

ORIGINAL ARTICLE

Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses

Gut microbiota and immune responses

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Summary

Introduction Among sensitized infants, those with high, as compared with low levels, of salivary secretory IgA (SIgA) are less likely to develop allergic symptoms. Also, early colonization with certain gut microbiota, e.g. *Lactobacilli* and *Bifidobacterium* species, might be associated with less allergy development. Although animal and *in vitro* studies emphasize the role of the commensal gut microbiota in the development of the immune system, the influence of the gut microbiota on immune development in infants is unclear.

Objective To assess whether early colonization with certain gut microbiota species associates with mucosal and systemic immune responses i.e. salivary SIgA and the spontaneous Toll-like receptor (TLR) 2 and TLR4 mRNA expression and lipopolysaccharide (LPS)-induced cytokine/chemokine responses in peripheral blood mononuclear cells (PBMCs).

Methods Fecal samples were collected at 1 week, 1 month and 2 months after birth from 64 Swedish infants, followed prospectively up to 5 years of age. Bacterial DNA was analysed with real-time PCR using primers binding to *Clostridium difficile*, four species of bifidobacteria, two lactobacilli groups and *Bacteroides fragilis*. Saliva was collected at age 6 and 12 months and at 2 and 5 years and SIgA was measured with ELISA. The PBMCs, collected 12 months after birth, were analysed for TLR2 and TLR4 mRNA expression with real-time PCR. Further, the PBMCs were stimulated with LPS, and cytokine/chemokine responses were measured with Luminex.

Results The number of *Bifidobacterium* species in the early fecal samples correlated significantly with the total levels of salivary SIgA at 6 months. Early colonization with *Bifidobacterium* species, lactobacilli groups or *C. difficile* did not influence TLR2 and TLR4 expression in PBMCs. However, PBMCs from infants colonized early with high amounts of *Bacteroides fragilis* expressed lower levels of TLR4 mRNA spontaneously. Furthermore, LPS-induced production of inflammatory cytokines and chemokines, e.g. IL-6 and CCL4 (MIP-1 β), was inversely correlated to the relative amounts of *Bacteroides fragilis* in the early fecal samples.

Conclusion Bifidobacterial diversity may enhance the maturation of the mucosal SIgA system and early intense colonization with *Bacteroides fragilis* might down-regulate LPS responsiveness in infancy.

Keywords *Bacteroides fragilis*, bifidobacteria, *Clostridium difficile*, gut microbiota, infant, lactobacilli, SIgA, TLR2, TLR4

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Introduction

A reduced microbial pressure in westernized countries is postulated to underlie the increase in allergy development

during the past decades. Alterations in the early gut microbiota may precede allergy development [1, 2]. Children developing allergy are compared with those who remain non-allergic, not as often colonized with

bifidobacteria and enterococci but more frequently colonized with clostridia including *Clostridium (C.) difficile* early in life [1, 2]. *Bifidobacterium* colonization at species level might also be associated with allergy [3–5]. Furthermore, children who developed allergy during their first 5 years were already, during the first week of life less often colonized with lactobacilli group I, comprising *Lactobacillus (L.) rhamnosus*, *L. casei* and *L. paracasei*, as compared with children not developing allergy [3]. However, not all studies demonstrate a relationship between the early gut microbiota and allergy development [6]. Whether pre- and postnatal administration of probiotic bacteria associates with a decreased incidence of allergic disease is unclear. Less IgE-associated eczema [7] and less allergy development up to 2 years [8] but not 5 years of age [9] are reported in probiotic-treated infants. However, other studies failed to find any effect of probiotic administration on allergic symptoms [10]. In order to understand whether the early gut microbiota is associated with allergy development, the possible mechanisms explaining how the early gut microbiota influence infant immune responses and thus subsequent allergy development need to be investigated.

Animal studies have emphasized the importance of the gut microbiota in educating the immune system. The gut-associated lymphoid tissue (GALT) in germ-free (GF) animals is underdeveloped [11, 12] with few IgA⁺ B cells [13]. In addition, the spleen of GF mice contains fewer numbers of CD4⁺ T cells [14]. Colonization with lactobacilli strains increases the numbers of IgA⁺ B cells [13] and a polysaccharide from *Bacteroides fragilis* (PSA) could restore the proportion of splenic CD4⁺ T cells [14]. Also, PSA administration increases IL-10 production [15]. The early colonization appears to be of particular importance as *Bifidobacterium (B.) infantis* could restore Th1 responses in neonatal but not in adult ex-GF mice [16]. Additionally, the serum levels of IgA appear to increase in piglets colonized shortly after birth but remain low in GF piglets [17].

Studies in humans indicate that the early colonization with certain bacteria influences systemic immune responses. For example, *Bacteroides fragilis* colonization in infancy appears to increase the number of circulating IgA and IgM antibody-producing cells [18]. Furthermore, infants who received a mixture of probiotic strains from birth had higher plasma levels of C-reactive protein, total IgA, total IgE and IL-10 at 6 months than infants in the placebo group [19]. Although the composition of the gut microbiota at 6 months does not appear to influence the salivary IgA levels at that age [20], it is conceivable that the microbiota that colonize the gut shortly after birth might influence immune development.

The innate immune compartment responds to different microbial-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) expressed on immune

cells, e.g. dendritic cells and mucosal epithelia [21]. The capacity to respond to PRR signals is important for adaptive immune responses such as the IL-12-dependent direction of naïve T cells into Th1 cells [22]. The PRR Toll-like receptor (TLR) 2 responds to MAMPs such as lipoteichoic acid from Gram-positive (G⁺) bacteria, while the TLR4 recognizes the endotoxin lipopolysaccharide (LPS) from Gram-negative (G⁻) bacteria together with a complex with CD14 and MD-2 [23]. Soluble CD14 appears to be higher in the plasma of infants early colonized with *Staphylococcus (S.) aureus* than in the plasma of non-colonized infants [24]. However, it is not known how the early infant gut microbiota, consisting of both G⁺ and G⁻ bacteria, influence TLR responsiveness.

High salivary secretory IgA (SIgA) may protect sensitized children from developing allergic symptoms and non-allergic children tend to have higher salivary SIgA levels than allergic children [25]. Recently, we also demonstrated that Swedish children have less SIgA early in life compared with Estonian age-matched children (S. Tomicic, T. Voor, M. C. Jenmalm, B. Björkstén, M. F. Böttcher, unpublished data). As Estonian children are frequently colonized with lactobacilli [26], we hypothesize that the early gut microbiota, notably lactobacilli and bifidobacteria at species level, could influence the maturation of salivary SIgA production. The possible allergy-protective effects from the increased pre- and postnatal microbial exposure in farming environments might increase the expression of several PRRs, e.g. TLR2 and TLR4 [27, 28]. Thus, we also hypothesized that early exposure to certain intestinal microbes, the G⁻ *Bacteroides fragilis* and the G⁺ *C. difficile*, bifidobacteria and lactobacilli, modulates TLR expression and LPS responsiveness. In addition, as *Bacteroides fragilis* colonization/PSA administration induces IFN- γ production in GF mice [14], we hypothesized that early *Bacteroides fragilis* colonization influences spontaneous and PHA-induced IFN- γ production in infants.

Materials and methods

Study population

The birth cohort, comprising 123 Swedish children born between March 1996 and October 1999, has been described in detail elsewhere [29]. Briefly, the children were born at term and had an uncomplicated perinatal period. Inclusion in the present study was based on availability of fecal samples at 1 week, 1 month and/or at 2 months of age. In all, 64 infants were included. From the majority of these children, blood samples collected at 12 months and/or salivary samples, collected at 6, 12 months, 2 and/or 5 years, were also available. 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. Clinical examinations

Table 1. Demographic data of the subjects

	Subjects, <i>n</i> = 64
Female subjects	32 (50%)
Born with Caesarean section	3 (5%)
Any atopic heredity	50 (78%)
Exclusively breastfed ≥ 3 months	53 (83%)*
Oral antibiotics ≤ 3 months	2 (3%)
Pets	11 (17%)
Number of family members	3 (3–8) [†]
Household area/individual (m ²)	29 (18–75) [†]

*Missing data for one subject regarding exclusive breastfeeding.

[†]Missing data for three subjects regarding number of family members and thus household area/individual.

of the children were performed at 3 or 6 and 12 months and at 2 and 5 years. On these occasions, skin prick tests were performed, and questionnaires were completed regarding, for example, symptoms of allergy and use of antibiotics. As development of allergic disease in relation to their early gut microbiota has been investigated in a previous study in these children [3], this will not be discussed here.

The majority of the children had a history of atopic disease in the immediate family (78%, Table 1). In total, three children were delivered by a caesarean section. Most infants were exclusively breastfed during their first 3 months (83%) and only two infants received antibiotics during this time.

Analysis of bacterial DNA in the fecal samples

The analysis of the bacterial DNA has been described previously [3]. In brief, fecal samples were collected into sterile plastic containers by the parents when the infants were 1 week (collected at day 5 or 6), 1 month and 2 months old. The samples were stored at -70°C until analysis.

The Qiaamp DNA Stool Mini KitTM (Qiagen, Hilden, Germany) was used for the isolation of DNA from 180 to 220 mg faeces and the protocol included for increasing the bacterial DNA over human DNA was used. The concentration of nucleic acids was measured with BIO-RAD Smart-spec (Bio-Rad laboratories, Hercules, CA, USA) at 260 nm using BIO RAD trUView Disposable Cuvettes (Bio-Rad laboratories).

Bacterial DNA was analysed with real-time PCR using primers binding to *C. difficile*, *B. adolescentis*, *B. longum*, *B. bifidum*, *B. breve*, lactobacilli group I (comprising *L. rhamnosus*, *L. casei* and *L. paracasei*), lactobacilli group II (comprising *L. gasseri* and *L. johnsonii*) and *Bacteroides fragilis*. The sequences and concentrations of primers are described in [3]. 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. Reference bacterial DNA, used as the standard and posi-

tive control, was purchased from LGC Standards (Borås, Sweden) and Biotechon Diagnostics (Potsdam, Germany). The SYBR Green real-time PCR was performed using 96-well detection plates in ABI prism 7000 (Applied Biosystems, Stockholm, Sweden). All samples were performed in triplicate. Each well contained 2 $\times$ Power SYBR Green mastermix (Applied Biosystems, Stockholm, Sweden), forward and reverse primer (MWG-Biotech, Edersburg, Germany), DNA and water. The Absolute Quantification protocol in 7000 System software version 1.2.3f2 (Applied Biosystems, Stockholm, Sweden) was used and the amplification was performed using the default program of 40 cycles, which also included melting curve analysis. The software calculated the amount of specific bacterial DNA from the standard curve, constructed from known amounts of reference bacterial DNA (5 ng diluted in a 10-fold dilution series down to 50 fg). To avoid detecting false positives, triplicates with C_T values above 35 were considered as negative. 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 detection limit was $5 \times 10^{-6}\%$ specific bacterial DNA of total nucleic acids. Negative samples were assigned a value 10 times below the detection limit i.e. $5 \times 10^{-7}\%$ specific bacterial DNA of total nucleic acids, and used in the correlation analyses.

Analysis of total secretory immunoglobulin A in saliva

Saliva was collected at 6 and 12 months and at 2 and 5 years of age from the buccal cavity using a hand-pump connected to a thin plastic tube and immediately frozen at -20°C . Before analysis of SIgA, the samples were heated at 56°C for 30 min and then centrifuged at 5000 g for 15 min. Total SIgA was analysed with ELISA using an anti-human secretory component antibody (Dakopatts, Täby, Sweden) as a coating antibody, as described previously [25]. Human IgA (Sigma Immunochemicals, Stockholm, Sweden) was diluted in seven steps for the standard curve. The SIgA, bound to the coating antibodies, was detected with alkaline phosphatase-conjugated goat anti-human IgA antibodies (Sigma Immunochemicals) and the FAST pNPP substrate. The detection range was 16–1000 ng/mL for SIgA.

Cell preparation

Venous blood samples were drawn into heparinized tubes (Becton Dickinson, Stockholm, Sweden) at 12 months of age. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll Paque density gradient (Pharmacia Biotech, Uppsala, Sweden). The cells were thereafter cryopreserved according to the standard methodology in

10% dimethyl sulphoxide (Sigma-Aldrich, Stockholm, Sweden), 50% fetal calf serum and 40% RPMI-1640 (Life Technologies AB, Täby, Sweden).

Reverse transcription-polymerase chain reaction of mRNA and quantification of gene expression

Two hundred and fifty microlitres of cell suspension [1×10^6 viable cells/mL (as checked by Trypan blue exclusion) in AIM-V serum-free medium (Life Technologies AB) with 20 μ M mercaptoethanol (Sigma-Aldrich)] was cultivated for 24 h at 37 °C with 5% CO₂ (Forma CO₂-incubator model 3862, Forma Scientific Inc., Marietta, OH, USA), with no added stimulus. Thereafter, the cells were lysed with RLT lysis buffer (Qiagen, Hilden, Germany). The cell lysates were stored in -70 °C until RNA isolation. Total RNA was isolated using the RNeasyTM 96 Protocol (Qiagen) according to the manufacturer's instructions. Briefly, the cells were lysed by RLT lysis buffer, mixed with ethanol and applied to RNeasyTM 96-well plates. Contaminants were washed away by buffers and the RNA was eluted in 2 $\times$ 30 μ L of RNase-free water.

RNA was converted to complementary DNA (cDNA) using the High Capacity Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, RNA was mixed with MultiScribe reverse transcriptase, random primers, dNTPs, reverse transcription buffers and RNase-free water in 40 μ L reactions and run for 10 min at 25°, followed by 37 °C for 120 min. The gene expression analysis was performed with quantitative real-time PCR. Taqman[®] Gene Expression and Taqman[®] Fast Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). The id's were TLR2 Hs00610101_m1 and TLR4 Hs00152939_m1. Primers, probes, Master Mix, water and cDNA were mixed and the samples were run on an Applied Biosystems 7500 fast real-time PCR system (Foster City, CA, USA). The thermal cycling conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. rRNA was used as the internal control, i.e. the amount of the expressed gene was calculated relative to the amount of rRNA in each sample. Standards were used to create a standard curve from which the amounts were calculated in each run using the standard curve method as described in User Bulletin no 2 (Applied Biosystems, Foster City, CA, USA). The inter-assay variation was < 6% for both genes and the slopes of the standard curves varied between -3.3 and -3.9. Each sample was run in duplicate and a CV of maximum 15% was allowed.

Cytokine and chemokine analysis

After thawing, 0.25 mL of 1×10^6 viable cells/mL cell suspension in AIM-V serum-free medium was cultured at 37 °C with 5% CO₂. The cells were either cultured with

10 ng/mL LPS *Salmonella enterica* serotype *thyphimurium* (Sigma-Aldrich) or with medium alone. The viability was checked by trypan blue exclusion. After 24 h, the supernatants were collected and stored at -70 °C until analysis. The cell supernatants were analysed using a multiplex assay kit, according to the manufacturer's instructions (Human cytokine 9-plex panel, Bio-Rad Laboratories, Hercules, CA, USA). The assay detects the following analytes: IL-6, CXCL8 (IL-8), IL-10, IL12p70, IL-17, IL-1 β , CCL2 (MCP-1), CCL4 (MIP-1 β) and TNF. The samples were analysed on a Luminex¹⁰⁰ instrument (Biosource, Nivelles, Belgium), and the data were analysed with the software StarStation 2.0 (Applied Cytometry Systems, Sheffield, UK). The lower detection limit was 10 pg/mL for IL-6 and CCL4, 6 pg/mL for IL-10, CXCL8 and TNF, 2 pg/mL for IL-12p70, 3 pg/mL for IL-17, 4 pg/mL for IL-1 β and 18 pg/mL for CCL2. Comparisons of LPS-induced cytokine and chemokine responses were made after the control value, i.e. responses from cells cultured in medium alone, was withdrawn.

For IFN- γ analyses, 1 mL of cell suspension [1×10^6 viable cells/mL in AIM-V serum-free medium supplemented with 20 μ M mercaptoethanol (Sigma-Aldrich)] was grown in duplicate alone or with 2 μ g/mL phytohaemagglutinin (PHA) (Sigma-Aldrich). The culture conditions and analyses have been described in more detail previously [30]. After 1 and 6 days of culture, the supernatants were collected and stored at -20 °C. IFN- γ was measured using an ELISA kit (CLB Pelikine CompactTM, Research Diagnostics Inc., Flanders, NJ, USA). The detection limit was 25 pg/mL. Spontaneous IFN- γ production was detected after 6 days, but not after 1 day of culture.

Statistics

Spearman's rank coefficient was calculated to investigate whether the relative amount of bacterial DNA and/or the number of *Bifidobacterium* species in fecal samples correlated with the concentrations of total salivary SIgA and the relative mRNA expression of TLR2 and TLR4. It was also calculated to determine whether the bacterial amounts influenced the production of cytokines and chemokines from PBMCs after microbial stimulation. The Mann-Whitney *U* test was performed to evaluate whether infants colonized with the specific bacteria had different levels of total SIgA in saliva compared with uncolonized infants. In addition, the Mann-Whitney *U* test was performed to determine whether the colonized and uncolonized groups expressed different levels of TLR2 and TLR4 mRNA and produced different levels of IFN- γ at 12 months. Fisher's exact test was performed to determine whether colonization with *Bacteroides fragilis* was associated with spontaneous IFN- γ production. Many statistical tests were performed and thus *P*-values close to 0.05 might indicate false significance. As this study was not

sufficiently powered to detect very low P -values, we only report significant values observed at several time-points, or significant values observed on one occasion with at least a tendency at another time-point. This approach would decrease the risk of including false significance. The study should be viewed as exploratory, and consequently $P < 0.05$ was chosen to indicate statistical significance.

Results

Gut microbiota during the first 2 months

Bifidobacteria and *Bacteroides fragilis* were commonly present already in 1-week-old infants, whereas the other bacteria tended to be more frequently detected as the infants grew older (Table 2). Lactobacilli occurred in lower amounts than bifidobacteria and *Bacteroides fragilis*. Few infants were colonized with *C. difficile*.

Total salivary secretory immunoglobulin A associates with the early gut microbiota

The number of *Bifidobacterium* species in faeces collected 1 week, 1 month and 2 months after birth correlated with total salivary SIgA at 6 months ($r = 0.51$ – 0.58 , $P = 0.02$ – 0.045 , Fig. 1 exemplifies the 1-month colonization) but not in older children. When analysing the different *Bifidobacterium* species separately, it was shown that infants colonized with *B. adolescentis* at 1 and 2 months had significantly higher levels of SIgA at 6 months [Fig. 2a and median; 12.0 (4.3 – 19.7) $\mu\text{g/mL}$, respectively] compared with non-colonized infants [Fig. 2a and median; 5.3 (2.0 – 10.0) $\mu\text{g/mL}$, respectively]. Furthermore, the intensity of 1- and 2-month *B. adolescentis* colonization (expressed as percentage *B. adolescentis* DNA of all fecal

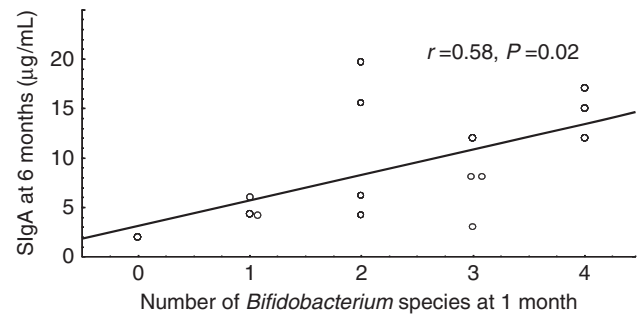


Fig. 1. Total salivary secretory IgA (SIgA) at 6 months is associated with a more diverse *Bifidobacterium* flora. The y-axis shows total SIgA ($\mu\text{g/mL}$) at 6 months and the x-axis shows the number of *Bifidobacterium* species at 1 month.

nucleic acids) was associated with higher SIgA at 6 months ($r = 0.66$ and 0.55 , $P = 0.007$ and 0.03 , respectively). In addition, SIgA levels at 6 months tended to be higher in infants colonized with *B. breve* at 1 week and 1 month [median; 9.0 (6.2 – 17.0) $\mu\text{g/mL}$ and Fig. 2a, respectively] than in non-colonized infants [median; 4.3 (2.0 – 15.5) $\mu\text{g/mL}$, $P = 0.02$ and Fig. 2a, respectively]. The intensity of *B. bifidum* colonization at 1 week, 1 month and 2 months after birth was correlated with the SIgA production at 12 months ($r = 0.41$ – 0.47 , $P = 0.01$ – 0.04 , Fig. 3 exemplifies 1-month colonization).

The levels of SIgA at 6 months and 5 years were significantly associated with the colonization with lactobacilli group I at 1 month after birth (Figs 2a and d). Also, increased levels of SIgA at 5 years were associated with colonization with *C. difficile* at 1 and 2 months after birth ($P = 0.008$ – 0.03 , Fig. 2d). The few individuals colonized with this bacterium could greatly bias the results, however. The salivary SIgA levels at 2 years of age were not associated with the early gut microbiota (Fig. 2c).

Early colonization with *Bacteroides fragilis* associates with spontaneous Toll-like receptor 4 mRNA expression, lipopolysaccharide responsiveness and spontaneous interferon- γ production at 12 months of age

The expression of TLR4 mRNA was negatively correlated to the intensity of *Bacteroides fragilis* colonization during the first week and month after birth ($r = -0.47$, $P = 0.02$ and Fig. 4). None of the other analysed bacteria associated with the spontaneous expression of TLR2 or TLR4 (data not shown).

In concordance with the TLR4 mRNA findings, the intensities of *Bacteroides fragilis* colonization at 1 week, 1 and 2 months were all inversely related to the LPS-induced production of IL-6 and CCL4 (MIP-1 β) ($r = -0.62$ to -0.79 , $P = 0.002$ – 0.04 and $r = -0.75$ to -0.86 , $P = 0.001$ – 0.005 , Fig. 5 exemplifies 1-month colonization). Also, LPS-induced IL-8 and IL-17 were inversely related to the intensity of *Bacteroides fragilis* colonization at 1 week

Table 2. Proportion of infants (%) colonized with *Bifidobacterium*, *Lactobacillus*, *Clostridium difficile* and *Bacteroides fragilis* during the first 2 months of life

Bacteria	1 week, $n = 52$	1 month, $n = 56$	2 months, $n = 56$
<i>Bifidobacterium longum</i>	88	91	91
<i>Bifidobacterium adolescentis</i>	71	66	75
<i>Bifidobacterium bifidum</i>	56	63	63
<i>Bifidobacterium breve</i>	40	43	50
Lactobacilli group I	31	57	57
Lactobacilli group II	33	43	54
<i>Clostridium difficile</i>	6	11	21
<i>Bacteroides fragilis</i>	60	55	55

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.

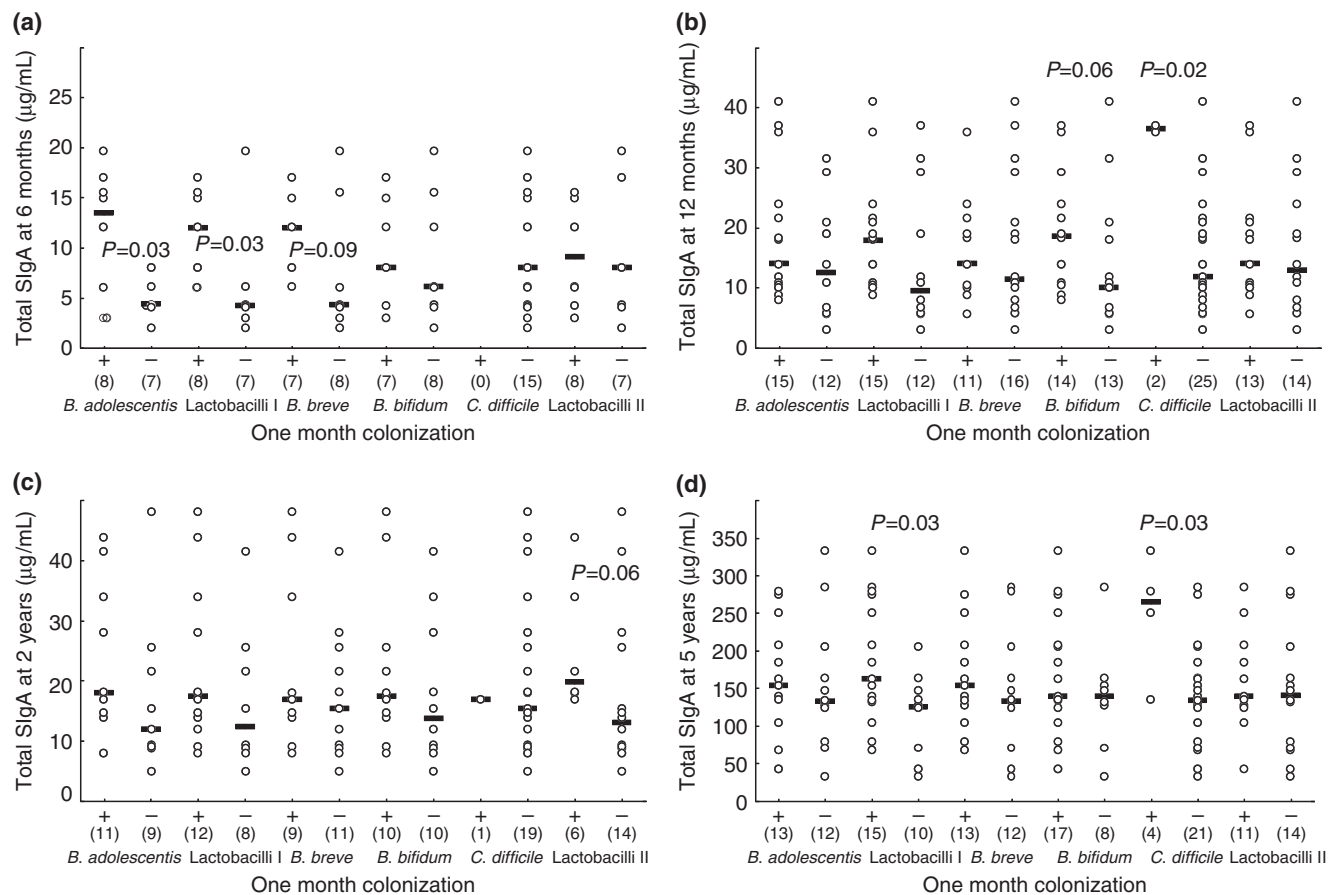


Fig. 2. Colonization with bifidobacteria, lactobacilli and *Clostridium difficile* at 1 month of age in relation to salivary secretory IgA (SigA) at age 6 months (a), 12 months (b), 2 years (c) and 5 years (d). The positive sign denotes colonized infants and the negative sign those lacking the bacteria. The numbers in brackets show the number of infants from whom both fecal and salivary samples were available. The short lines demonstrate the median values. Colonization with *Bacteroides fragilis* and *Bifidobacterium longum* did not influence the SigA production and these bacteria are therefore not shown.

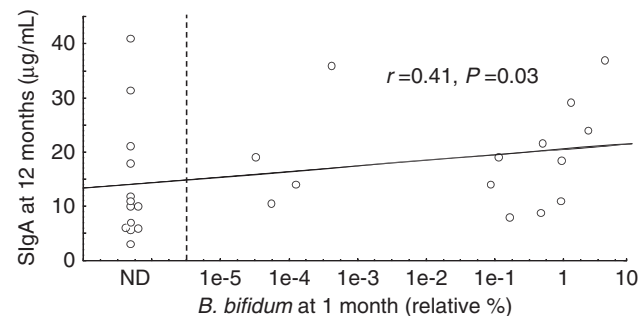


Fig. 3. Total salivary secretory IgA (SigA) at 12 months is associated with the intensity of *Bifidobacterium bifidum* colonization. The y-axis shows the total salivary SigA ($\mu\text{g/mL}$) at 12 months and the x-axis shows the intensity of *B. bifidum* colonization (expressed as percent *B. bifidum* DNA of total amount nucleic acids) at 1 month. The dotted line shows the detection limit at $5 \times 10^{-6}\%$ *B. bifidum* DNA of total amount of nucleic acids. ND, not detectable.

after birth ($r = -0.75$, $P = 0.008$ and $r = -0.68$, $P = 0.02$). In contrast, the intensity of *B. adolescentis* colonization at 2 months correlated or tended to correlate to TNF, IL-10 and IL-12p70 ($r = 0.64$, $P = 0.03$, $r = 0.67$, $P = 0.03$ and

$r = 0.55$, $P = 0.08$, respectively) with similar tendencies at 1 month ($r = 0.54$, $P = 0.07$, $r = 0.46$, $P = 0.13$ and $r = 0.58$, $P = 0.05$, respectively). None of the other investigated bacteria yielded any conclusive significant correlations, i.e. significant on one occasion with at least a tendency at another time-point.

Early *Bacteroides fragilis* colonization was also investigated for its possible role in modulating IFN- γ production at 12 months. Infants colonized with *Bacteroides fragilis* at 1 week and 1 month did not produce IFN- γ spontaneously more often at 12 months (9/22 and 7/20) than infants not colonized with *Bacteroides fragilis* (2/10 and 2/13). All the infants who produced IFN- γ spontaneously at 12 months and were uncolonized at 1 week and 1 month had become colonized with *Bacteroides fragilis* at age 2 months, however. Thus, among the 21 infants colonized with *Bacteroides fragilis* at 2 months, 10 produced IFN- γ spontaneously at 12 months compared with no spontaneous IFN- γ producers among the 14 uncolonized infants ($P = 0.019$). There was no difference in IFN- γ production after PHA stimuli between the colonized and the uncolonized infants.

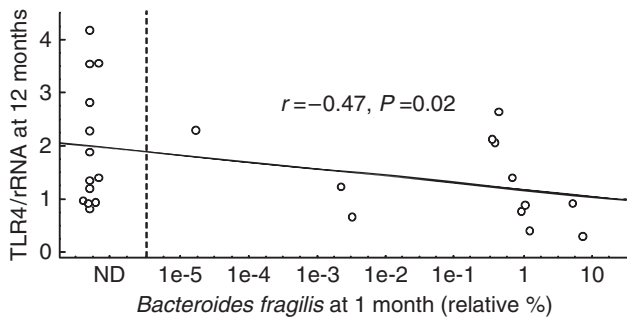


Fig. 4. The spontaneous expression of Toll-like receptor (TLR) 4 mRNA at 12 months is inversely correlated to the intensity of *Bacteroides fragilis* colonization at 1 month after birth. The y-axis shows the relative TLR4 mRNA expression/rRNA in peripheral blood mononuclear cells collected 12 months after birth and the x-axis shows the relative amount of *Bacteroides fragilis* at 1 month. The dotted line shows the detection limit at $5 \times 10^{-6}\%$ *Bacteroides fragilis* DNA of the total amount of nucleic acids. ND, not detectable.

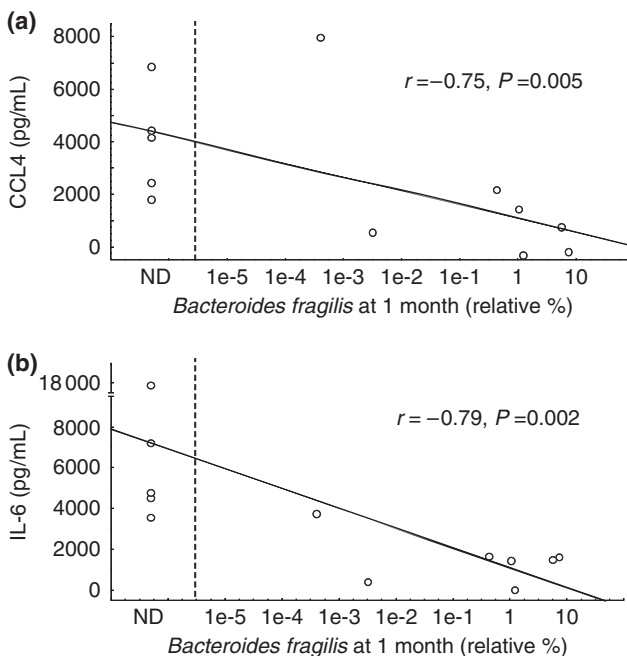


Fig. 5. The lipopolysaccharide (LPS)-induced production of CCL4 (MIP-1 β) (a) and IL-6 (b) from peripheral blood mononuclear cells (PBMCs), collected 12 months after birth, is inversely correlated to the intensity of *Bacteroides fragilis* colonization 1 month after birth. The y-axis shows the concentration of CCL4 (a) and IL-6 (b) in supernatants from PBMCs collected 12 months after birth and stimulated with LPS for 24 hours. The x-axis shows the relative percent of *Bacteroides fragilis* at 1 month. The dotted line shows the detection limit, $5 \times 10^{-6}\%$ *Bacteroides fragilis* DNA of the total amount of nucleic acids. ND, not detectable.

Discussion

The *Bifidobacterium* flora during the first 2 months after birth was associated with enhanced production of salivary SIgA at 6 and 12 months. The present study, in which bifidobacteria were identified at the species level, suggests that the diversity of *Bifidobacterium* species might have a

larger impact on the maturation of IgA responses rather than the number of bacteria within the *Bifidobacterium* genera [20, 31]. The period of colonization shortly after birth might be of particular importance for maturation of immune responses, as others have reported that colonization with bifidobacteria at 6 months did not associate with salivary SIgA levels at that age [20].

Early colonization with a strain of *Escherichia coli* can induce fecal antigen-specific SIgA levels already in the first weeks after birth [32] and early colonization with toxigenic *S. aureus* influences plasma IgA levels at 4 months, but not at 18 months [31], thus further supporting the role of early colonization for maturation of IgA responses. As early colonization with bifidobacterial species was not associated with salivary SIgA levels at 2 and 5 years, other factors are probably more important for later induction of salivary SIgA responses. Recurrent respiratory infections may stimulate IgA formation as suggested by the observation that infants exposed to more than three infections during infancy have higher SIgA levels than less infected infants (A. Sandin, B. Björkstén, M. F. Böttcher, M. C. Jennalm, L. Bråbäck, unpublished data). Whether the gut microbiota and recurrent infections account for early and late SIgA formation, respectively, needs to be further studied. Although early *Bacteroides fragilis* colonization associates with the number of IgA-producing cells [18], we found no association between *Bacteroides fragilis* colonization and salivary SIgA.

Both T cell-dependent and T cell-independent IgA class switching occurs in the human gut (reviewed in [33]). Interestingly, the gut microbiota has been shown to activate intestinal epithelial cells (IECs) through TLRs, leading to the production of a proliferation-inducing ligand (APRIL) and thymic stromal-derived lymphoprotein (TSLP) [34]. The TSLP in turn influences dendritic cells to produce additional APRIL, which is important for T cell-independent IgA₂ class switching [34]. Furthermore, IgA production is profoundly reduced in GF mice [13]. Also, some studies indicate that GALT IgA-producing B cells can migrate to other parts of the mucosal tissue, such as salivary glands (reviewed in [35]). Thus, it is conceivable that lactic acid bacteria in the gut may influence IgA production in the saliva. Alternatively, the gut microbiota species studied are epiphenomena correlating with unknown factors that the infants are exposed to, e.g. in the oral mucosa, and this is what affects the SIgA production in saliva. We previously showed that bifidobacterial diversity was associated with house dust exposure to microbes (endotoxin) [3].

As higher levels of SIgA might protect sensitized infants from allergy development [25], it is tempting to speculate that a more diverse *Bifidobacterium* flora early in life could offer some protection against allergy development by increasing salivary SIgA. Studies from our group also indicate that salivary SIgA levels, already at

age 3, 6 and 12 months, are lower among 2-year-old sensitized infants with allergic symptoms compared with asymptomatic sensitized infants ([25], S. Tomicic, T. Voor, M. C. Jenmalm, B. Björkstén, M. F. Böttcher, unpublished data). Thus, early induction of SIgA levels could be of relevance for allergy development. We previously showed that infants developing allergy were less often colonized with *B. adolescentis* during their first 2 months of life [3]. However, we could not conclusively show that development of allergy up to 5 years of age was associated with less *Bifidobacterium* diversity in early infancy [3]. In addition, others showed that *B. bifidum* colonization was more common among infants who developed allergy at 6 months [36] and *B. adolescentis* colonization could be more common in allergic compared with non-allergic infants and children [5, 37]. Thus, the role of the *Bifidobacterium* flora in association with allergy development is controversial and needs further investigation. Speculatively, the observed association between the early *Bifidobacterium* flora and SIgA levels during the first year could indicate that an overall more diverse gut microbiota increases salivary SIgA. Actually, a more diverse early gut microbiota is more common among non-allergic infants compared with allergic infants [38].

The colonization with lactobacilli group I (*L. rhamnosus*, *L. casei* and *L. paracasei*) at 1 month was also associated with higher levels of SIgA in saliva at 6 months and 5 years. This is further supported by the observations that Swedish infants appear to be less often colonized with lactobacilli and also have lower levels of SIgA in saliva than Estonian infants ([26], S. Tomicic, T. Voor, M. C. Jenmalm, B. Björkstén, M. F. Böttcher, unpublished data).

The TLR4 mRNA expression was lower in infants colonized with high relative amounts of the G⁻ bacterium *Bacteroides fragilis* at 1 week and 1 month after birth. Furthermore, LPS-induced CCL4 (MIP1 β), CXL8 (IL-8), IL-6 and IL-17 levels were inversely correlated to the intensity of *Bacteroides fragilis* colonization. Partially supporting our findings, it has been observed that increased home dust exposure to components from G⁻ bacteria, i.e. 3-hydroxy fatty acids, is inversely associated with the production of IL-6 and TNF after mitogen stimulation [39]. Lappalainen also showed that exposure to muramic acid, from G⁺ bacteria, correlated with IL-6 and TNF production [39]. Supporting this, we observed that the relative amounts of the G⁺ *B. adolescentis* tended to correlate with the production of TNF, IL-10 and IL-12p70 after LPS stimulation. Yet, the spontaneous expression of TLR4 mRNA was not associated with early *B. adolescentis* colonization, possibly implying that these associations were found by chance. The spontaneous TLR2 mRNA expression was not associated with colonization with any of the investigated bacteria. This could indicate that these early gut microbiota species do not associate with TLR2 expression and the childhood TLR2

expression may be more influenced by prenatal microbial exposure [27] than the early gut microbiota.

High LPS exposure induces endotoxin (LPS) tolerance [40] and endotoxin-tolerant mice have decreased splenic TLR2 and TLR4 [41]. Speculatively, the observed decreased LPS responsiveness in infants colonized with high levels of *Bacteroides fragilis* could thus be due to endotoxin tolerance caused by the high exposure to the G⁻ *Bacteroides fragilis*. Also, PSA, which is another component from *Bacteroides fragilis*, has shown immunomodulatory properties [14, 15]. This component is able to balance the production of inflammatory cytokines, e.g. TNF and IL-17, in mice with induced colitis [15]. As PSA increases the production of IL-10, this has been proposed to be the explanation for regulating inflammatory responses [15]. We could not show that infants colonized with high amounts of *Bacteroides fragilis* produce higher levels of IL-10 after LPS stimuli, however. The decreased TLR4 mRNA expression associated with intense *Bacteroides fragilis* colonization could add to the regulation of inflammatory cytokines. Spleen cells from ex-GF mice colonized with *Bacteroides fragilis*/PSA produce more IFN- γ after α CD3/ α CD28 stimulation than spleen cells from GF mice [14]. In a different system, we showed that infants colonized with *Bacteroides fragilis* during their first 2 months of life more often produced IFN- γ spontaneously than uncolonized infants. However, early colonization with *Bacteroides fragilis* was not associated with higher PHA-induced IFN- γ production. As spleen cells from conventional and ex-GF mice colonized with *Bacteroides fragilis* produce similar levels of IFN- γ after α CD3/ α CD28 stimuli [14], the other gut microbiota species of infants may influence their PHA-induced IFN- γ production. In our previous paper, we could not find any relationship between early *Bacteroides fragilis* colonization and allergy development [3], postulating that the immune responses associated with *Bacteroides fragilis* colonization in infants plays a minor role in the development of allergy.

In conclusion, the early *Bifidobacterium* flora was associated with salivary SIgA levels during the first year of life in this exploratory study. Thus, bifidobacterial diversity may enhance the maturation of the mucosal SIgA system. In addition, early colonization with elevated amounts of *Bacteroides fragilis* was associated with decreased LPS responsiveness, indicating that *Bacteroides fragilis* could influence systemic immune responses.

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