

ORIGINAL ARTICLE

Temporal variations in early gut microbial colonization are associated with allergen-specific immunoglobulin E but not atopic eczema at 2 years of age

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Experimental
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Summary

Background Intestinal microbiota undergoes substantial development during the first 2 years of life, important for intestinal immunologic development and maturation influencing systemic immune responses.**Objective** We aimed to investigate, using a prospective study design, whether allergen-specific IgE (sIgE) and atopic eczema are associated with variations in gut microbial colonization patterns in an unselected population during the first 2 years of life.**Methods** Faeces from 94 infants were repeatedly sampled from 10 days, 4 months, 1 and 2 years postnatal and analysed for 12 different bacterial species by quantitative real-time PCR. Venous blood samples from the infants were collected at 2 years of age and were analysed for sIgE for 12 specific allergens. The temporal gut colonization patterns for 42 sIgE-positive (sIgE ≥ 0.35 kU/L) and 52 sIgE-negative children (sIgE < 0.1 kU/L) were then compared. The association between colonization pattern and phenotype as atopic eczema according to UK Working Party (UKWP) criteria were also described.**Results** Subjects with atopic sensitization had lower levels of *Escherichia coli* at 4 months and 1 year, higher levels of *Bifidobacterium longum* at 1 year and lower levels of *Bacteroides fragilis* at 2 years. For *E. coli* and *B. longum*, the differences were only transient and had disappeared by 2 years of age. For other species, there were no differences in colonization patterns, and we found no association between colonization pattern and atopic eczema.**Conclusions and Clinical Relevance** We found temporal and transient variations in gut microbial colonization patterns associated with differences in sIgE sensitization at 2 years of age. A full understanding of the principles and mechanisms that underlie intestinal microbial colonization and diversity and host-microbiota relationships will be pivotal for the development of therapeutic approaches that manipulate the intestinal microbiota to maintain human health. [Registration number: ISRCTN28090297]**Keywords** atopic sensitization, eczema, gut microbiota, infancy, prospective design, transient changes

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Introduction

The complex gut microbial flora (microbiota) interaction with the innate and adaptive immune system in the gut mucosa is the initial trigger of immune responses in the neonate, which initiates during birth [1–3]. The appropriate development and maturation of neonate immunity responses occur in a dynamic interaction between host and gut microbiota in a mutually beneficial relationship [4, 5]. An emerging understanding of the development and diversity of gut microbiota and its impact on immu-

nity development in the host has provided new insights into how the commensal gut microbiota contributes to human intestinal and systemic health and disease [3, 6]. Intestinal bacterial colonization is necessary to initiate an activation of gut-associated T cells in a cross-talk between components in the microbiota and pattern-recognizing receptors in intestinal epithelial and dendritic cells [7]. Dendritic cells are the main antigen-presenting cells driving differentiation of effector CD4⁺ T cells to Th1, Th2 or Treg cells in intestinal germinal centres (Peyer's patches) [8]. The general gut microbial

colonization pattern is well described with aerobic or facultative aerobic microbes (*Enterococcus*, *Streptococcus* and *Enterobacteriaceae*) dominating from early infancy. With reduced oxygen supply, anaerobic microbes (*Bifidobacterium*, *Bacteroides* and *Clostridium*) become more abundant [9, 10]. The microbiota colonization pattern and development are influenced by exposure factors such as vaginal or caesarean delivery mode, maternal contact, feeding habits, maternity care and number of siblings. The main sources of colonizing bacteria are influenced by cultural and geographic differences regarding factors as diet and lifestyle [2, 5, 11]. Although the precise nature of the complex microbial interaction required for protection from allergy remains to be identified, it has been suggested that a disturbed microbial intestinal colonization (dysbiosis), causing insufficient activation of 'healthy' immune responses in gut mucosa, also leads to concurrent systemic immunological deviations and possible disease in early infancy [9, 12–14]. Candidate bacteria promoting possible immune-modulating effects have been investigated in studies on probiotic supplementation in and after pregnancy, both to mothers and children, but data on causal relationships between defined bacterial species and allergic disease have so far been confusing [15–17]. It is well supported that gut microbial diversity is equally important for intestinal immune system development and maturation and that reduced diversity in the early gut microbiota is associated with atopic eczema [18, 19]. It has also been shown that high microbial diversity may enhance the maturation of the protective mucosal IgA system [20]. In the ALLERGYFLORA project, however, no highly significant differences in colonization by various groups of culturable intestinal bacteria were found during the first year of life between infants with or without atopic eczema in the first 18 months of life [11].

It has recently been proposed that there are indications of a possible 'window of opportunity' for microbiota-induced immune modulation. This shows that the actual microbiota composition in an infant at one chosen point of time should be evaluated in connection with the temporal gut microbial colonization patterns, under the assumption that diverse colonization patterns at defined time periods in life may be associated with immune-modulatory effects and development [1, 9, 15]. In earlier prospective studies, the colonization characteristics of the gut microbiota have been described either at one defined point of time, such as in the Koala Study and in the study by Wang et al. [18, 21], or for a rather short period of time as in the study by Sjögren et al. [14] suggesting associations between gut microbiota composition and atopic sensitization and allergy. To our knowledge, no study has followed a population of unselected infants prospectively with repeated sampling of fecal material throughout the first 2 years of life to describe the temporal gut colonization pattern and at the same time investigated the possible association with both systemic immune responses and atopic eczema in infancy.

In this study we aimed, using a prospective study design, to investigate whether intestinal colonization pattern for 12 candidate bacteria during early infancy in an unselected population was associated with atopic sensitization status as specific IgE (sIgE) in venous blood and atopic eczema at 2 years of age.

Methods

Participants and questionnaire

The Prevention of Allergy among Children in Trondheim (PACT) study is a large population-based study in Norway focusing on the impacts on childhood allergy of systematic and structured interventions to reduce tobacco exposure, to increase consumption of *n*-3 polyunsaturated fatty acids and to reduce indoor dampness, described in detail elsewhere [22]. We undertook this sub-study, the Immunology and Microbiology study in PACT (IMPACT), from 2001 to 2006, as described elsewhere [23].

Family doctors and midwives in Trondheim aimed to recruit 720 women during ordinary early pregnancy consultations. All participants were recruited from the control cohort in the PACT study. All data were collected for both groups before the end-point study and before we knew who were sensitized or were having atopic eczema. The 52 participants in the control group were chosen as a random selection of participants with sIgE < 0.1 kU/L. The criteria for the sensitized group was sIgE ≥ 0.35 kU/L, resulting in a 'loss' of participants with sIgE between 0.1 and 0.35 kU/L, reducing the control group to a total of 42 (Fig. 1). They were not specifically matched for any characteristics with the 'cases'. The only inclusion criterion was that their parent had signed a written consent and that they could complete a questionnaire in Norwegian language; there were no exclusion criteria. No instructions regarding dietary intervention or lifestyle advice were presented to the doctors and midwives in their contact with the participants in this study.

The questionnaire on clinical end-points of allergic rhinoconjunctivitis, asthma and eczema was the modified ISAAC form completed at 6 weeks after delivery, and at 1 and 2 years of age of the children [24, 25]. A paediatrician and a dermatologist also assessed the clinical diagnosis of allergic disease for all infants. Eczema is by far the most common atopic manifestation during the first 2 years of life, and was classified according to the UK Working Party (UKWP) criteria [26]. Venous blood for quantification of sIgE was collected at 2 years of age.

Serum analysis (sIgE)

In this study, atopic sensitization at 2 years of age defined as sIgE ≥ 0.35 kU/L in serum from venous blood samples was used as the primary outcome variable. Elevated

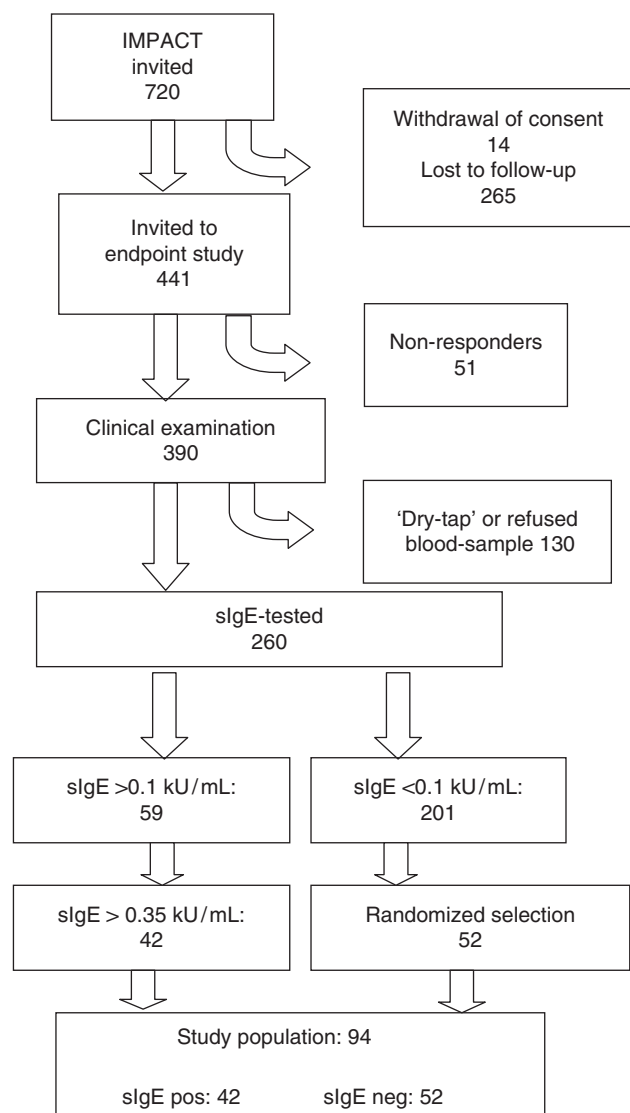


Fig. 1. 720 unselected pregnant women were invited to participate in the study with a follow-up until the children were 2 years of age. In an end-point study, venous blood from a total of 260 unselected children were sampled and analysed for sIgE. The final population consisted of 42 sIgE-positive and 52 sIgE-negative infants from this unselected population.

concentrations of sIgE are an indicator of immunologic deviation in an individual, and can be detected both in healthy and diseased persons. For the blood sampling, a cutaneous local anaesthetic (Emla[®]) was used, and only one attempt to draw blood was granted by the Regional Ethical Committee. Atopic sensitization was assessed as elevated sIgE (≥ 0.35 kU/L) in serum for antigens in an assay testing for mite (*Dermatophagoides pteronyssinus*), mould (*Cladosporium herbarum*), cat and dog dander, birch, timothy (grass) and mugwort pollens, cow's milk, hen's egg white, codfish, hazelnut and peanut allergies. The sIgE analyses were performed in a laboratory at the University Hospital in Trondheim with no information on the infant's intestinal colonization patterns or clinical

status. An Immulite 2000 Allergen-specific IgE system was used (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA).

Analyses of the microbiota

Faeces were collected from the napkin and transferred to a transport media by the parents and stored immediately at -18°C at home before being transported to permanent storage at -80°C until analysis. A total of four stool samples were collected at 10 days postnatal and when the child was 4 months and 1 and 2 years old. We had to limit the choice of bacteria to analyse to make the study feasible according to analytic capacity and funding. The choice of bacteria was based on earlier reports on differences in allergy prevalence and the composition of gut microbiota [12, 13, 27] and from clinical trials on the effect of probiotics [28, 29]. Predominant phylogroups in the human microbiota are *actinobacterida*, *firmicutes*, *bacterioidetes* and *enterobacteria*, and so we decided to include the *Bifidobacteriae* phylae and the species *Bifidobacterium breve*, *Bifidobacterium lactis* and *Bifidobacterium longum*. We included three species of *Lactobacillae* *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, two species of *Clostridiae* *Clostridium difficile* and *Clostridium perfringens*, in addition to *Bacteroides fragilis*, *Escherichia coli* and *Helicobacter pylori* (Table 1). The microbiota in the stool samples were analysed by quantitative real-time PCR using the primers and probes described in Table 1. The samples were thawed on ice and mixed with a whirly mixer. Then, 100 μL was added to 400 μL of 4 M guanidinium thiocyanate, and the DNA purification was carried out as described previously [30]. The real-time quantitative PCR reaction was performed using DNA polymerase (DyNAzyme II Hot Start DNA Polymerase Finnzymes Oy, Espoo, Finland) with a 1 μL template in a 25 μL reaction volume, as described by Skånseng et al. [31]. Briefly, quantifications were made by first correcting for the differences in amplification efficiencies, and then by normalizing the amounts detected for the different bacterial groups relative to the total microbiota, as determined by the Bacteria amlicon [30].

Statistics

The SPSS for Windows[®] version 16.0 (SPSS Inc., Chicago, IL, USA) program was used to describe population characteristics. Matlab version 7.10 (The MathWorks, Natick, MA, USA) was used for all other purposes. The missing values for bacterial concentrations were filled in according to the principle of mean imputation [32]. In the case of a single missing value, the imputed value was given as the mean of all values below the known detection limit. Several missing values were imputed by an iterative procedure. Furthermore, imputation was performed

Table 1. Real-time quantitative PCR amplicons (PACT 2010)

Target bacteria	Primers and probes	Eff.	References
Bacteria	TCC TAC GGG AGG CAG CAG T FAM-CTG ATT ACC GCG GCT GCT GGC AC-TAMRA GGA CTA CCA GGG TAT CTA ATC CTG TT	1.07	[37]
<i>Bacteroides fragilis</i>	GAA AGC ATT AAG TAT TCC ACC TG FAM-TGA AAC TCA AAG GAA TTG ACG GGG-TAMRA CGG TGA TTG GTC ACT GAC A	0.91	[38]
<i>Escherichia coli</i>	GTG TGA TAT CTA CCC GCT TCG C FAM-TCG GCA TCC GGT CAG TGG CAG T-TAMRA AGA ACG GTT TGT GGT TAA TCA GGA	1.15	[39]
<i>Bifidobacterium breve</i>	GTG GTG GCT TGA GAA CTG GAT AG FAM-TGA TTC CTC GTT CTT GCT GT-MGB CAA AAC GAT CGA AAC AAA CAC TAA A	0.85	[40]
<i>Bifidobacterium lactis</i>	AGA ACC ACG GCG GCG TC FAM-TGC GCT CGC CGA CG-MGB CGC GGT CTT CTC GAG CAC T	0.91	This study
<i>Bifidobacterium longum</i>	TGG AAG ACG TCG TTG GCT TT FAM-CGC ACC CAC CGC A-MGB ATC GCG CCA GGC AAA A	0.82	[40]
<i>Bifidobacterium</i> spp.	GGG ATG CTG GTG TGG AAG AGA FAM-TCA AAC CAC CAC GCG CCA-MGB TGC TCG CGT CCA CTA TCC AGT	0.90	[40]
<i>Lactobacillus reuteri</i>	ATG GCT TTT GTT TGA AAG ATG GC FAM-TGG CTA TCA CTC TGG GAT G-MGB CCT TAC CAA CTA GCT AAT GCA CCG	1.00	This study
<i>Lactobacillus rhamnosus</i>	CAT AAA TCC AAG AAC CGC ATG G FAM-CTT GGC TGA AAG ATG-MGB CAC GCC GAC AAC AGT TACT CT GC	0.62	This study
<i>Clostridium difficile</i>	ATA TCA GAG ACT GAT GAG FAM-TGG AGA ATC TAT ATT TGT AGA AAC-MGB TAG CAT ATT CAG AGA ATA TTG T	0.99	[41]
<i>Clostridium perfringens</i>	TTC TAT CTT GGA GAG GCT ATG CAC TAT TTT FAM-TAG ATA CTC CAT ATC ATC CTG CTA ATG TTA CTG CCG TTG A-TAMRA TTT CAA ACT TAA CAT GTC CTG CGC	1.07	[30]
<i>Lactobacillus plantarum</i>	TGG ACC GCA TGG TCC GAG FAM-TCC CGC GGC GTA TTA-MGB GTG AGC CGT TAC CCC ACC AT	0.58	This study
<i>Helicobacter pylori</i>	CGT GGC AAG CAT GAT CCA T FAM-TCA GGA AAC ATC GCT TCA ATA CCC ACT T-TAMRA GGG TAT GCA CGG TTA CGA GTT T	0.94	[42]

PACT, Prevention of Allergy among Children in Trondheim.

within each age category. A detailed algorithm of the imputation method is given in the additional material.

Quantitative PCR data usually imply the imputation of missing data that complicates the statistical procedures. In order to test the significance of these data, permutation-based approaches are commonly used, which is an exact statistical test, even for data with a complex distribution structure [33]. The *P*-values for group differences within each age category were calculated by permutation testing. To allow for possible systematic differences between genders, permutations were performed within each gender. The *P*-values were calculated according to a two-sided test. More specifically, the algorithm given in the appendix was used and $N = 10^6$

permutation replications were performed. The group information was not used in the imputation method, further ensuring that this permutation testing method was an exact statistical test.

Approvals

The Regional Committee for Medical Research Ethics for Central Norway approved the study (Ref. 120–2000). The study was granted a license by the Norwegian Data Inspectorate to process personal health data and one of the parents of each child signed a written informed consent form (Ref. 2003/953–3 KBE/–). Current controlled trials registration number: ISRCTN28090297.

Results

Population characteristics

The final population consisted of 42 sIgE-positive and 52 sIgE-negative infants from an unselected population as presented in the flowchart (Fig. 1). There were no differences in maternal age, educational level or birth weight of the children. In the group with $\text{sIgE} \geq 0.35$ kU/L, there were more boys, less premature births, less primiparous mothers and somewhat more caesarean sections. In this group, there were more atopy in the families, more antibiotic treatment, somewhat more breast feeding and less parental smoking. There were no differences between the groups regarding pets in the household or in prevalence of eczema last 12 months according to the UKWP criteria (Table 2).

Specific immunoglobulin E sensitization

Of the 390 children who attended the end-point examination at 2 years of age, we succeeded in drawing venous blood from 260 of them (Fig. 1). Of these children, 42 (16.2%) had $\text{sIgE} \geq 0.35$ kU/L for at least one allergen. The sIgE analyses showed that among the $\text{sIgE} \geq 0.35$ kU/L infants the most common allergens to which they were sensitized were egg (71.4%), milk (26.4%), hazelnut (19.1%), peanut (16.7%), dog (14.4%), cat (14.2), fish (9.5%) and grass/timothy (4.7%). None were sensitized for mite, cladosporium or mugwort.

Table 2. Characteristics of the study population

	sIgE > 0.35 kU/mL (n = 42)	sIgE < 0.1 kU/mL (n = 52)
Mean (SD)		
Age of mother (years)	30.7 (4.2)	29.0 (4.4)
Education of mother (years)	15.2 (2.5)	14.8 (2.2)
Education of father (years)	14.8 (2.8)	14.0 (2.9)
Birth weight (g)	3708 (649)	3713 (600)
%		
Gender (male)	64.3	53.8
Premature*	2.4	13.5
Primiparous	38.1	46.9
Caesarean section	13.3	9.3
Atopy in family [†]	81.0	65.3
Eczema UKWP	23.8	23.1
Antibiotic treatment	54.8	32.7
Breast-fed ≥ 4 months*	66.7	62.8
Smoking mother at the start of pregnancy	26.8	34.7
Smoking father at the start of pregnancy	20.5	25.5
Pets at home at the start of pregnancy	23.1	26.1

*Assessed 1 year postnatally.

[†]Mother, father and/or sibling ever had asthma, allergic rhinoconjunctivitis or eczema.

UKWP, UK Working Party.

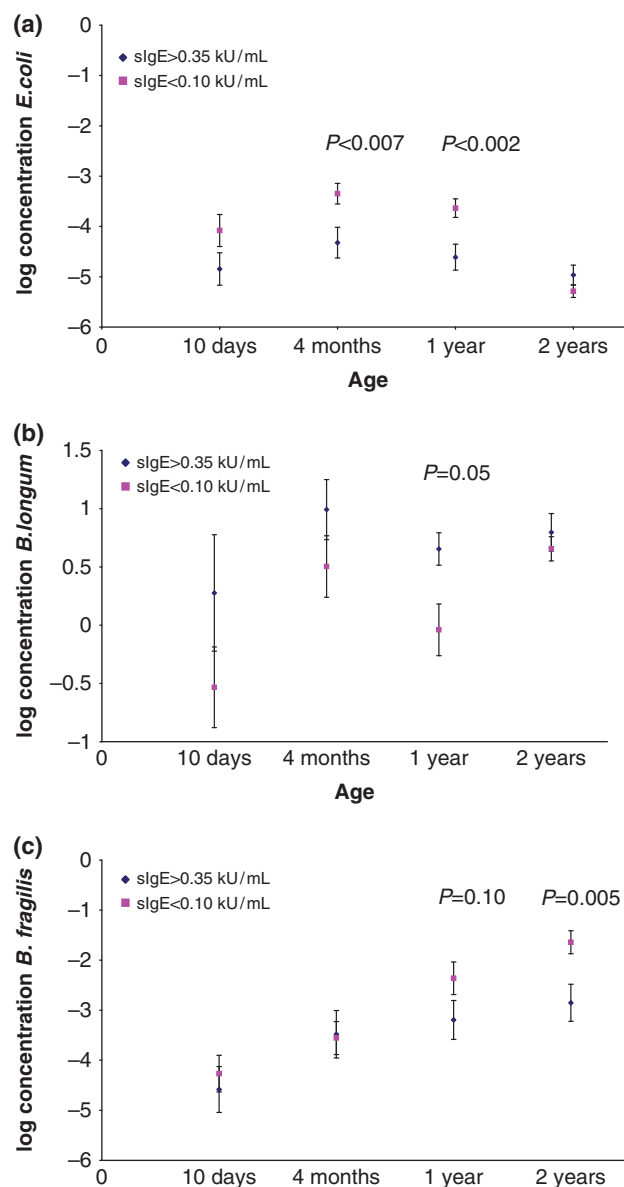


Fig. 2. Mean microbial concentration in faeces from sensitized ($\text{sIgE} > 0.35$ kU/mL) and non-sensitized ($\text{sIgE} < 0.10$ kU/mL) children from 10 days to 2 years of age. Values are presented showing average log concentration with SEM (standard error of mean) for (a) *Escherichia coli*, (b) *Bifidobacterium longum* and (c) *B. fragilis* at 10 days, 4 months, 1 and 2 years after birth.

Microbiota characteristics

Postnatal fecal samples were collected for all 94 participants at 10 days (19.1% missing), 4 months (11.7% missing), 1 year (14.9% missing) and 2 years (7.4% missing) (data not shown). Among the non-sensitized infants at 2 years of age, there was an increased concentration of *E. coli* significant both at 4 months and at 1 year and of *B. fragilis* only at 2 years of age. Among the sensitized infants, there was an increased concentration of *B. longum* significant at 1 year (Fig. 2). For *E. coli* and

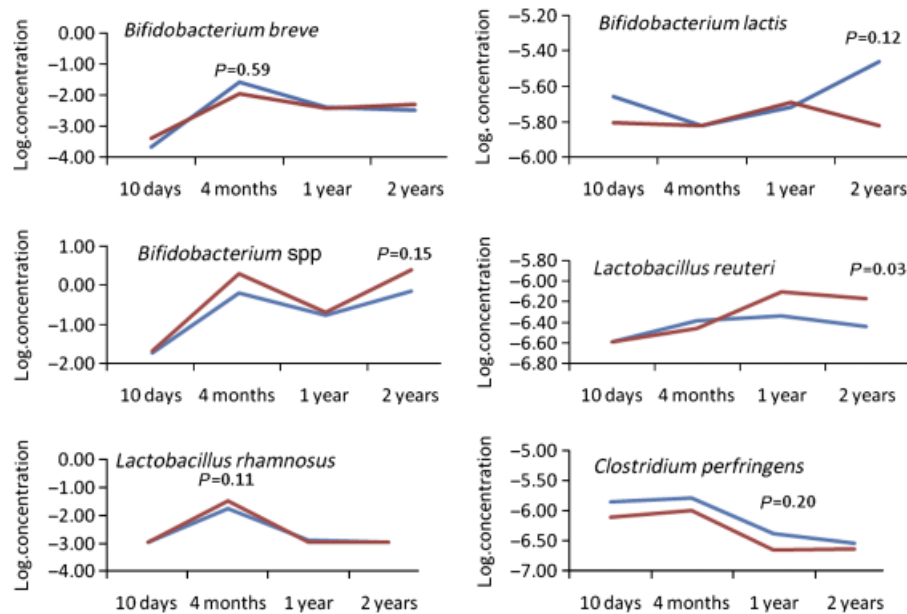


Fig. 3. The microbial colonization patterns for 6 microbial species, from 10 days to 2 years of age. Of 94 children in the study group, 42 children (44.7%) were sensitized (sIgE > 0.35 kU/mL) at 2 years of age (red line), and 52 (55.3%) were not sensitized (blue line). The x-axis shows the age when faecal samples were collected and the y axis shows the logarithmic concentration of bacteria. P-values are given for the maximal difference between the groups. For *L. reuteri*, there was an increased colonization among sensitized children at 2 years of age, but the concentration was very low and the significance was not applicable. For *L. plantarum* and *H. pylori*, the concentrations were below detection limit. Generally, low levels for lactobacilli in both groups indicate equal and very low intake of probiotics in this study.

B. Longum, the differences had disappeared again at 2 years of age. For the total *Bifidobacteriaceae* phylae (*Bifidobacterium* spp.) and the species *B. breve* and *B. Lactis*, we found no differences between sensitized and non-sensitized children (Fig. 3). Similarly, for the *Lactobacillae* (*L. reuteri*, *L. rhamnosus* and *L. plantarum*), Clostridia (*C. difficile* and *C. perfringens*) and *H. pylori*, we found no differences in colonization between sensitized and non-sensitized children at any point of time (Fig. 3).

When the children were classified with eczema according to the UKWP criteria, we found no difference in colonization pattern for any bacterial species at any point of time (Fig. 4).

Discussion

In a population of 94 infants up to 2 years of age, we found intestinal colonization patterns for *E. coli*, *B. longum* and *B. fragilis* to be associated with sIgE sensitization at 2 years of age, while there were no association between colonization patterns and atopic eczema. This indicates that systemic immune responses as elevated sIgE in serum are influenced by the gut microbiota stimulating the immunologic maturation and development in the intestinal mucosa. Importantly, these results only describe a probable association between intestinal colonization pattern and IgE sensitization, and mention nothing about how changes in gut microbiota

may be associated with the pathogenesis in eczema and allergic disease. In fact, several studies have demonstrated a dietary (probiotic) effect on the prevalence of eczema in childhood, but with no concurrent change in IgE sensitization [12, 16]. The clinical effects may be by other mechanisms, and modulations of inflammatory mediator composition have been suggested.

There was no difference in the prevalence of atopic eczema between the sensitized and non-sensitized children, so this is unlikely as an explanation of the differences in sIgE sensitization. The population characteristics in our study indicate some differences regarding risk factors for atopic sensitization, such as male gender, caesarean section, breast-feeding, antibiotic treatment and atopy in the family. Genetic factors certainly have an impact on atopic sensitization, and early exposure to maternal microflora through delivery procedures and breast-feeding are well-known factors for microbial exposure in infancy.

There are some discrepancies with other studies regarding microbial colonization pattern and atopic sensitization, but this should be interpreted with caution, as atopic sensitization may have been defined differently in other studies, both regarding IgE sensitization and clinical criteria for atopic eczema. We found that an increased level of *E. coli* at 4 months and 1 year was associated with low sIgE at 2 years. No association was observed between microbiota and atopic eczema in agreement with the

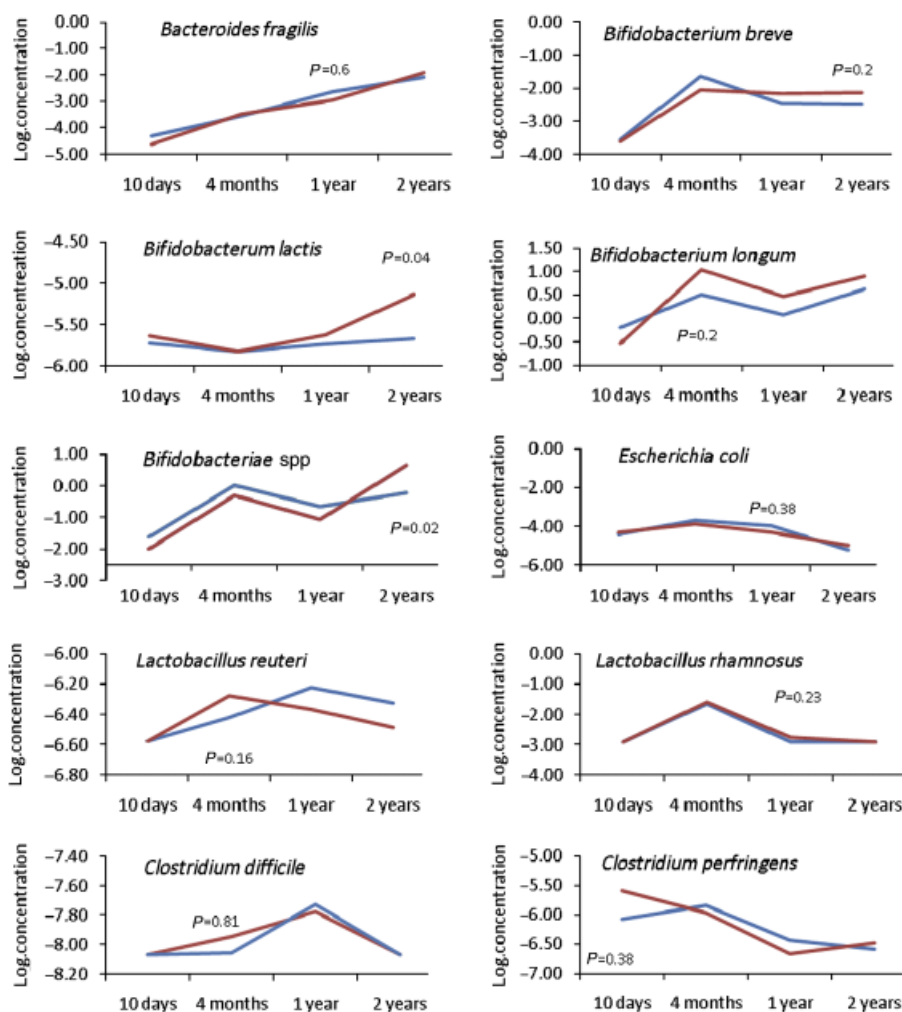


Fig. 4. The microbial colonization patterns for 10 microbial species, from 10 days to 2 years of age. Of the 94 children in the study group, 22 children (23.4%) had eczema according to clinical diagnosis of eczema (UKWP criteria) for the last 12 months (red line), and 72 (76.6%) had no eczema (blue line). The x-axis shows the age when faecal samples were collected and the y axis shows the logarithmic concentration of bacteria. P-values are given for the maximal difference between the groups. For *B.lactis* and *Bif.SPP* there is an increased colonization among diseased children at 2 years of age. For *L.plantarum* and *H.pylori*, the faecal concentrations were below detection limit.

ALLERGYFLORA project [11], but opposed to the KOALA study [21], where colonization with *E. coli* at 1 month after birth was associated with atopic eczema but not with IgE at 2 years. As for *Bifidobacterium* flora, there was an increased colonization with *B. longum* among children with high sIgE at 1 year of age but no differences for the other strands or when all *Bifidobacterium* (*Bifidobacterium* spp.) are described. We found no association with atopic eczema. This may be a controversial finding in that *Bifidobacteriae* in earlier reports has been associated with prevention of atopy and a healthy gut microbiota [9]. *B. fragilis* colonized the gut to a significantly higher level in the sIgE-negative children at 2 years of age. In the study by Sjögren et al. [20], no relationship was found between *B. fragilis* colonization up to 2 months and allergy development up to 5 years of age, postulating that the immune responses associated with *B. fragilis* coloni-

zation in infants play a minor role in the development of allergy. We found, however, that a difference actually seems to occur, but only in a later time period, and that this may be associated with atopic sensitization. The results demonstrated that an observational period of up to 2 years of age could lead to quite different conclusions. This indicates that differences in colonization pattern for specific bacterial species both can be transient and may occur at different ages. Interventional studies with probiotics has demonstrated both an effect on allergy and eczema in infancy, and a modulating effect on the infant's immune system when introduced to women in and after pregnancy or to children in defined periods early in life [16, 34–37]. Our findings support an assumption that there might be a 'window of opportunity' for a microbial colonization to have an impact on immune modulation [9, 15].

Although it may be tempting to look for specific associations between microbial phylotypes and immunologic deviation, it is intriguing to speculate that the associations we observe at least partly reflect a reduced gut microbial diversity among the sensitized children. Sufficient diversity in the microbiota during infancy might be important for the prevention of atopic eczema [18]. Reduced microbial diversity with high counts of *Bacteroides*, *Clostridium*, *Enterobacteriaceae* and *Staphylococcus* early in life has been associated with a disturbed immunologic development in the gut mucosa and an increased risk for atopic sensitization and disease [2, 9, 18]. In concordance with other studies, we found that infant strain diversity levels increased towards maternal diversity levels over time [6, 9].

Regarding colonization with other *Lactobacteria* and *Bifidobacteria*, the concentrations were very low and equal in both groups probably indicating low probiotic intake.

The main features of our study were the high number of participants, the unselected population and the prospective design over a relatively long period of time with repeated stool samples. Still, the limitations of the study should be acknowledged. First, atopic eczema was the only common clinical allergic manifestation in this group. Hayfever and asthma are very uncommon at this young age, and a reexamination at school age might reveal associations between microbiota in infancy and later development of respiratory allergy. Secondly, transient food allergy and concurrent IgE sensitization appearing and disappearing before 2 years of age was also not considered in the present study. All children with sIgE < 0.1 kU/L were considered non-sensitized and eligible for the control group. Finally, it must be emphasized that we found few significant differences between the two groups, and only regarding sIgE sensitization. The findings were however consistent for at least two consecutive sampling points, strengthening the correlation between colonization pattern and sIgE level. The significance of these findings remains to be confirmed in other studies.

In all studies identifying bacteria using PCR technology, it has been a challenge to deal with detection thresholds, which inevitably result in missing values. Furthermore, the data are usually not normally distributed. These challenges were managed to some extent by replacing the missing values by imputed values, thereby increasing the power and ensuring a more precise estimation of the variance for each individual. Permutation tests were used to deal with data with a complex distribution structure.

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Clinical relevance

A full understanding of the principles and mechanisms that underlie intestinal microbial colonization and diversity and host-microbiota relationships will be pivotal for the development of therapeutic approaches that manipulate the intestinal microbiota to maintain human health.

Conclusion

The many discrepancies in reported findings from different studies indicate that there is considerable complexity involved in this area. This study confirmed the existence of temporal and transient periods for differences in microbial quantities of *E. coli* and *B. longum* and temporal colonization for *B. fragilis*, during the first 2 years of life associated with sIgE. It is controversial whether this is a causal relationship, a consequence of exposure and genetic polymorphisms or whether our findings indicate that the sensitized children may have a reduced gut microbial diversity with possible implications regarding pathogenesis in atopic sensitization. This study revealed the importance of sampling gut microbiota over a relatively long time span to gain insight into its possible immune modulating effects.

Differences in microbial colonization for an extended microbiome and gut microbial diversity in different populations, as well as interactions regarding environmental exposure and host genetics, need to be further investigated.

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The authors declare no competing interests.

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