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

Gut flora and immune responses.

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ABSTRACT

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 LPS-induced cytokine/chemokine responses in peripheral blood mononuclear cells (PBMCs).

Methods: Faecal samples were collected at one week, one month and two months after birth from 64 Swedish infants, followed prospectively to five years of age. Bacterial DNA was analyzed 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 six and twelve months and at two and five years and SIgA was measured with ELISA. The PBMCs, collected twelve months after birth, were analyzed 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 faecal samples correlated significantly with the total salivary SIgA levels at six 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-1β), were inversely correlated to the relative amounts of *Bacteroides fragilis* in the early faecal samples.

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

Key words

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

Abbreviations

APRIL	A proliferation-inducing factor
B.	*Bifidobacterium*
C.	*Clostridium*
cDNA	complementary deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
G-	Gram negative
G+	Gram positive
GALT	Gut associated lymphoid tissue
GF	Germ-free
IL	Interleukin
L.	*Lactobacillus*
LPS	Lipopolysaccharide
MAMPs	Microbial associated molecular pattern
mRNA	messenger ribonucleic acid
PBMCs	Peripheral blood mononuclear cells
PRR	Pattern recognition receptor
PSA	Polysaccharide A
RT PCR	Reverse transcriptase polymerase chain reaction
SIgA	Secretory IgA
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSLP	Thymic stromal-derived lymphoprotein

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 to 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 develop allergy during their five years of life were already during the first week of life less often colonized with lactobacilli (*L.*) group I, comprising *L. rhamnosus*, *L. casei* and *L. paracasei*, as compared to 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 decreased incidence of allergic disease is unclear. Less IgE-associated eczema (7) and less allergy development up to two years (8) but not five years of age (9) are reported in probiotic-treated infants. However, others studies find no any association between probiotic administration and allergy development (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, also 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 infantis* could restore Th1 responses in neonatal but not in adult ex-germ-free mice (16). Additionally, the serum levels of IgA appears 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 influence 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 six months than infants in the placebo group (19). Although the composition of the gut microbiota at six months does not appear to influence the salivary IgA levels at that age (20), it is conceivable that the microbiota that colonizes 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 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, influences 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 to Estonian age-matched children (Tomicic *et al* unpublished). 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 IFNγ production in GF mice (14), we hypothesized that early *Bacteroides fragilis* colonization influences spontaneous and PHA-induced IFNγ production in infants.

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 faecal samples at one week, one month and/or at two months of age. In all, 64 infants were included. From the majority of these children blood samples collected at twelve months, and/or salivary samples, collected at six months, twelve months, two years and/or five 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 of the children were made at three or six and twelve months and at two and five years. At these occasions, skin prick tests were performed, and questionnaires were completed regarding, in 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.

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

Analysis of bacterial DNA in the faecal samples

The analysis of the bacterial DNA has been described previously (3). In short, faecal samples were collected into sterile plastic containers by the parents when the infants were one week (collected at day five or six), one month and two months old. The samples were stored at -70ºC until analysis.

Qiamp DNA Stool Mini Kit™ (Qiagen, Hilden, Germany) was used for the isolation of DNA from 180-220 mg faeces and the included 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).

Bacterial DNA was analyzed 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*. 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 standard and positive 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 Biosystem, Stockholm, Sweden). 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 Absolute Quantification protocol in 7000 System software version 1.2.3f2 (Applied Biosystems) was employed 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 (5ng diluted in 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 $5*10^{-6}$ % specific bacterial DNA of total nucleic acids. Negative samples were assigned a value ten times below the detection limit *i.e.* $5*10^{-7}$ % specific bacterial DNA of total nucleic acids, and used in the correlation analyses.

Analysis of total secretory IgA in saliva

Saliva was collected at six and twelve months and at two and five 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 minutes and then centrifuged at 5000g for 15 minutes. Total SIgA was analyzed with ELISA using an anti-human secretory component antibody (Dakopatts AB) as coating antibody, as previously described (25). Human IgA (Sigma Immunochemicals) 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 FAST pNPP substrate. The detection range was 16 to 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 standard methodology in 10% dimethyl sulfoxide (Sigma-Aldrich, Stockholm, Sweden), 50% foetal calf serum and 40 % RPMI-1640 (Life Technologies AB, Täby, Sweden).

Reverse transcription (RT) PCR of mRNA and quantification of gene expression

250 µL of cell suspension ($1x10^6$ viable cells/mL (as checked by Trypan blue exclusion) in AIM-V serum free medium (Life Technologies AB) with 20 µM mercaptoethanol (Sigma-Aldrich)) was cultivated for 24h in 37°C with 5% CO2 (Forma CO2-incubator model 3862, Forma Scientific Inc., Marietta, Ohio, 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 RNeasy™ 96 Protocol (Qiagen, Hilden, Germany) according to manufacturer´s instructions. Briefly, the cells were lysed by RLT lysis buffer, mixed with ethanol and applied to RNeasy™ 96 well plates. Contaminants were washed away by buffers and the RNA was eluted in 2 x 30µl of RNase free water.

RNA was converted to complementary DNA (cDNA) using High Capacity Archive Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer´s instructions. Briefly, RNA was mixed with MultiScribe reverse transcriptase, random primers, dNTPs, reverse transcription buffers and RNAse free water in 40µl 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. The assay id:s were TLR2 Hs00610101_m1 and TLR4 Hs00152939_m1. Primers, probes, Master Mix, water and cDNA was mixed and the samples were run on an Applied Biosystems 7500 Fast Real-Time PCR system. The thermal cycling conditions were 95°C for 20s, followed by 40 cycles of 95°C for 3s and 60°C for 30s. rRNA was used as internal controls, *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). The inter-

assay variation was <6% for both genes and the slopes of the standard curves varied between -3.3 to -3.9. Each sample was run in duplicates 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 were cultured in 37°C with 5% CO_2. 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 24h the supernatants were collected and stored at -70°C until analysis. The cell supernatants were analysed with 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-1β, CCL2 (MCP-1), CCL4 (MIP-1β), and TNF. The samples were analysed on a Luminex[100] instrument (Biosource, Nivelles, Belgium) and the data was 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 IL12p70, 3 pg/mL for IL-17, 4 pg/mL for IL-1β, 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 IFNγ analyses, 1 mL of cell suspension ($1*10^6$ viable cells/mL in AIM-V serum free medium supplemented with 20μM mercaptoetanol (Sigma-Aldrich)) was grown in duplicates alone or with 2μg/mL phytohaemagglutamin (PHA) (Sigma-Aldrich). The culture conditions and analyses have been described in more detail a previous paper (30). After one and six days of culture, the supernatants were collected and stored at -20ºC. Interferon γ was measured with an ELISA kit (CLB Pelikine Compact[TM], Research Diagnostics Inc., Flandern,

NJ, USA). The detection limit was 25 pg/mL. Spontaneous IFNγ production was detected after six days, but not one day of culture.

Statistics

Spearman's rank coefficient was calculated to investigate whether relative amount of bacterial DNA and/or number of *Bifidobacterium* species in faecal samples correlated with concentrations of total salivary SIgA and the relative mRNA expression of TLR2 and TLR4. It was also calculated to understand whether the bacterial amounts influenced the production of cytokines and chemokines from PBMCs after microbial stimulation. 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 un-colonized infants. In addition, Mann-Whitney U test was calculated to understand whether the colonized and un-colonized groups expressed different levels of TLR2 and TLR4 mRNA and produced different levels of IFNγ at 12 months. Fisher´s exact test was calculated to understand whether colonization with *Bacteroides fragilis* was associated with spontaneous IFNγ production. Many statistical tests were performed and thus p-values close to 0.05 might be false significances. 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 at one occasion with at least a tendency at another time point. This approach would decrease the risk of including false significances. The study should be viewed as exploratory and consequently p<0.05 were chosen as statistically significant.

RESULTS

Gut microbiota during the first two months

Bifidobacteria 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 (table 2). Lactobacilli occurred in lower amounts than bifidobacteria and *Bacteroides fragilis*. Few infants were colonised with *C. difficile*.

Total salivary secretory IgA associates with the early gut microbiota.

The number of *Bifidobacterium* species in faeces collected one week, one month and two months after birth correlated with total salivary SIgA at six months (r=0.51 to 0.58, p=0.02 to 0.045, Fig. 1 exemplifies the one month colonization) but not in older children. When analyzing the different *Bifidobacterium* species separately, it was shown that infants colonized with *B. adolescentis* at one and two months had significantly higher levels of SIgA at six months (Fig. 2a and median; 12.0 (4.3-19-7) µg/mL, respectively) compared to non-colonized infants (Fig. 2a and median; 5.3 (2.0-10.0) µg/mL, respectively). Furthermore, the intensity of one and two month *B. adolescentis* colonization (expressed as percentage *B. adolescentis* DNA of all faecal nucleic acids), was associated with higher SIgA at six months (r=0.66 and 0.55, p=0.007 and 0.03, respectively). In addition, SIgA levels at six months tended to be higher in infants colonized with *B. breve* at one week and one month (median; 9.0 (6.2-17.0) µg/mL and Fig 2a, respectively) than in non-colonized infants (median; 4.3 (2.0-15.5) µg/mL, p=0.02 and Fig 2a, respectively). The intensity of *B. bifidum* colonization at one week, one month and two months after birth was correlated with the SIgA production at twelve months (r=0.41 to 0.47, p=0.01 to 0.04, Fig. 3 exemplifies one month colonization).

The levels of SIgA at six months and five years were significantly associated with the colonisation with lactobacilli group I at one month after birth (Fig. 2a and d). Also,

increased levels of SIgA at five years were associated with colonization with *C. difficile* at one and two months after birth (p=0.008 to 0.03, Fig. 2d). The few individuals colonized with this bacterium could greatly bias the results, however. The salivary SIgA levels at two years of age were not associated with the early gut microbiota (Fig. 2c).

Early colonization with *Bacteroides fragilis* associates with spontaneous TLR4 mRNA expression, LPS responsiveness and spontaneous IFNγ 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 analyzed bacteria associated with the spontaneous expression of TLR2 or TLR4 (data not shown).

In concordance with the TLR4 mRNA findings, the intensity of *Bacteroides fragilis* colonization at one week, one month and two months were all inversely related to the LPS induced production of IL-6 and CCL4 (MIP-1β) (r=-0.62 to -0.79, p=0.002 to 0.04 and r= -0.75 to -0.86, p=0.001 to 0.005, Fig. 5 exemplifies one month colonization). Also, LPS induced IL-8 and IL-17 were inversely related to the intensity of *Bacteroides fragilis* colonization at one week after birth (r=-0.75, p=0.008 and r=-0.68, p=0.02). In contrast, the intensity of *B. adolescentis* colonization at two 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 one 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 at one occasion with at least a tendency at another time point.

Early *Bacteroides fragilis* colonization was also investigated for its possible role in modulating IFNγ production at twelve months. Infants colonized with *Bacteroides fragilis* at one week and one month did not produce IFNγ spontaneously more often at twelve months

(9/22 and 7/20) than infants not colonized with *Bacteroides fragilis* (2/10 and 2/13). All the infants who produced IFNγ spontaneously at twelve months and were un-colonized at one week and one month had become colonized with *Bacteroides fragilis* at age two months, however. Thus, among the 21 infants colonized with *Bacteroides fragilis* at two months 10 produced IFNγ spontaneously at twelve months compared to no spontaneous IFNγ-producers among the 14 un-colonized infants (p=0.019). There was no difference in IFNγ production after PHA stimuli between the colonized and un-colonized infants.

DISCUSSION

The *Bifidobacterium* flora during the first two months after birth was associated with enhanced production of salivary SIgA at six and twelve months. The SIgA levels at six months were mainly associated with the diversity of the *Bifidobacterium* flora and colonization with *B. adolescentis,* whereas the colonization with *B. bifidum* was associated with the SIgA levels at twelve months. The present study, in which bifidobacteria were identified at 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 colonization shortly after birth might be of particular importance for maturation of immune responses, as others have reported that colonization with bifidobacteria at six months did not associate with salivary SIgA levels at that age (20). Early colonization with a strain of *E. coli* can induce faecal antigen-specific SIgA levels already 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 two and five years, other factors are probably more important for later induction of salivary SIgA responses. Recurrent respiratory infections 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 (Sandin *et al* unpublished). Whether the gut microbiota and recurrent infections account for early and late SIgA formation respectively, needs to be further studied. Early *Bacteroides fragilis* colonization associates with number of IgA producing cells (18) however 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 IgA_2 class switching (34). Furthermore, IgA production is profoundly reduced in germ-free mice (13). Also, some studies indicate that GALT IgA-producing B cells can migrate to other part 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 saliva. Alternatively, the studied gut microbiota species are epiphenomena correlating with unknown factors that the infants are exposed to, *e.g.* in the oral mucosa, and this is what effects 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 early *Bifidobacterium* flora could prevent allergy development partially by increasing salivary SIgA. Studies from our group also indicate that salivary SIgA levels, already at age three, six and twelve months, are lower among two year-old sensitized infants with allergic symptoms compared to asymptomatic sensitized infants ((25), Tomicic *et al* unpublished). 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 two months of life (3). However, we could not conclusively show that development of allergy up to five 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 six months (36) and *B. adolescentis* colonization could be more

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common in allergic compared to 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 to allergic infants (38).

The colonization with lactobacilli group I (*L. rhamnosus, L. casei* and *L. paracesei*) at one month was also associated with higher levels of SIgA in saliva at six months and five 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), Tomicic unpublished).

The TLR4 mRNA expression was lower in infants colonized with high relative amounts of the G- bacterium *Bacteroides fragilis* at one week and one month after birth. Furthermore, LPS-induced CCL4 (MIP1β), 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 be an additive effect to the regulation of the inflammatory cytokines. Spleen cells from ex-germfree mice colonized with *Bacteroides fragilis/*PSA produce more IFNγ after αCD3/αCD28 stimulation than spleen cells from GF mice (14). In a very different system, we showed that infants colonized with *Bacteroides fragilis* during their first two months more often produced IFNγ spontaneously than un-colonized infants. However, early colonization with *Bacteroides fragilis* was not associated with higher PHA-induced IFNγ production. As spleen cells from conventional and ex-GF mice colonized with *Bacteroides fragilis* produce similar levels of IFNγ after αCD3/αCD28 stimuli (14), the other gut microbiota species of infants may influence their PHA-induced IFNγ 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 play a minor role in the development of allergy.

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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.

ACKNOWLEDGMENTS

Table 1. Demographic data of the subjects.

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

[a] = missing data for one subject regarding exclusive breastfeeding.
[b] = missing data for three subjects regarding number of family members and thus household area/individual.

Table 2. **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.**

Bacteria	1 week n=52	1 month n=56	2 months n=56
B. longum	88	91	91
B. adolescentis	71	66	75
B. bifidum	56	63	63
B. breve	40	43	50
Lactobacilli group I	31	57	57
Lactobacilli group II	33	43	54
C. difficile	6	11	21
Bacteroides fragilis	60	55	55

Figure legends:

Fig. 1. Total salivary secretory IgA (SIgA) at six months is associated with a more diverse *Bifidobacterium (B.)* flora. The y-axis shows total salivary secretory IgA (SIgA) (µg/ml) at six months and the x-axis shows the number of *Bifidobacterium* species at one month.

Fig. 2. Colonization with bifidobacteria, lactobacilli and *Clostridium difficile* at one month of age in relation to salivary secretory IgA (SIgA) at age six months (a), twelve months (b), two years (c) and five years (d). The positive sign denote colonized infants and the negative sign those lacking the bacteria. The numbers in brackets show the number of infants from whom both faecal and salivary samples were available. The short lines demonstrate the median values. Colonization with *Bacteroides fragilis* and *B. longum* did not influence the SIgA production and these bacteria are therefore not shown.

Fig. 3. Total salivary secretory IgA (SIgA) at twelve months is associated with the intensity of *B. bifidum* colonization. The y-axis shows total salivary secretory IgA (SIgA) (µg/ml) at twelve months and the x-axis shows intensity of *B. bifidum* colonization (expressed as percent *B. bifidum* DNA of total amount nucleic acids at one month. The dotted line shows the detection limit at $5*10^{-6}$ % *B. bifidum* DNA of total amount nucleic acids. n.d. = not detectable.

Fig. 4. The spontaneous expression of TLR4 mRNA at twelve months is inversely correlated to the intensity of *Bacteroides fragilis* colonization at one month after birth. The y-axis shows the relative TLR4 mRNA expression/rRNA in PBMCs collected twelve months after birth and the x-axis shows the relative amount of *Bacteroides*

fragilis at one month. The dotted line shows the detection limit at $5*10^{-6}$ % *Bacteroides fragilis* DNA of total amount nucleic acids. n.d. = not detectable.

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

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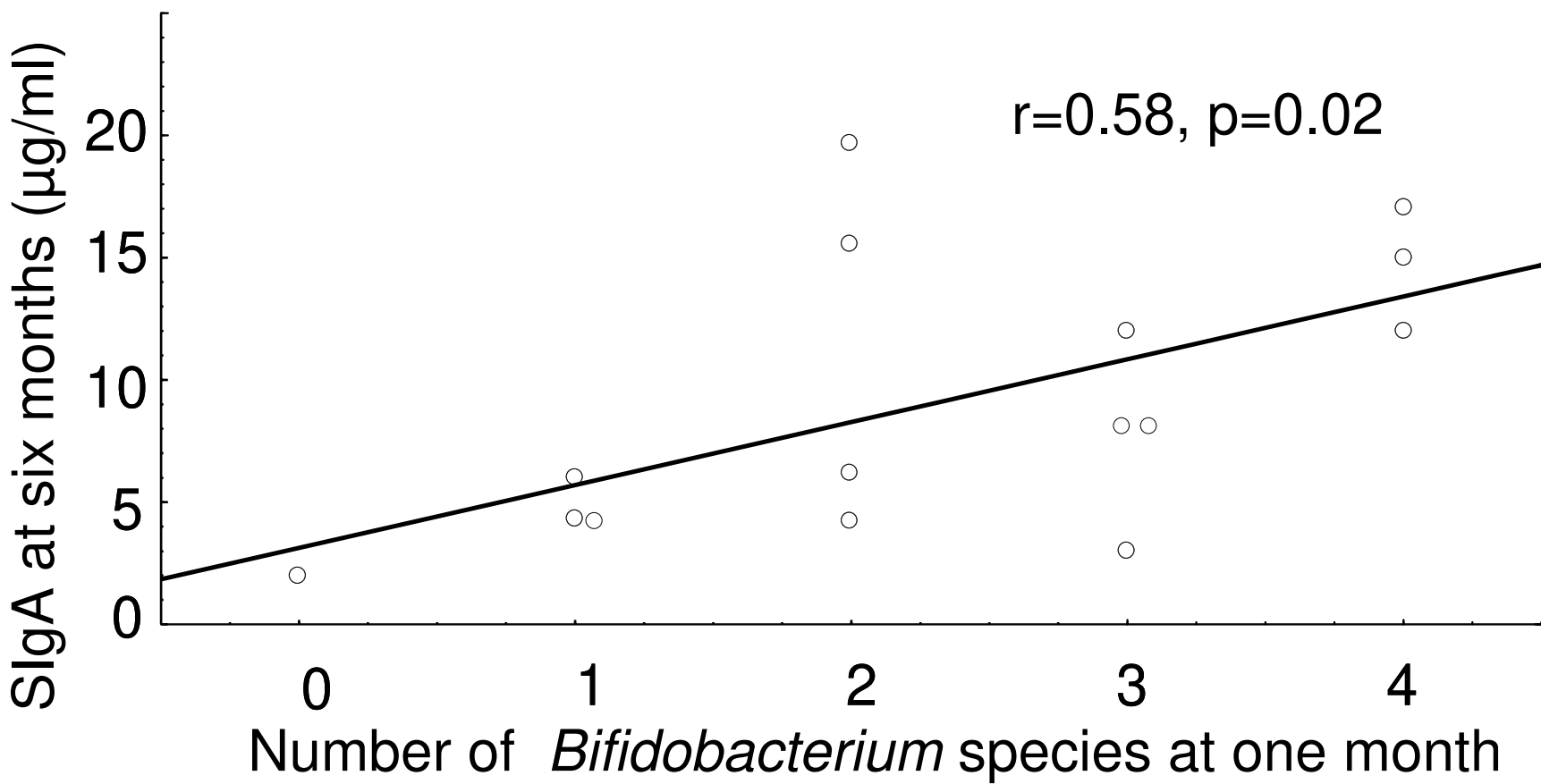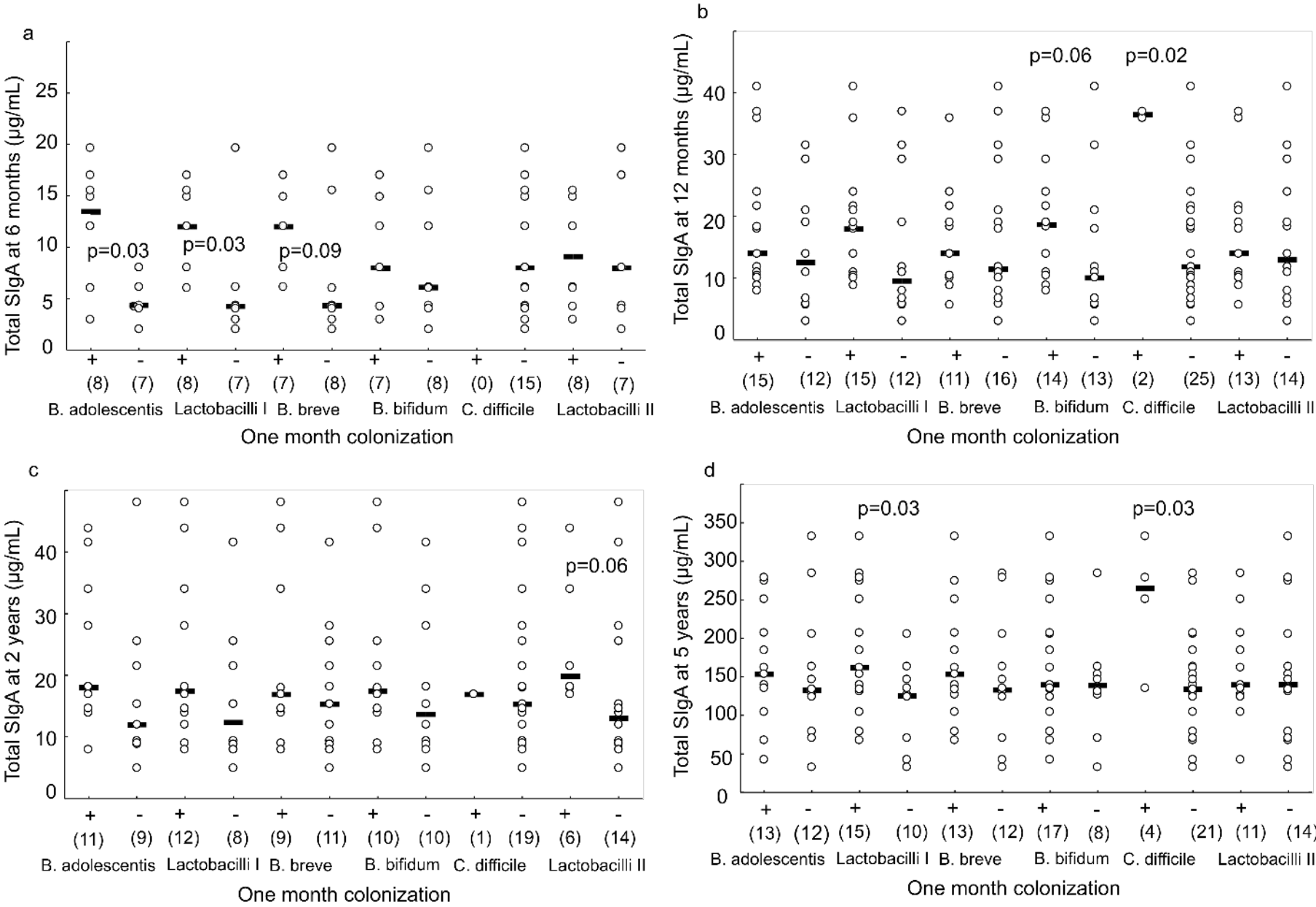(41) Zhong B, Ma HY, Yang Q, Gu FR, Yin GQ, Xia CM. Decrease in toll-like receptors 2 and 4 in the spleen of mouse with endotoxic tolerance. Inflamm.Res. 2008;57:252-259.
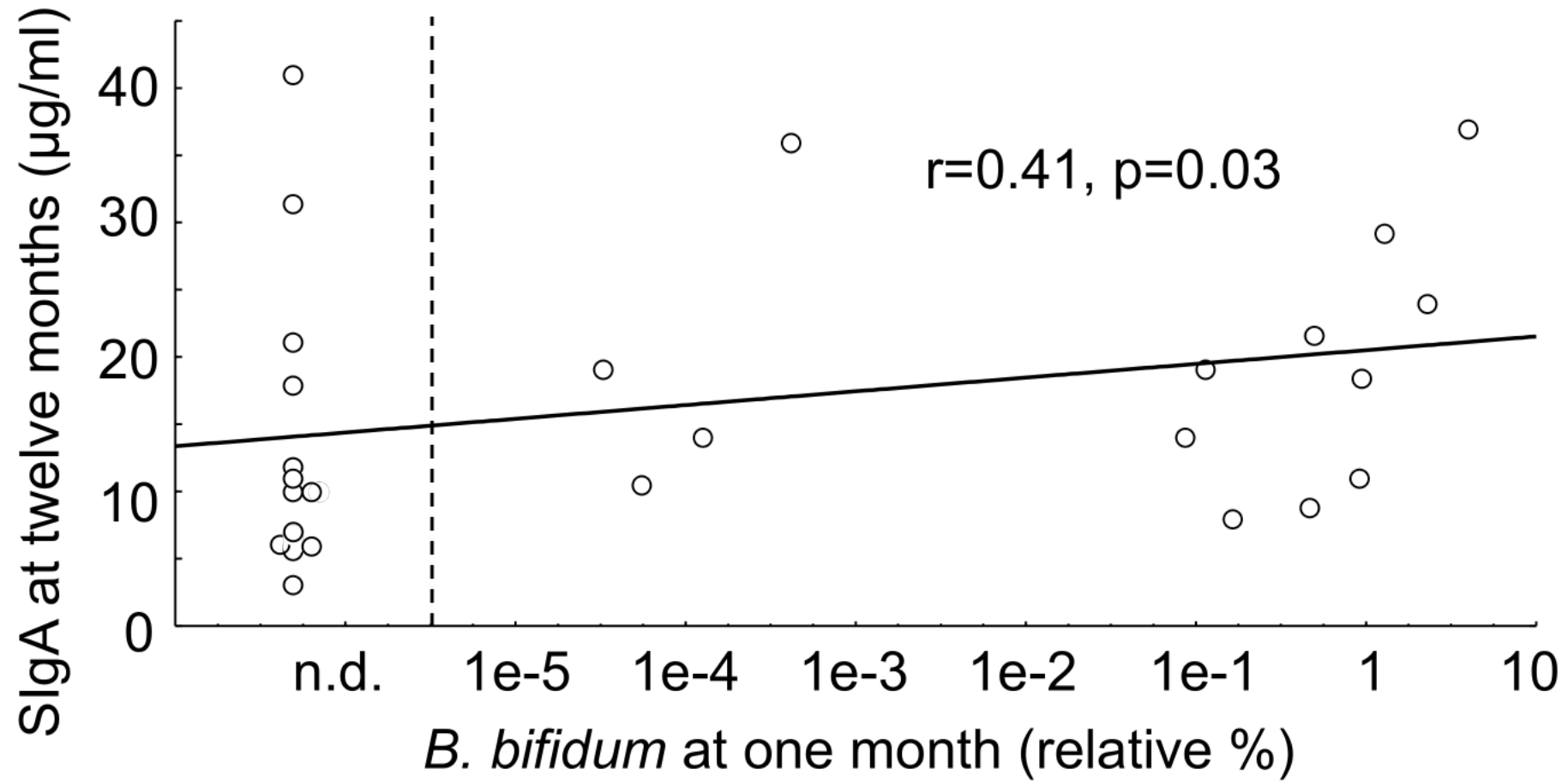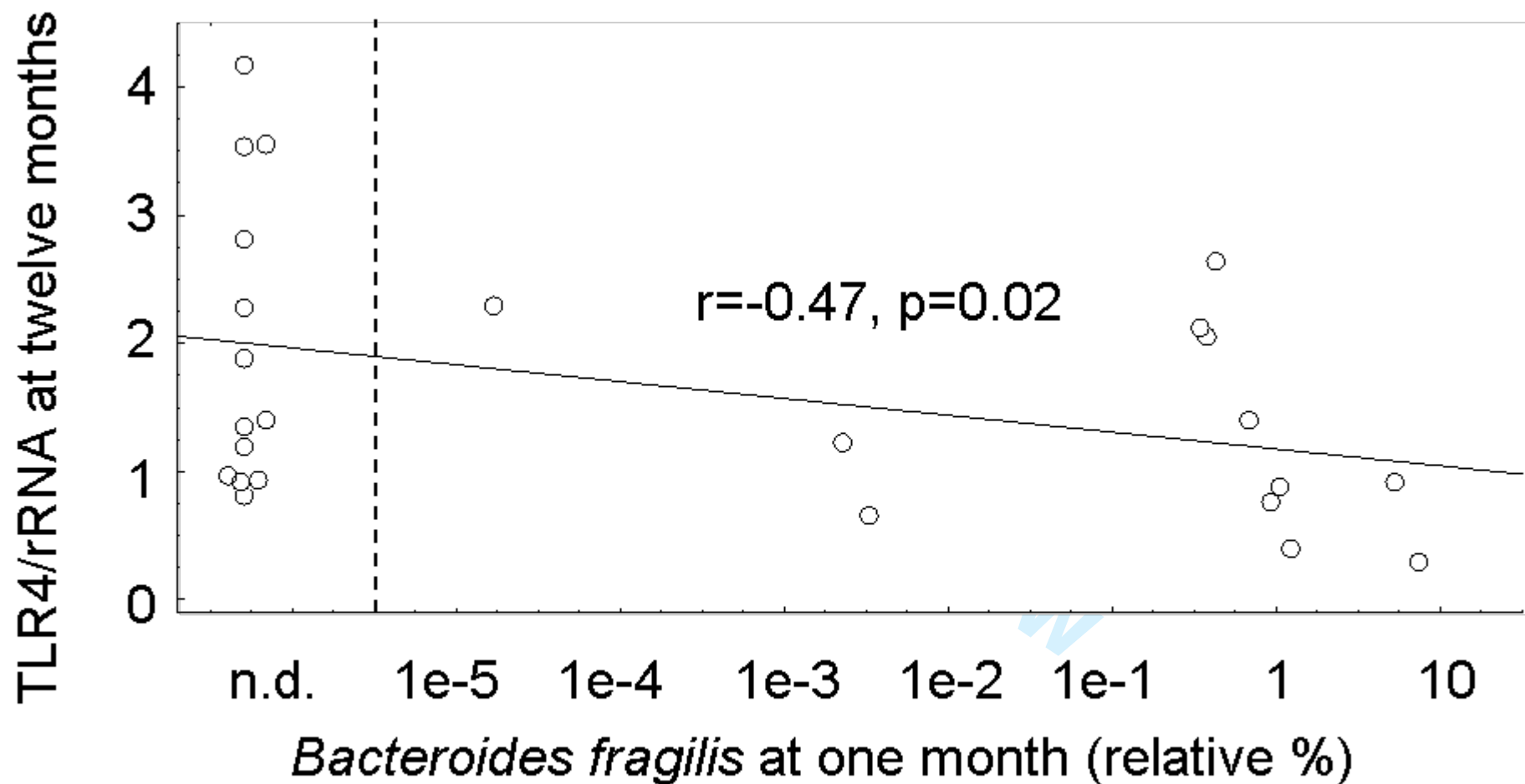
Figure 1

Figure 2

Figure 3

Figure 4

a

CCL4 (pg/mL) vs *Bacteroides fragilis* at one month (relative %)

r=-0.75, p=0.005

b

IL-6 (pg/mL) vs *Bacteroides fragilis* at one month (relative %)

r=-0.79, p=0.002

Figure 5