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Ylva Sjögren, Maria Jenmalm, Malin Böttcher, Bengt Björkstén and E Sverremar-Ekström, Altered early infant gut microbiota in children developing allergy up to 5 years of age, 2009, CLINICAL AND EXPERIMENTAL ALLERGY, (39), 4, 518-526.
http://dx.doi.org/10.1111/j.1365-2222.2008.03156.x

Altered early infant gut microbiota in children developing allergy up to five years of age.

Gut microbiota and allergy development.

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Word count.
4432

ABSTRACT

Background: Early colonization with bifidobacteria and lactobacilli is postulated to protect children from allergy, while *Clostridium difficile* colonization might be associated with allergic disease. Previous studies of the infant gut microbiota in relation to subsequent allergy development have mostly employed culture dependent techniques, studied genera of bacteria and the follow up period was limited to two years.

Objective: To relate gut microbiota in early infancy, notably bifidobacteria and lactobacilli at species level, to allergy development during the first five years of life and study if environmental factors influence the early infant gut microbiota.

Methods: Faecal samples were collected at one week, one month and two months after birth from 47 Swedish infants, followed prospectively to five years of age. Bacterial DNA was analysed with Real-time PCR and related to allergy development, family size as well as endotoxin and *Fel d 1* levels in house dust samples. Primers binding to *Clostridium difficile,* four species of bifidobacteria, two lactobacilli groups and *Bacteroides fragilis* were used. Children regarded as allergic manifested allergic symptoms and were skin prick test positive during their first five years while non-allergic children were neither.

Results: Children who developed allergy were significantly less often colonized with lactobacilli group I (*Lactobacillus (L.) rhamnosus, L. casei, L. paracasei)*, *Bifidobacterium adolescentis* and *Clostridium difficile* during their first two months. Infants colonized with several *Bifidobacterium* species had been exposed to higher amounts of endotoxin and grew up in larger families than infants harbouring few species.

Conclusion: A more diverse gut microbiota early in life might prevent allergy development and may be related to the previously suggested inverse relationship between allergy, family size and endotoxin exposure.

Key words

Gut microbiota, infant, allergy, siblings, endotoxin, Fel d 1, bifidobacteria, lactobacilli, *Clostridium difficile*.

Abbreviations

AB	Asthma bronchialis
AD	Atopic dermatitis
ARC	Allergic rhinitis/conjunctivitis
B.	*Bifidobacterium*
C.	*Clostridium*
DNA	Deoxyribonucleic acid
EU	Endotoxin units
Fel d 1	major allergen of cat (*Felis domesticus*)
G-	Gram negative
G+	Gram positive
L.	*Lactobacillus*
PCR	Polymerase chain reaction
SPT	Skin prick test

INTRODUCTION

The commensal gut microbiota, comprising 500-1000 different species of bacteria [1] is essential for oral tolerance induction and gut homeostasis [2, 3]. Colonization of the neonate starts immediately after birth and early colonizers are e.g. *Escherichia coli, Streptococcus*, *Bifidobacterium (B.)* and *Bacteroides* (reviewed in [4])**.** Studies have shown differences in the gut microbiota of children raised in affluent and non-affluent societies. For example, a lower proportion of Swedish children harbour lactobacilli, compared to Estonian and Ethiopian children [5, 6]. Also, Swedish children are colonized somewhat later and have a less diverse enterobacterial microbiota than Pakistani infants [7]. Furthermore, colonization with clostridia, including *Clostridium (C.) difficile,* has been associated with allergy development up to two years in several studies [8-10]. Children not developing allergy during their first two years have instead been shown to be more frequently colonized with enterococci and bifidobacteria as infants [9]. However, the influence of the infant gut microbiota on later allergy development needs to be further elucidated.

Type of delivery, diet and country of birth influence the infant gut microbiota [5-7, 11, 12]. Yet, other factors might also contribute to the colonization of the infant gut. Interestingly, number of siblings associates weakly with amount of bifidobacteria, in faeces from one month old infants [12]. Exposure to endotoxin, which is present in the outer membrane of Gram-negative (G-) bacteria, is higher in Estonian compared to Swedish homes [13]. Possibly, children living in an environment with high exposure to endotoxin might harbour a more diverse gut microbiota as suggested previously [5]. Also, as the endotoxin levels in house dust correlate with pet keeping [14], high levels of pet allergens could be viewed as a marker of bacterial exposure. These factors are particularly interesting to relate to the infant gut microbiota, as allergy development is inversely associated with number of siblings and exposure to endotoxin [13, 15, 16].

As species differences among bifidobacteria in allergic and non-allergic children have been reported previously [17], further studies of bacterial diversity within various genera of bacteria are of interest. Most previous studies have employed cultivation dependent techniques [9, 17]. Recently, sequencing of bacterial genomes has made it possible to study presence and amounts of bacteria with molecular methods. The 16SrDNA and 23SrDNA of bacteria, containing both variable and conserved regions, are often targeted for primer and probe design thus facilitating the analysis of bacteria at species level [18]. One method using these primers is real-time PCR, a method which is more sensitive and less time-consuming than cultivation techniques [18].

Hence, we hypothesize that children who develop allergy have an altered early infant gut microbiota, at species level, compared to children who do not develop allergy. Furthermore, the gut microbiota is influenced by household factors known to be inversely associated with allergy development *i.e.* family size and endotoxin and *Fel d 1* exposure. Therefore, the presence and amounts of certain gut microbes in early infancy were related to subsequent allergy development up to five years of age and to family size as well as house dust levels of endotoxin and *Fel d 1*. Real-time PCR was employed for analysis of the following bacterial species; *C. difficile, B. bifidum, B. longum, B. adolescentis, B. breve, Bacteroides fragilis,* Lactobacilli group I (*L. rhamnosus, L. paracasei*, *L. casei*) and Lactobacilli group II (*L. gasseri, L. johnsonii* group). The Lactobacilli and *Bifidobacterium* species were chosen due to their abundance in early faecal samples from infants [19, 20] and their postulated role in allergy development [17]. Also, *C. difficile* has been associated with allergy development [10]. Furthermore, a component from *Bacteroides fragilis* has recently been shown to be a potent immunomodulator [21] and thus this bacteria was also included.

MATERIAL AND METHODS

Study population

The study population, including 123 Swedish children, has been described in detail by Voor et al [22]. The children were born between March 1996 and October 1999 in Linköping, Sweden. All were born at term and they had an uncomplicated perinatal period. Inclusion in this study was based on availability of faecal samples at one week, one month and/or at two months of age and known allergy status up to the age of five. In all, 47 infants were included. Sixteen infants developed allergy during their first five years of life, while 31 remained non-allergic throughout the study period (Table 1). The allergic children had shown symptoms of allergic disease and at least one positive skin prick test (SPT) during their first five years of life, while the non-allergic children had not shown symptoms of allergic disease, nor had a positive SPT. The study was approved by the Regional Ethics Committee for Human Research at Linköping University. The parents of all children gave their informed consent in writing.

Table 1. Demographic data of the subjects. None of the differences between the non-allergic and allergic group were statistically significant (p>0.05).

	Subjects n=47	Non-allergic n=31	Allergic n=16
Female subjects	23 (49%)	14 (45%)	9 (56%)
Born with Caesarean section	3 (6%)	3 (10%)	0 (0%)
Any atopic heredity	37 (79%)	22 (71%)	15 (94%)
Allergic mother	13 (28%)	8 (26%)	5 (31%)
Exclusively breastfed ≥ 2 months	45 (96%)	31 (100%)	14 (88%)
Oral antibiotics ≤ 2 months	2 (4%)	2 (6%)	0 (0%)
Pets	7 (15%)	6 (19%)	1 (6%)
Number of family members	3 (3-8) [+]	3 (3-8)	3 (3-5) [+]
Household area/individual (m^2)	28 (18-47) [+]	28 (18-47)	27 (20-38) [+]

[+] Three children were excluded due to unavailable data on family members. Median values of family members and household area/individual are shown with the range in brackets.

A clinical examination of the babies was made at three, six and twelve months and at two and five years. At these occasions, skin prick tests were performed, and questionnaires were completed regarding symptoms of allergy, use of antibiotics and family size. Atopic dermatitis was defined as pruritic chronic or chronically relapsing dermatitis with typical morphology and distribution. Asthma was defined as three or more episodes of bronchial obstruction during the last 12-month period, of which at least one should be verified by a physician. Allergic rhinitis/conjunctivitis was defined as rhinitis and/or conjunctivitis following at least twice in one hour after allergen exposure and not in relation to an obvious infection e.g. fever or a cold.

Skin prick tests, with fresh skimmed cow's milk and thawed egg white, were performed at all follow ups. From 12 months, the children were tested with cat allergen extract and at two and five years also with birch- and timothy extracts (ALK, Hørsholm, Denmark). The SPT was considered to be positive if the mean diameter of the wheal reaction was ≥3 mm.

Only three children were SPT positive at the first clinical examination at three months of age. Therefore it is not likely that the allergic children were allergic already when the faecal samples were collected (at one week, one month and two months after birth). At five years of age, nine of the allergic children were SPT positive against inhalant allergens while seven of the allergic children manifested earlier food sensitization. Fifteen children had atopic dermatitis (AD) during their first five years of life. At age five, six children had allergic rhinitis/conjunctivitis (ARC) either in combination with asthma (AB) (three) or without (three), and one child had asthma in combination with atopic dermatitis. Thus, seven children were included in the group ARC/AB ever, and nine children were included in the group AD only.

The demographic data were similar in infants who developed and did not develop allergy (Table 1). The majority of the children, in the total cohort, had a history of atopic disease in the immediate family (79%). In total, three children were delivered with caesarean section. During their first two months, when the faecal samples were collected, almost all (96%) in the total cohort were exclusively breastfed and only two infants received antibiotics.

Extraction of DNA from faecal samples

Faecal samples were collected at home when the infants were one week (collected at day five or six), one month and two months old. Approximately one gram voided stool was collected into sterile plastic containers by the parents and frozen at -20ºC. The stool sample was brought in a plastic bag with ice to the research nurse who stored the sample at -70ºC until analysis.

Qiamp DNA Stool Mini Kit™ (Qiagen, Hilden, Germany) was used for the isolation of DNA from the faecal samples. According to the manufacturer's instructions, 180-220 mg frozen faeces were subjected to DNA isolation procedures. A protocol for increasing the bacterial DNA over human DNA was used. The concentration of nucleic acids was measured with BIO-RAD Smartspec (Bio-Rad laboratories, Hercules, CA, USA) at 260 nm using BIO RAD trUView Disposable Cuvettes (Bio-Rad laboratories, Hercules, CA, USA).

Reference bacterial DNA

Reference DNA from *B. adolescentis* DSM 20083, *B. bifidum* DSM 20456, *B. breve* DSM 20213, *B. infantis* DSM 20088, *B. longum* DSM 20219, *C. difficile* DSM 21296, *L. gasseri* DSM 20243 and *L. rhamnosus* DSM 20711 were purchased from BIOTECHON Diagnostics (Potsdam, Germany). Also reference DNA from *B. adolescentis* ATCC 15703D, *C. difficile* ATCC 9689D, *B. breve* ATCC 15700D and *B. infantis* ATCC 15697D were

purchased from LGC Promochem (Borås, Sweden). The bacterial DNA was solved in 1X TE buffer (pH 8) or water according to the manufacturer´s instructions and stored at -20^{0}C.

Real-time PCR for quantification of bacterial DNA

For concentrations and sequences of primers see table 2. The primers were used due to their specificity in binding to the specific bacterial DNA, as well as for their suitability in SYBR Green PCR chemistry. The primers for *B. longum*, *Bacteroides fragilis,* Lactobacilli group I and Lactobacilli group II were designed by us. The primer for *B. longum* was designed to detect the different suggested subspecies of *B. longum* including *B. infantis* [23]. Since different Lactobacilli species have shown similarities in the 16SrDNA-23SrDNA we choose to design primers directed towards two of these groups [24], here referred to as Lactobacilli group I and Lactobacilli group II. Primer design was performed by running published sequences, retrieved from Nucleotide Database at National Center for Biotechnology Information (NCBI), of the above bacteria's genes for 16SrRNA and 23SrRNA in Primer Express software version (Applied Biosystems) using TaqMan MGB Quantification protocol. This resulted in primers suitable for SYBR green PCR chemistry *i.e.* with a melting temperature of 58-60° and a GC content of 30-80%. Suggested forward and reverse primers were then tested for their specificity using Basic Local Alignment Serach Tool (BLAST) under NCBI (www.ncbi.nlm.nih.gov/BLAST). Primer sets binding to the appropriate bacterial DNA were then tested with the reference bacteria and optimization of primer concentrations was performed. The concentrations giving the lowest C_T value combined with no primer-dimer, as seen by melting curve analysis, were chosen. Regarding some of the already published primers, adjustments of primer-concentrations needed to be done to fit SYBR Green chemistry (Table 2).

Table 2. Sequences and concentrations of primer sets used. The specific primers amplified regions in the 16SrRNA for *B. bifidum, B. breve, B. longum* or *C. difficile* and in the 16S-23SrRNA intragenic spacer for *B. adolescentis,* Lactobacilli gr. I, Lactobacilli gr. II or *Bacteroides fragilis.*

Bacteria	Sequence (5´→ 3´)	Conc. (nM)	Amplicon length
B. bifidum (39)	F: CCACATGATCGCATGTGATTG	F: 250	278 bp
	R: CCGAAGGCTTGCTCCCAAA	R: 250	
B. breve (39)	F: CCGGATGCTCCATCACAC	F: 200	288 bp
	R: ACAAAGTGCCTTGCTCCCT	R: 50	
B. longum[t]	F: CAGTTGATCGCATGGTCTTCTG	F: 400	54 bp
	R: CGCGACCCCATCCCATA	R: 650	
B. adolescentis (40)	F: ATAGTGGACGCGAGCAAGAGA	F: 300	71 bp
	R: TTGAAGAGTTTGGCGAAATCG	R: 150	
Lactobacilli gr. I[t]	F: AAGAAATTCGCATCGCATAACC	F: 150	62 bp
	R: TGCGCCCTTTGTAACTTAACC	R: 650	
Lactobacilli gr. II[t]	F: GGAAGGCGAAAGATGATGGA	F: 650	62 bp
	R: GCTTGGCTTTCTCGATGACTTCT	R: 400	
C. difficile (41)	F: TTGAGCGATTTACTTCGGTAAAGA	F: 500	157 bp
	R: CCATCCTGTACTGGCTCACCT	R: 500	
Bacteroides fragilis[t]	F: CCGTTATTCTCCACTCCGATACC	F: 400	67 bp
	R: TCATGTCAAAGATCGTTTGATTACAC	R: 400	

[t] = this study, F=forward primer, R=reverse primer, bp=base pair. Conc.=concentration.

The SYBR Green real-time PCR was performed using 96-well detection plates (ABgene, Epsom, UK) together with adhesive films (ABgene, Epsom, UK) in ABI prism 7000 (Applied Biosystem, Stockholm, Sweden). The Absolute Quantification protocol in 7000 System software version 1.2.3f2 (Applied Biosystems) was employed together with a standard curve set up from known amounts of reference bacterial DNA. The standards were diluted in 10-fold dilution series and ranged from 5 ng to 50 fg. All samples were performed in triplicates. Each well contained 2xPower SYBR Green mastermix (Applied Biosystems, Stockholm, Sweden), forward and reverse primer (MWG-Biotech, Edersburg, Germany), DNA and water. The amplification was performed as follows: 2 min at 50°, 10 min at 95° followed by 40 cycles of 15s at 95°C (denaturation) and 1 min at 60° (annealing and

extension). To determine the specificity of the PCR a melting curve analysis was performed by slow heating from 60-99°C with continuous fluorescence collection.

To avoid detecting false positives, triplicates with C_T values above 35 were considered as negative. The Absolute Quantification protocol in 7000 System software calculated the amount of specific bacterial DNA from the standard curve. The amount of the specific bacterial DNA was then related to the total amount of nucleic acids in each sample. The specific bacterial DNA is thus expressed as percent specific bacterial DNA of total nucleic acids and referred to as relative amounts of specific bacterial DNA. The limit of detection was $5*10^{-6}$ % specific bacterial DNA of total nucleic acids.

Endotoxin and *Fel d 1* analyses

Dust samples were collected from carpets in the children´s homes once when the children were 3-12 months old. The collection procedures as well as analyses are further described in [13]. In short, the analyses were performed under sterile conditions with a chromogenic Limulus Amebocyte Lysate assay. The lowest limit of detection of endotoxin was 0.50 EU/mg dust and values below this detection limit was assigned the value 0.25 EU/mg. *Fel d 1* were analysed by ELISA (Indoor Biotechnologies, Cardiff, UK) and the lowest detection limit was 4 ng/g dust.

Statistics

Fisher**'**s exact test was performed to evaluate whether presence of bacteria at various occasions differed between children developing and not developing allergy and for evaluation of demographic markers. Mann-Whitney U test was performed to evaluate whether the two populations had different amounts of specific bacterial DNA in their faeces and whether infants with high (3-4) compared to low (0-2) number of *Bifidobacterium* species had been

exposed to different endotoxin levels. In addition, Mann-Whitney U test (with Monte Carlo permutation as correction for ties) was used to evaluate whether the allergic and non-allergic group differed regarding harbouring the different bacteria at several occasions. Spearman's rank coefficient was calculated to investigate whether relative amount of bacterial DNA correlated with endotoxin units, *Fel d 1* levels and number of family members. Any association between number of *Bifidobacterium* species and number of family members were calculated with Cuzick's non-parametric test for trend. The two children, who received antibiotics, while the samples were collected, were excluded from the analyses of environmental factors and gut microbiota. Many statistical tests were performed and thus some p-values close to 0.05 might be false significances. The study is unfortunately not sufficiently powered to detect very low p-values. Importantly, it should be viewed as exploratory and consequently $p<0.05$ were chosen as statistically significant. Noteworthy, future larger studies are of importance to investigate if these results could be replicated.

RESULTS

Presence and amounts of bacteria

Bifidobacteria were detected in all infants in at least one of the three samples collected during the first two months of life. The most commonly detected *Bifidobacterium* species were *B. longum* and *B. adolescentis* (Table 3), and *B. longum* also occurred in the highest amounts (Fig. 1). *Bifidobacterium breve* was the least common of the *Bifidobacterium* species studied. Few infants were colonised with *C. difficile*. *Bifidobacterium longum*, *B. adolescentis* and *Bacteroides fragilis* were commonly present already in one week old infants, whereas the other bacteria tended to become more frequently detected as the infants grew older. Lactobacilli occurred in lower amounts than bifidobacteria (Fig. 1). The two infants who received antibiotics harboured few *Bifidobacterium* species. However, DNA from *Bacteroides fragilis* constituted a major fraction of the nucleic acids purified from the faecal samples of these children (close to one percent). Two of the three children who were born with caesarean section harboured *C. difficile.* The samples from the two children who were not exclusively breastfed did not appear to differ from the samples from exclusively breastfed children.

B. adolescentis and Lactobacilli group I were more common in early infant faecal samples from non-allergic compared to allergic children.

At one week of age, 23/25 (92%) of the non-allergic infants harboured *B. adolescentis* and 12/25 (48%) harboured Lactobacilli group I, while only 8/13 (62%) and 1/13 (8%) of the infants who developed allergy during their first five years were colonized with these bacteria ($p=0.03$ and $p=0.02$ respectively, Table 3). Also, *Bifidobacterium adolescentis*, Lactobacilli group I and *C. difficile* were more often detected in the three samples, obtained during the

Table 3. **Proportion of infants colonized with *Bifidobacterium, Lactobacillus, C. difficile* and *Bacteroides fragilis* during the first two months of life. The numbers in the headers show the number of infants with available faecal samples at the different time points. The figures, after the different bacteria, show the percent of infants who were colonized with the bacteria. Infants who developed allergy during their first five years of life are in group A whereas infants who remained non-allergic throughout the study period are in group NA.**

Bacteria	1 week (%)			1 month (%)			2 months (%)		
	All n=38	NA n=25	A n=13	All n=42	NA n=28	A n=14	All n=41	NA n=27	A n=14
B. longum	84	80	92	90	86	100	90	89	93
B. adolescentis	82	**92**[a]	**62**[a]	69	75	57	83	85	79
B. bifidum	61	60	62	71	75	64	71	67	79
B. breve	37	40	31	43	43	43	49	56	36
≥3 *Bifidobacterium* sp.	55	60	46	60	61	57	76	78	71
Lactobacilli group I	34	**48**[b]	**8**[b]	62	71	43	61	67	50
Lactobacilli group II	34	32	38	43	36	57	54	48	64
C. difficile	5	8	0	12	14	7	27	37	7
Bacteroides fragilis	61	56	69	57	54	64	59	59	57

[a]p =0.03, [b]p =0.02

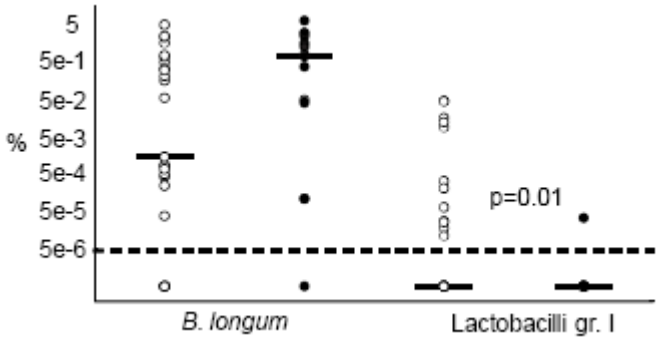

Fig. 1. Relative amounts of B. longum and Lactobacilli gr. I at one week after birth in relation to allergy development up to five years of age. *Open circles denote those infants who did not develop allergy (n=25) and filled circles show those who developed allergy during their first five years of life (n=13). The results are expressed as percent specific bacterial DNA of total amount nucleic acids. The short lines demonstrate the median values and the dotted line illustrates the detection limit. The p-value indicates the difference between the non-allergic and allergic group regarding the relative amounts of Lactobacilli gr. I.*

first two months of life, from non-allergic infants than in the samples from allergic infants (p=0.045, p=0.02 and p=0.03 respectively, Fig. 2). The colonization pattern of the other bacteria was similar in infants who did and did not develop allergy (Table 3). The number of stool samples, with three or more *Bifidobacterium* species, was also similar in the two groups

(Table 3). With the exception of Lactobacilli group I at one week (Fig. 1), the relative amounts of the different bacteria did not differ in samples from infants who did and did not become allergic.

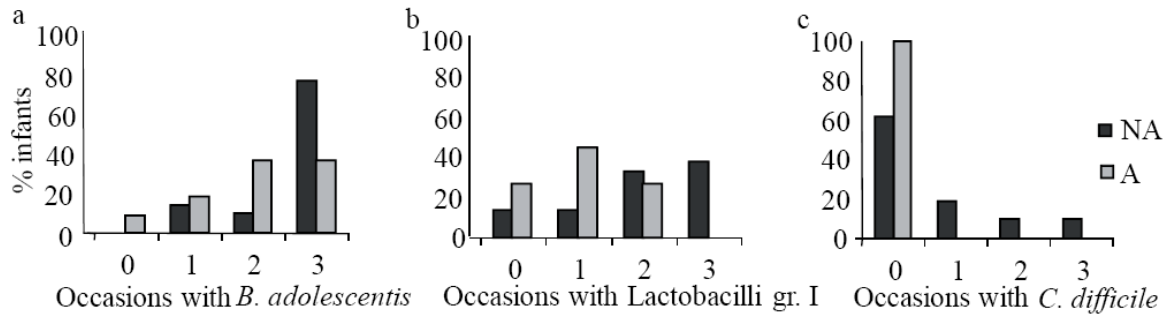

Fig. 2. Number of occasions with detectable B. adolescentis, Lactobacilli group I and C. difficile during the first two months of life in relation to allergy development up to age five. *The y-axis shows the percentage of infants who did (A, n=11) or did not develop allergy (NA, n=21) during their first five years of life. The bacteria were studied on three occasions during the first two months and the x-axis illustrates the number of occasions with detectable bacteria. As calculated with Mann-Whitney U test, the non-allergic children harboured B. adolescentis, Lactobacilli gr. I and C. difficile on more occasions than the allergic group (p=0.045, p=0.02 and p=0.03 respectively). None of the other investigated bacteria occurred at different occasions between the allergic and the non-allergic group. Children are only included if faecal samples from all occasions were available.*

The allergic children were divided into two groups depending on their allergic symptoms during their first five years (see study population). Nine children were included in the atopic dermatitis only group (AD only) and seven children in the allergic rhinitis/conjunctivitis and/or asthma ever group (ARC/AB ever). There was no difference regarding the presence of any of the investigated bacteria between the AD only and the ARC/AB ever group. As shown for the total allergic group, the AD only tended to be less frequently colonized with *B. adolescentis* and Lactobacilli group I one week after birth compared with the non-allergic group (p=0.06 and p=0.03, respectively). No other bacteria differed in prevalence between the AD only group and the non-allergic group. Interestingly, none of the bacteria investigated occurred differently between the ARC/AB ever group compared with the non-allergic group (p= 0.16 and 0.36 for *B. adolescentis* and Lactobacilli

gr. I). However, the small numbers in each group when separating the children into the AD only group (nine children) and the AB/ARC ever group (seven children) could greatly bias the results.

Environmental factors influence the early infant gut microbiota.

The number of family members correlated significantly with the number of *Bifidobacterium* species in faecal samples collected at one week and two months after birth (p=0.04 and p=0.02, Table 4). Colonization with several (3-4) *Bifidobacterium* species, at one week and two months of age tended to be associated with exposure to higher levels of endotoxin than colonization with low numbers (0-2) of *Bifidobacterium* species (Fig. 3). This was particularly obvious during the first week of life (Fig. 3b). There was no similar correlation with the *Fel d 1* levels in house dust.

Table 4. Number of faecal *Bifidobacterium* species (*B.* sp) during the first two months in relation to number of family members. The association between number of family members and number of *B.* sp was calculated with Cuzick's non-parametric test for trend.

	1 week	1 month	2 months
family members	**mean *B.* sp. (n infants)**	**mean *B.* sp. (n infants)**	**mean *B.* sp. (n infants)**
3	2.5 (16)	2.6 (19)	2.7 (20)
4	2.5 (8)	2.9 (7)	3.1 (7)
5	3.0 (8)	2.7 (9)	3.1 (7)
6	3.5 (2)	3.5 (2)	3.5 (2)
8	4.0 (1)	4.0 (1)	4.0 (1)
p-value	**0.04**	0.17	**0.02**

In one- and two-month faecal samples the relative amounts of *B. longum* correlated or tended to correlate with both endotoxin (r=0.41, p=0.02 and r=0.35, p=0.06) and *Fel d 1* levels (r=0.38, p=0.03 and r=0.52, p=0.004). The relative amounts of *C. difficile* in two-month faecal samples correlated negatively with *Fel d 1* levels (r=-0.53, p=0.003), and also tended to be inversely associated with *Fel d 1* and endotoxin in one-month faecal samples

(r=-0.32 p=0.07 and r=-0.34, p=0.06 respectively). *B. bifidum* in faecal samples collected at one month and two months was instead dependent on number of family members (r=0.46 p=0.006 and r=0.43, p=0.01 respectively). However, in one month samples, *B. adolescentis* was negatively associated with number of family members (r=-0.34, p=0.04). Yet, the many statistical tests performed, in combination with a limited sample size, could lead to false significances.

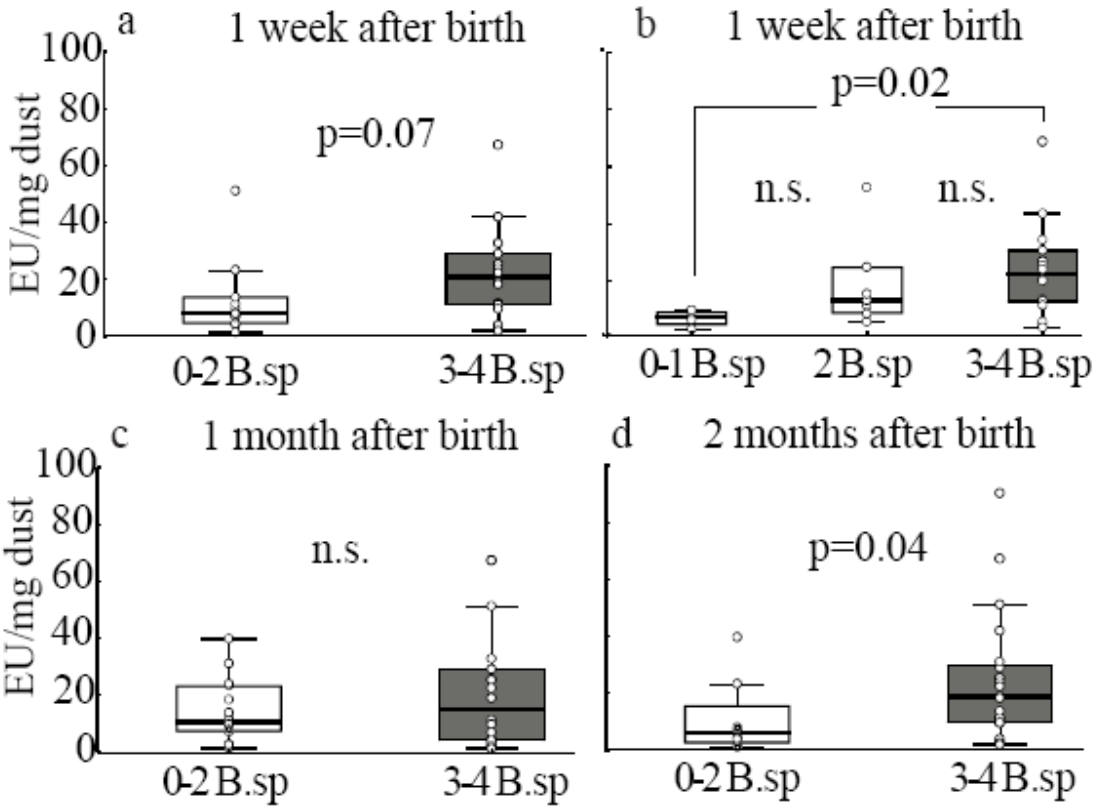

Fig. 3. Number of faecal Bifidobacterium species in relation to endotoxin levels in house dust (EU/mg dust). *The white boxes symbolize samples with two or less Bifidobacterium species (B. sp.) and the grey boxes those with three or more. In figure a and b, the number of B. sp. is measured in faecal samples collected at one week after birth. In figure c and d, the faecal samples were collected at one month and two months, respectively.*

DISCUSSION

Infants who harboured Lactobacilli group I and *B. adolescentis* at one week after birth were less likely to develop allergic disease during the first five years of life. *Bifidobacterium longum,* which includes *B. infantis,* was the most prevalent *Bifidobacterium* species in the infant faecal samples, which is in accordance with previous findings [20, 25]. Also *B. adolescentis* was common, which is in agreement with some [26] but not all previous studies [20, 25]. Both Lactobacilli group I and Lactobacilli group II were present in the early faecal samples. Others have shown that Swedish infants harbour lactobacilli species, belonging to these groups, during their first two months [19].

In most previous studies the early infant gut microbiota has been related to allergy development during the first two years of life [8-10]. At this age, allergic manifestations are mostly limited to the skin and gut. The children in our study were followed up to the age of five, *i.e.* to an age when respiratory allergy and sensitization to inhaled allergens have become the major clinical manifestation of allergy. Also, although our study size is somewhat limited, allergy is well-defined, as both sensitization and symptoms were used to classify the children as allergic and non-allergic. Interestingly, infants who harboured Lactobacilli group I at one week of age less frequently developed allergic disease than infants lacking these bacteria at the same time. Also, non-allergic children were more likely to harbour Lactobacilli group I on several occasions during their first two months of life. When the allergic children were grouped based on symptoms, it was shown that children who only suffered from atopic dermatitis were less often colonized with Lactobacilli gr. I, one week after birth, compared to the non-allergic children. These findings could be supported by clinical studies, in which different strains of probiotic lactobacilli reduced the incidence of infant eczema [27] and IgE-associated eczema [28]. However, bacterial colonization in the seven children with respiratory allergy did not differ from bacterial colonization in the non-

allergic children. It is difficult to interpret the role of Lactobacilli group I on respiratory allergies as only one child developed respiratory allergy without previous atopic dermatitis. Furthermore, only seven children had developed allergic asthma and rhinitis/conjunctivitis at age five. Children belonging to the AD only group, at age five, might develop respiratory allergy later as a substantial proportion of sensitized children with atopic eczema develop respiratory allergy before age seven [29].

Bifidobacterium adolescentis was more commonly detected in infants not developing allergic disease, which is in contrast with two previous reports on allergic and non-allergic infants [17] and children [30]. In contrast to these studies, our study population was investigated in early infancy, before the onset of allergic disease. There may be a time window of opportunity, only open very early in life, when microbial stimuli can influence the development of the immune system [31]. Results from animal experiments support this theory. In a murine model, *B. infantis* could restore oral tolerance in neonatal ex-germ-free mice but not in adult mice [2]. Another study on allergic and non-allergic infants did not find any association between allergic disease and colonization with *B. adolescentis* [32]. Yet, in contrast to the study by Stsepetova [30], *B. pseudocatenulatum* were more frequently detected in the allergic infants [32]. Studies show that different *Bifidobacterium* species elicit different immune responses from diverse immune cells [33, 34]. Speculatively, the colonization with several different species of commensal flora might lead to balanced immune responses contributing to the development of a fully functional immune system protecting children from allergy development. However, under certain conditions *i.e.* when the gut microbiota is less diverse, certain species could appear to have a detrimental role in allergic disease. This could also be the case for *C. difficile*. Previous studies suggest that colonization early in life with *C. difficile* is more common among infants who develop allergic disease than among infants who do not [10]. We could not confirm this, as infants

who developed allergy were less likely to be colonized with *C. difficile* during their first two months. The low rate of *C. difficile* colonization combined with the relatively few number of allergic children may explain the lack of a reverse association in our study. Nevertheless, several studies, showing more clostridia, also indicate less other commensal bacteria in children developing or suffering from allergy [8, 9, 35]. Therefore, it might rather be a gut microbiota with lower diversity that is associated with allergy development. Interestingly, a large recent study did not find any relationship between the presence of different bacteria and allergy development up to 18 months of age [11], but indeed showed that infants who developed allergy had a lower diversity in their gut microbiota [36]. In order to further understand the influence of the gut microbiota on immune responses and allergy development additional prospective studies and molecular studies are needed.

Lifestyle factors have been inversely associated with allergy development, and could possibly influence the gut microbiota and how it establishes early in life. Interestingly, we show that certain household factors are associated with the diversity of the bifidobacterial flora. The number of *Bifidobacterium* species at one week and two months correlated significantly with the number of family members. Higher counts of bifidobacteria [12] and later acquisition of *Clostridium* species in infants with siblings [11] have been reported previously. Thus, family members and siblings may rapidly contaminate the infant with a commensal microbiota. This is further supported by experiments in rats showing that ex-germfree rats encounter a normal gut microbiota almost as quickly when they are reared with conventional rats as when they are inoculated with gut microbiota from conventional rats [37].

Exposure to high levels of endotoxin in house dust was also associated with a more diverse *Bifidobacterium* microbiota at one week and two months after birth. Endotoxin is a component in G- bacteria and should probably be viewed as a marker of total bacterial

exposure in the homes and not as a factor directly related to the G+ bifidobacterial microbiota. However, postnatal colonization with the G- and facultative aerobic enterobacterial microbiota, is important to establish an appropriate environment for anaerobic bacteria, such as bifidobacteria and *Bacteroides* [4]. Today, staphylococci are acquired the first days after birth among Swedish infants, while it takes up to two months before most infants have achieved an enterobacterial microbiota [38]. Speculatively, high bacterial exposure may lead to early colonization with appropriate facultative aerobic bacteria, which in turn would facilitate colonization of commensal anaerobic bacteria. The positive association between endotoxin and *Fel d 1* levels in house dust with the relative amount of *B. longum,* and the inverse relationship with *C. difficile,* would support this possibility. Many statistical tests were performed and p-values close to 0.05 might be false significances. Yet, there are several indications in this study that larger families and more microbial exposure lead to a gut flora with higher diversity. These data are also in line with the finding that certain bacteria were less frequently detected in infants developing allergy. However, future studies, with different and larger cohorts, are needed to verify these results. It will also be necessary to perform more detailed studies of how a diverse microbiota, or certain species of bacteria, influences infant immune responses. How these immune responses relate to allergy development also needs to be further explored.

In conclusion, children who develop allergic disease were less often colonized with Lactobacilli group I, *B. adolescentis* and *C. difficile* during their first two months of life. As family size and endotoxin exposure appeared to influence the *Bifidobacterium* flora, a more diverse gut microbiota early in life could, to some extent, explain the inverse association between these household factors and allergy development.

ACKNOWLEDGMENTS

We are grateful to all children and parents participating in this study and to the following people at Linköping University Hospital; research nurse Lena Lindell, laboratory technologist Ann-Marie Fornander and Dr Sara Tomicic for excellent assistance and Dr Karel Duchén for clinical evaluation of the children. We are also grateful to Tiina Rebane at Tartu University Clinics, Jan-Olov Persson at Stockholm University for statistical help and Elisabeth K. Norin at Karolinska Institutet for critically reading the manuscript.

This work was supported by the Ekhaga foundation, the Cancer and Allergy foundation, Mjölkdroppen, the Magnus Bergvall foundation, the Swedish Research Council (57X-15160-05-2 and 74X-20146-01-2), the National Swedish Association against Allergic Diseases, the National Heart and Lung Association and the Swedish Foundation for Health Care Sciences and Allergy Research.

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