the mechanisms underlying the different allergic diseases such as asthma, eczema and allergic rhinoconjunctivitis (ARC) are heterogeneous [5-7]. Also, the importance of and relationship with the intestinal microbiota may differ between the different diseases. Previously, low gut microbial diversity during the first month of life has been associated with subsequent eczema [2, 8-10] and sensitization [2, 3, 8], but still there are no studies reporting low gut microbial diversity preceding asthma development. This is probably primarily due to the fact that most of the clinical follow-ups have been performed in infancy [2, 8-10], when allergic asthma and rhinoconjunctivitis still are uncommon. It might also be a consequence of methodology limitations. The microbial detection sensitivity of terminal restriction fragment length polymorphism (T-RFLP) [8, 10] and denaturing gradient gel electrophoresis (DGGE) [3, 9], which were employed in all studies except one [2], is low, since the median number of peaks/bands detected in these studies was much lower than the expected number of bacterial species. Recently, by employing high-throughput 16S rRNA gene sequencing, we could confirm that low gut microbial diversity during the first month of life was associated with subsequent sensitization and eczema at two years of age [2]. In contrast to previous studies, we could also show that the differences in diversity were attributed to a specific bacterial phylum, Bacteroidetes, and the bacterial genus *Bacteroides.*

A follow-up of this cohort at seven years of age, when respiratory allergic diseases are as common as eczema, gave us the opportunity to assess whether microbial diversity and the relative abundance of dominant bacteria in stool during the first year of life are also associated with development of asthma and allergic rhinoconjunctivitis, and if the importance of the gut microbiota composition during the first month of life lasts until school age. We also hypothesized that the importance of and relationship with the intestinal microbiota differ between the different allergic manifestations.

Methods

Subjects and sample collection

The children included in this study were part of a larger study in South Eastern Sweden between 2001 and 2005, evaluating allergy prevention in infants with family history of allergic disease until two years of age with the probiotic *Lactobacillus reuteri* ATCC 55730 [11]. In this study the infant received *L. reuteri* or placebo daily from day 1-3 until twelve months of age. Children admitted to the neonatal ward during the first week of life were excluded. Stool samples were collected from the infants at age 5-7 days and at one month and twelve months of age. The samples were immediately frozen at -20°C following collections and later stored at -70°C. At two years of age, a follow-up with microbial analyses with barcoded 16S rDNA 454-pyrosequencing was performed, relating microbial diversity in these stool samples with the development of IgE-associated eczema during the first two years of life [2]. All 20 infants with IgE-associated eczema and stool samples available from all three sampling occasions were included in these analyses, and 28 infants without any allergic manifestation were randomly selected as controls. In total 47 of these 48 children have now completed the present seven-year follow-up. The child who dropped out did not have any allergic manifestation at two years of age. Seventeen children belonged to the probiotic and 30 to the placebo group in the original study. All infants were breastfed for at least one month, and no infant received antibiotics before one month of age. A written informed consent was obtained from both parents before inclusion. The Regional Ethics Committee for Human Research at Linköping University approved the study (M171-07). The study is registered at ClinicalTrials.gov (ID NCT01285830).

Clinical investigations

A clinical follow-up was performed by research nurses at seven years of age (± 3 months). Before the visit, the parents completed a questionnaire based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire for 6-7 year old children (http://isaac.auckland.ac.nz/Index.html), supplemented with questions regarding gastrointestinal symptoms, antibiotic and probiotic intake during the last month, family size, pets and parental smoking. Data pertaining infancy was collected in the two-year follow-up [11]. The visits included structured interviews related to symptoms of allergic disease, physical examination, spirometry and measurement of fractional exhaled nitric oxid (FE_{NO}). Spirometry was performed with Jaeger Masterscope version 4.5 (Erich Jaeger GmbH, Würzburg, Germany). Forced expiratory volume at 1 second ($FEV_{1.0}$), and the functional vital capacity (FVC) were assessed. The FVC% was calculated from the ratio $FEV_{1.0}$/FVC. A FVC%<80% was regarded as pathological. Reversibility test with $FEV_{1.0}$ measurement before and after inhalation of a β-agonist (1 mg Terbutaline) was regarded as positive if $FEV_{1.0}$ increased ≥12% (http://www.ginasthma.com). The FE_{NO} was measured at a constant flow of 50 mL/s with NIOX-MINO (Aerocrine AB, Stockholm, Sweden). The cut off level for a pathological FE_{NO} was 20 ppb, which is the 95% percentile in 7-9 year old children [12]. Skin prick tests were done on the volar aspects of the forearm with egg white, fresh skimmed cow milk (lipid concentration 0.5%) and standardized cat, dog, birch, peanut, mite (Der p) and timothy extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as positive and albumin diluent as negative control. The test was regarded as positive if the mean diameter of the wheal was ≥3mm.

Diagnostic criteria

The child should have had symptoms of and/or have been treated for the actual allergic disease during the last twelve months. Thus, children with allergic disease before school age who did not have any symptoms during the last twelve months were defined as healthy. Asthma diagnosis required at least one of following two criteria: 1. Doctor diagnosis and asthma symptoms and/or medication during the last twelve months; 2. Wheeze or nocturnal cough and a positive reversibility test and/or pathological FE_{NO} value. In Sweden most children with asthma are asymptomatic when visiting the doctor, since they are efficiently treated with inhaled corticosteroids. If the asthma diagnosis was based on doctors diagnosis, medical records of the child was always reviewed to confirm that the diagnosis were consistent with the GINA criteria (http://www.ginasthma.com). The diagnosis of ARC was based on standard ISAAC question (http://isaac.auckland.ac.nz/Index.html) and required watery discharge at least twice in contact with the same allergen and no signs of infection. Urticaria was defined as allergic when appearing at least twice in conjunction with a certain allergen. Eczema was defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution, as suggested by Hanifin and Rajka [13]. Eczema was classified as IgE-associated if the infant had also a positive skin prick test.

16S rDNA sequencing and bioinformatics

DNA extraction, 16S rDNA PCR amplification with primer pair 341F-805R targeting V3-V4, PCR product purification, and 454 sequencing were performed as described previously [2]. De-noising, chimera removal and complete linkage clustering of sequences into Operational Taxonomic Units (OTUs) were performed with AmpliconNoise [2]. 318,215 high quality, typically 198 bp long, sequence reads remained, with 828 to 12,909 reads per sample (mean = 2257). These corresponded to 3048 unique sequences and 1856 OTUs, clustered at 97% similarity level. Taxonomic annotations were conducted by BLAST searching the OTUs against a local BLAST database of 16S rDNA sequences from the Ribosomal Database Project (RDP) v. 10.10 [14]. OTUs lacking hits of of ≥ 95% identity over an alignment of length ≥ 180 bp were classified as "no_match". If multiple best hits (same score) were found, the taxonomy was set to the most-detailed level of taxonomy shared by the best hits [2].

Statistical analyses

The online version of Fast Unifrac (http://bmf2.colorado.edu/fastunifrac/) [15]was used to calculate weighted sample distances by mapping our OTU sequences with BLAST onto the Greengenes reference sequences (downloaded from the Fast Unifrac web page, May 2009) and using the corresponding Greengenes tree. A Principal Coordinates Analysis (PCoA) plot based on all pair-wise sample distance was created on the Fast Unifrac web page. Our OTU sequences were mapped onto 154 Greengenes sequences. The Shannon diversity index was employed to measure the biodiversity in samples. Briefly, it is a test that takes in account the richness and the evenness of the species, typically with a value between 1.5-3.5 [16]. It was calculated as $-\Sigma \log(p_i)p_i$, where p_i denotes the frequency of OTU $_i$ [17]. Calculations of the index were made with the *R* software (http://www.r-project.org/) and the *R* package vegan (http://cran.r-project.org/web/packages/vegan/), and differences in diversity were tested with Mann-Whitney U-test, since the levels were not normally distributed. Evenness was calculated with Pielou's evenness index as $-\Sigma \log(p_i)p_i / \log(S_{obs})$, where S_{obs} denotes the number of observed OTUs in the sample. Since these levels are influenced by sequencing depth, and sequencing depth differed between samples, we subsampled (with replacement) 1400 reads from each sample, counted the occurrences of the corresponding OTUs, and performed the diversity calculations on these counts. Only four (out of 141) samples had fewer than 1400 reads and were excluded from this part of the analysis. Statistical significance testing over- and under-representation of the bacterial lineages was made at phylum, class and genus (3% dissimilarity) levels with Mann-Whitney U-test, and p-values were converted to False Discovery Rate values (q-values) to correct for multiple testing [18].

The X^2 test was employed for categorical data, unless the expected frequency for any cell was less than five, when Fisher´s exact test was employed. Student´s t test were employed for normally distributed continuous data. (SPSS 16.0, SPSS Inc, Chicago, IL, USA).

Results

At seven years of age, the prevalence of asthma was 17% (8/47), allergic rhinoconjuntivitis 28% (13/47), eczema 26% (12/47), allergic urticaria 9% (4/47), skin prick test reactivity 34% (14/41) and IgE-associated eczema 27% (11/41). Low total diversity as measured by the Shannon diversity index of the gut microbiota at one week and one month of age was associated with asthma diagnosis in children at seven years of age (Table 1, Fig. 1a). Allergic rhinoconjunctivitis, SPT reactivity (Table 1), eczema and IgE-associated eczema (Supplementary Table 1) at this age did not associate with the gut microbiota diversity during the first year of life, however. Neither did asthma have any significant association with total microbiota diversity later in infancy (at twelve months) nor any consistent association with the diversity of different bacterial phyla at any age (data not shown). Similar results were obtained when comparing children with asthma, allergic rhinoconjunctivitis, SPT reactivity, eczema and IgE-associated eczema with control children with no allergic manifestations (data not shown). The evenness of the microbial composition according to Pielou's test at one week and one month of age was lower in children with than without asthma (Fig. 1b). Also the number of bacterial OTUs in stool samples tended to be low at one month of age in the asthma group (Table 2). In order to evaluate whether sensitized infants who subsequently developed asthma also had a different gut microbiota composition than sensitized infants who did not, analyses were performed when only the 20 children with IgE-associated eczema at two years of age were included. Indeed, the seven children having IgE-associated eczema in infancy and subsequently developing asthma had a lower microbial diversity than those 13 children who did not (Supplementary Table 2), although the p-values reveal only a trend, probably due to the lost of statistical power ($p=0.06$ and $p=0.09$ at one week and one month, respectively). Thus, children with IgE-associated eczema in infancy who had developed asthma at seven years of age had a median of the diversity index of 1.25 (interquartile range;

0.84-1.45) at one month of age compared to 1.53 (1.42-1.72) if they did not have asthma and 1.67 (1.51-2.14) if they did not have IgE-associated eczema at two years of age. No such differences were seen for the other allergic manifestations (Supplementary Table 2). Despite the association to asthma, there was no significant correlation between FE_{NO} levels and microbial diversity (data now shown). However, the only child with pathological FE_{NO} levels (>20 ppm) had very low diversity indices (0.69 at one week and 0.72 at one month).

There were no significant differences in relative abundance of bacterial phyla, classes and genera between children with or without asthma (Table 3) or with and without ARC and eczema (data not shown). Neither did Principal Coordinates Analysis based on Unifrac sample distances reveal any clear separation of samples in relation to asthma (Supplementary Fig. 1) or any other of the allergic diseases (data not shown).

There were no differences regarding potential confounders such as sex, birth order, caesarean section, family history of allergic disease, breastfeeding, furred pets at home, antibiotics, infections and probiotic supplementation between the children with and without asthma (Table 4), nor between children with or without any other allergic manifestation (data not shown). Neither were there any significant associations between these factors and microbial diversity except for exclusive breastfeeding at one month, tending to be associated with low diversity at one month of age (p=0.05, data not shown). Excluding the seven children who were not breastfed exclusively at one month did not affect the comparison between asthmatic and non-asthmatic infants (p=0.001, data not shown), however, neither did exclusion of children who were delivered by caesarean section or were supplemented with probiotics, two other factors that might affect the gut microbial diversity at one month (p= 0.009 after excluding children delivered with caesarean section and p=0.03 after excluding children in the probiotic group, data not shown). No child received antibiotics during the first month of life. The number of reported infections during the first two years of life did not correlate significantly with total diversity values (data not shown).

Discussion

Employing high-throughput 16S rRNA gene based molecular microbiology, we could confirm and extend previous findings, showing that low intestinal diversity during the first month of life is associated with an increased risk of subsequent allergic disease [2, 3, 8-10] and that the effect remains in school age. In contrast to previous studies, however, our results indicate that early gut microbial diversity may be more associated with asthma development at school age than other allergic manifestations. Low gut microbial diversity has previously been associated with IgE-associated eczema at two years of age in the same cohort as the present one [2]. Interestingly, the present study indicates that the low gut microbiota diversity in these infants with IgE-associated eczema at two years of age primarily was confined to children subsequently developing asthma in school age. The absent correlation between the infant gut microbiota and eczema in our study supports the result from a previous study investigating the effect of the microbial diversity on an allergy development until school age [3] and indicates that other factors, e.g. skin barrier dysfunction due to filaggrin mutations, underlie persistent eczema [5]. There was no significant association between asthma and the relative abundance of any phylum or genus, nor any significant sample clustering in asthmatic infants. Thus, the total diversity seems to be more important than any particular microbial group for asthma development, although the lack of significant difference between individual phyla may also be due to low statistical power or in these analyses. Also, stool samples only reflect the microbiota in the luminal space of the colon and not the small intestine and the mucosa. Thus, there might be specific bacterial species important for prevention of asthma as well as ARC, which are not revealed in this study.

Previous studies have not revealed any relationship between microbial diversity and asthma development. This is probably primarily due to the fact that most of the clinical follow-ups have been performed in young children [2, 8-10], when allergic asthma and rhinoconjunctivitis still are uncommon. It might also be a consequence of methodology limitations. The sensitivity of our analyses was higher than in previous diversity studies [3, 8-10]. In the study by Bisgaard *et al.* [3], in which infant gut diversity was associated with sensitization but not asthma in school age, the mean of bands/samples, were only 8.5 (with DGGE) at 12 months of age, as compared to 69 OTUs/sample in our study. The community resolution might still not have been high enough in our study to reveal an association between specific bacterial species and asthma and ARC, however. Another important factor possibly affecting the results is the variation of the gut microbiota composition in different countries [19]. Whether our observations in Swedish children can be translated to children in other regions of the world needs to be further investigated.

It is noteworthy that the most important differences appeared the first months of life, supporting the theory that factors influencing the early of maturation of the immune system might be especially important for subsequent asthma development [20]. Furthermore, the results indicate that the immunological phenotype preceding asthma development in particular is established during the first month of life. Viral lower respiratory tract infections (LRTIs) have been suggested to be linked to asthma development among atopic children [7]. The incidence of recurrent wheeze, which often are caused by LRTIs in infancy, was 50% in the infants subsequently developing asthma at 7 years of age compared to 3% in those that did not. It is tempting to speculate that infants subsequently developing asthma are more prone to getting LRTIs, caused by respiratory syncytial virus or rhinoviruses, because of an attenuated maturation of the immune system as a consequence of low stimulation from the gut microbiota during the first months of life. Also, reduced mucosal barrier function may be linked to high susceptibility of LRTIs, amplification of Th2 responses and subsequent asthma development [7, 21]. Low salivary secretory IgA levels are associated with increased prevalence of late onset wheeze in sensitized infants [22], and interestingly, also low intestinal microbial diversity [23].

The present study does not explain why infants developing asthma have low gut microbial diversity. The differences were not due to antibiotic treatment, which may increase the risk for asthma development [24] as no child received antibiotics during the first month of life. Also, while caesarean section has been linked to asthma development and affects gut microbiota during the first month of life [25], the association between low diversity and asthma remained when including only children born with vaginal delivery. Still, the difference in diversity in neonates may be explained by other factors such as the biodiversity in the homes (mattresses, dust etc.) [26, 27], in the surrounding environment [28] and in family members (skin, mouth and gut) [29]. Also, hygienic practices may influence the microbial diversity and allergy development [30]. Recently, children whose parents "cleaned" their pacifier by sucking it were less likely to have asthma at 18 months of age than children whose parents did not use this cleaning technique [31]. Infants with low gut microbial diversity also had low microbial exposure via the respiratory mucosa. The maturation of the respiratory mucosal immune system depends at least partly on bacterial colonization of the lower airways [32]. Whether asthma, however, would be more related to the nature of microbial colonization of the airways than eczema and allergic rhinoconjunctivitis require further elucidation.

In conclusion, low total diversity of the gut microbiota during the first month of life was associated with asthma in children at seven years of age. The early gut microbial diversity seems to be most important for asthma development and did not apply to the other allergic manifestations in school age in our study, although this might be a consequence of the relatively few cases included.

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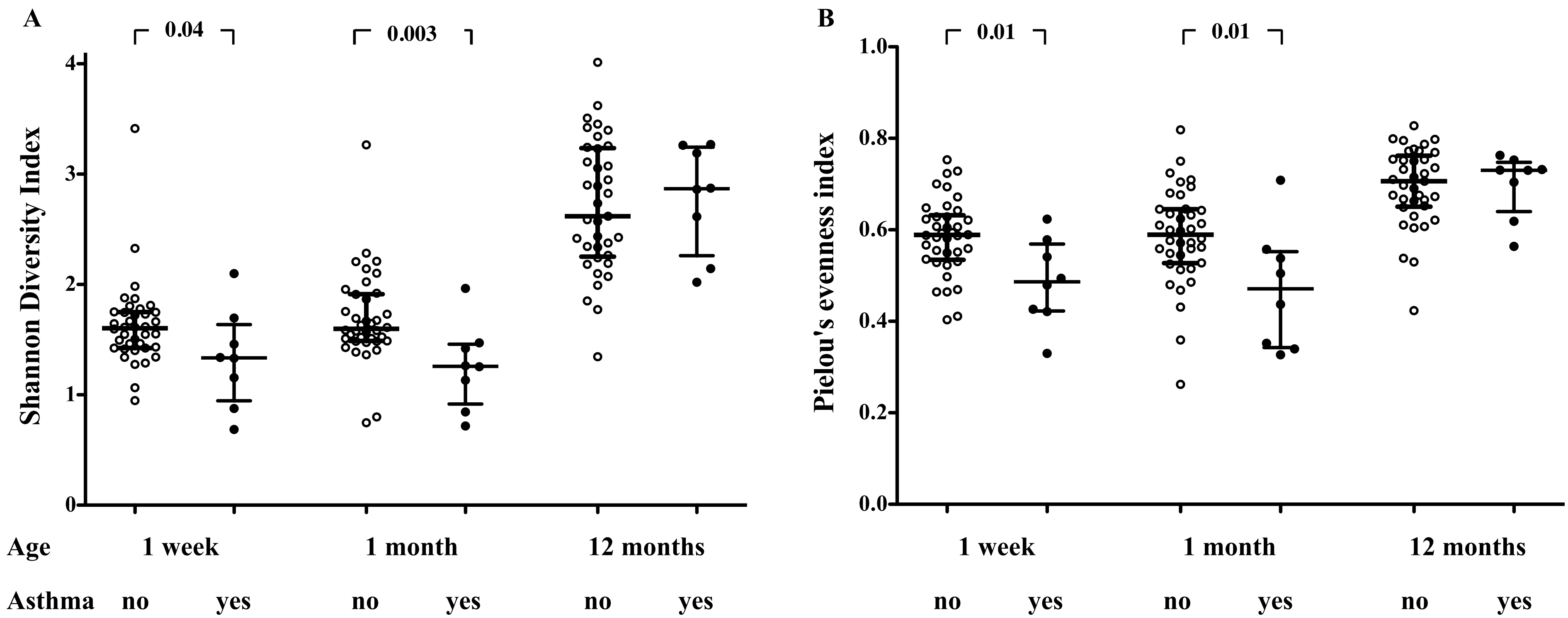

Fig 1.
The Shannon diversity index (a) and Pielou's evenness index (b) of the gut microbiota in stool samples at one week, one month and twelve months of age in infants with (black circles) and without (clear circles) asthma at seven years of age. The 25th, 50th and 75th percentiles are indicated. Groups were compared using Mann-Whitney U-test.

Tables

Table 1. The Shannon diversity index of the total microbiota during the first year of life in children with asthma, allergic rhinoconjunctivitis and positive skin prick test at seven years of age.

	Asthma at 7 years of age			**At 7 years of age**		
	Yes	**No**		**Asthma**	**Healthy****	
	n=8 median (iqr)	n=38 median (iqr)	P-value*	n=8 median (iqr)	n=23 median (iqr)	P-value*
1 week	1.34 (0.95-1.64)	1.60 (1.42-1.75)	**0.04**	1.34 (0.95-1.64)	1.60 (1.42-1.80)	**0.05**
1 month	1.26 (0.92-1.46)	1.60 (1.49-1.91)	**0.003**	1.26 (0.92-1.46)	1.58 (1.48-2.10)	**0.007**
12 months	2.87 (2.26-3.24)	2.62 (2.25-3.24)	0.79	2.87 (2.26-3.24)	2.82 (2.32-3.25)	0.96

	ARC at 7 years of age			**SPT pos at 7 years of age**		
	Yes	**No**		**Yes**	**No**	
	n=13 median (iqr)	n=33 median (iqr)	P-value*	n=14 median (iqr)	n=27 median (iqr)	P-value*
1 week	1.61 (1.25-1.75)	1.55 (1.42-1.74)	0.80	1.71 (1.38-1.75)	1.55 (1.42-1.79)	0.80
1 month	1.59 (1.42-1.89)	1.57 (1.44-1.83)	0.87	1.62 (1.42-1.88)	1.55 (1.47-1.92)	0.87
12 months	2.83 (2.22-2.98)	2.68 (2.28-3.26)	0.48	2.70 (2.32-3.21)	2.62 (2.22-3.24)	0.79

*Mann Whitney U-test. Iqr= interquartile range.

**Healthy= non-sensitised children without any allergic symptoms 0-7y.

Table 2. The median of all OTUs and taxonomic classified OTUs (bacterial genus)/infant in stool samples during the first year of life in children with and without asthma at seven years of age

	Asthma at 7 years of age					
	1 week		**1 month**		**12 months**	
	Yes n=8 median (iqr)	**No n=39 median (iqr)**	**Yes n=8 median (iqr)**	**No n=39 median (iqr)**	**Yes n=8 median (iqr)**	**No n=39 median (iqr)**
OTUs	15 (10-22)	16 (13-18)	14# (12-17)	18# (14-22)	51 (40-73)	47 (33-59)
Classified OTUs	15 (8-22)	15 (12-18)	14 (12-15)	17 (14-21)	50 (39-71)	47 (33-59)
Classified OTUs to genus level	13 (6-19)	12 (10-15)	11* (10-12)	14* (11-17)	39 (30-46)	33 (22-45)

iqr=interquartile range. #p=0.09, *p=0.06 with Mann Whitney U-test.

Table 3. The mean of the relative abundance of dominant phyla (bold), classes and genera (relative abundance >1% at any age) in stool samples obtained at various ages from infants who did or did not develop asthma at seven years of life.

	Asthma 1 week		**Asthma 1 month**		**Asthma 12 months**	
	Yes	**No**	**Yes**	**No**	**Yes**	**No**
	n=8 mean % (SD)	n=39 mean % (SD)	n=8 mean % (SD)	n=39 mean % (SD)	n=8 mean % (SD)	n=39 mean % (SD)
Actinobacteria	26 (32)	23 (24)	48 (36)	34 (27)	5 (5)	14 (17)
Bifidobacterium	25 (33)	22 (24)	47 (35)	32 (27)	4 (5)	13 (17)
Collinsella	<1	<1	<1	1 (2)	<1	<1
Proteobacteria	18 (22)	19 (18)	12 (12)	13 (13)	5 (9)	17 (21)
Enterobacteriaceae (unclassified)	18 (22)	11 (17)	11 (13)	6 (11)	<1	2 (4)
Bacteriodetes	7 (20)	14 (22)	5 (9)	17 (21)	12 (12)	10 (11)
Bacteroides	7 (19)	12 (19)	5 (9)	14 (20)	8 (10)	9 (11)
Parabacteroides	<1	2 (5)	<1	1 (4)	<1	<1
Prevotella	<1	<1	<1	<1	3 (8)	<1
Firmicutes	49 (36)	44 (28)	34 (36)	36 (25)	80 (15)	70 (18)
Bacilli class	33 (29)	29 (24)	7 (4)	15 (13)	2 (4)	7 (12)
Streptococcus	4 (5)	15 (16)	4 (4)	10 (12)	2 (3)	4 (7)
Enterococcus	18 (21)	6 (13)	1 (2)	3 (6)	<1	2 (10)
Lactobacillus	<1	1 (3)	1 (2)	1 (3)	<1	<1
Staphylococus	10 (12)	7 (9)	1 (2)	1 (2)	<1	<1
Clostridia class	15 (19)	14 (14)	27 (35)	18 (21)	71 (12)	58 (20)
Veillonella	3 (4)	5 (8)	1 (1)	2 (4)	2 (3)	2 (2)
Lachnospiraceae Incertae Sedis	3 (9)	<1	2 (5)	2 (6)	7 (5)	5 (5)
Peptostreptococcaceae Incertae Sedis	<1	1 (3)	<1	1 (2)	4 (4)	4 (4)
Erysipelotrichaceae Incertae Sedis	<1	<1	<1	2 (7)	3 (3)	4 (5)
Clostridium	<1	1 (3)	5 (9)	1 (6)	<1	1 (3)
Faecalibacterium	<1	<1	<1	<1	2 (3)	3 (4)
Ruminococcus	<1	<1	<1	<1	1 (1)	2 (3)
Anaerostipes	<1	<1	<1	<1	4 (6)	1 (1)
Anaerococcus	<1	<1	<1	1 (6)	<1	<1
(Unclassified) *Lachnospiraceae*	<1	<1	<1	<1	8 (8)	6 (6)
(Unclassified) *Erysipelotrichaceae*	<1	<1	<1	<1	3(5)	<1
(Unclassified) *Ruminococcaceae*	<1	<1	<1	<1	1 (1)	1 (1)
Verrucomicrobia	<1	<1	<1	1 (4)	2 (4)	2 (5)
Akkermansia	<1	<1	<1	1 (4)	2 (4)	2 (5)

No significant difference with Mann Whitney U-test

Table 4. The background factors and other allergic manifestations in children with and without asthma at seven years of age

	Asthma at 7 years of age		
	Yes	**No**	
	% (n/N)	**% (n/N)**	P*
Probiotic group	25 (2/8)	38 (15/39)	0.69
Boys	88 (7/8)	51 (20/39)	0.11
Older sibling	38 (3/8)	51 (20/39)	0.70
Maternal atopy	88 (7/8)	87 (34/39)	1.00
Asthma in family	75 (6/8)	46 (18/49)	0.25
Ceasarean section	13 (1/8)	23 (9/39)	0.68
Breastfeeding (exclusive) at 1 m	88 (7/8)	85 (33/39)	1.00
Breastfeeding (any) at 1 m	100 (8/8)	100 (39/39)	1.00
Breastfeeding (any) at 12 m	13 (1/8)	38 (15/39)	0.23
Furred pets at birth	0 (0/8)	8 (3/39)	1.00
Antibiotics 0-12 m	25 (2/8)	21 (8/39)	1.00
Infections 0-12m mean (sd)	5.3 (3.4)	5.6 (2.6)	0.71
Infections 12-24m mean (sd)	6.1 (2.9)	5.3 (4.0)	0.57
Day-care at 12 months of age	0 (0/8)	5 (2/39)	1.00
Day-care at 24 months of age	88 (7/8)	77 (30/39)	0.67
Parental smoking (prebirth)	0 (0/8)	15 (6/39)	0.57
Parental smoking at 7 y	0 (0/8)	13 (5/39)	0.57
Probiotics at 7 y (last month)	0 (0/8)	28 (11/39)	0.17
Family size at 7 y mean (sd)	4.3 (0.71)	4.3 (0.76)	0.82
Recurrent wheeze (≥3) at 2 y	50 (4/4)	3 (1/39)	0.002
IgE-associated eczema 2 y	88 (7/8)	37 (13/35)	0.02
Skin prick positive at 7 y	60 (3/5)	31 (11/36)	0.32
Allergic rhinoconjunctivitis at 7 y	50 (4/8)	23 (9/39)	0.19
Allergic urticaria at 7 y	13 (1/8)	8 (3/39)	0.54
Eczema at 7 y	38 (3/8)	23 (9/39)	0.40

* Chi2 test was employed for cathegorical variable. Fisher's exact test was used when the expected frequency for any cell was less than five. Student t-test was employed for continuous variables.

Supplementary tables

Supplementary Table 1. The Shannon diversity index of the total microbiota during the first year of life in children with and without eczema and IgE-associated eczema at seven years of age.

	Eczema at 7 years of age			IgE-associated eczema at 7 years of age		
	Yes	No		Yes	No	
	n=12 median (iqr)	n=34 median (iqr)	P-value*	n=11 median (iqr)	n=30 median (iqr)	P-value*
1 week	1.65 (1.36-1.75)	1.55 (1.40-1.74)	0.58	1.70 (1.34-1.75)	1.55 (1.43-1.79)	0.89
1 month	1.54 (1.41-1.66)	1.57 (1.46-1.92)	0.48	1.49 (1.40-1.63)	1.58 (1.48-1.92)	0.20
12 months	2.84 (2.37-3.16)	2.62 (2.17-3.25)	0.68	2.83 (2.34-3.19)	2.62 (2.21-3.24)	0.73

*Mann Whitney U-test. Iqr= interquartile range

Supplementary Table 2. The Shannon diversity index of the total microbiota during the first year of life in children with asthma, allergic rhinoconjunctivitis, eczema and positive skin prick test at seven years of age, when only the **20 children with IgE-associated eczema at two years** were included

	Asthma at 7 years of age			**ARC at 7 years of age**			**Eczema at 7 years of age**		
	Yes	**No**		**Yes**	**No**		**Yes**	**No**	
	n=7 median (iqr)	n=13 median (iqr)	P-value*	n=9 median (iqr)	n=11 median (iqr)	P-value*	n=10 median (iqr)	n=10 median (iqr)	P-value*
1 week	1.34 (0.88-1.70)	1.73 (1.44-1.77)	**0.06**	1.70 (1.33-1.78)	1.46 (1.34-1.75)	0.34	1.71 (1.46-1.75)	1.46 (1.09-1.79)	0.29
1 month	1.25 (0.84-1.45)	1.53 (1.42-1.72)	**0.09**	1.49 (1.34-1.89)	1.40 (0.80-1.60)	0.60	1.55 (1.37-1.74)	1.34 (0.79-1.58)	0.15
12 months	2.87 (2.14-3.26)	2.83 (2.27-3.23)	0.91	2.87 (2.63-3.13)	2.57 (2.07-3.34)	0.73	2.88 (2.54-3.21)	2.53 (2.06-3.29)	0.41

	SPT pos at 7 years			**IgE-associated eczema at 7 years**		
	Yes	**No**		**Yes**	**No**	
	n=6 median (iqr)	n=11 median (iqr)	P-value*	n=8 median (iqr)	n=9 median (iqr)	P-value*
1week	1.73 (1.50-1.75)	1.54 (1.43-1.79)	0.62	1.73 (1.42-1.75)	1.58 (1.46-1.80)	1.00
1 month	1.61 (1.40-1.76)	1.18 (0.79-1.62)	0.19	1.48 (1.33-1.65)	1.52 (0.81-1.83)	0.85
12 months	2.83 (2.34-3.19)	3.09 (1.85-3.37)	0.76	2.86 (2.50-3.23)	2.63 (2.03-3.28)	0.44

*Mann Whitney U-test. Iqr=interquartile range