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Decreased gut microbiota diversity, delayed Bacteroidetes colonization, and reduced Th1 responses in infants delivered by Caesarean section

Running title: Development of the intestinal microbiota

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Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal RNA; VD, vaginal delivered; CS, caesarian section.

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ABSTRACT

Objective: The early intestinal microbiota exerts important stimuli for immune development, and a reduced microbial exposure as well as caesarean section has been associated with the development of allergic disease. Here we address how microbiota development in infants is affected by mode of delivery, and relate differences in colonization patterns to the maturation of a balanced Th1/Th2 immune response.

Design: The postnatal intestinal colonization pattern was investigated in 24 infants, born vaginally (15;VD) or by caesarean section (9;CS). The intestinal microbiota were characterized using pyrosequencing of 16S rRNA genes at one week, and one, three, six, twelve, and 24 months after birth. Venous blood levels of Th1- and Th2-associated chemokines were measured at six, twelve and 24 months.

Results: Infants born through caesarean section had lower total microbiota diversity during the first two years of life. CS delivered infants also had a lower abundance and diversity of the Bacteroidetes phylum and were less often colonized with the Bacteroidetes phylum. Infants born through caesarean section had significantly lower levels of the Th1-associated chemokines CXCL10 and CXCL11 in blood.

Conclusion: Caesarean section was associated with a lower total microbial diversity, delayed colonization of the Bacteroidetes phylum and reduced Th1 responses during the first two years of life.

Summary box:

What is already known about this subject:

- The infant gut microbiota diversity increases during the first years of life.
- The microbiota composition differs between infants born by caesarean section or vaginal delivery with a delayed colonization of the genus *Bacteroides*.
- Bacterial colonization is necessary for the development of the immune system and immune regulation.
- An association between CS delivery and the development of allergic disease has been observed in several studies.

What are the new findings:

- The total microbiota diversity is lower in CS than VD infants through the first two years of life.
- The diversity of the Bacteroidetes phylum is lower in CS born infants during the first two years of life.
- Vaginal delivery is associated with increased circulating levels of Th1-associated chemokines during infancy.

How might it impact on clinical practice in the foreseeable future?

- Deeper knowledge of the impact of delivery mode on microbiota composition and immune regulation may lead to improved allergy preventive strategies.

Introduction

The gastrointestinal tract of the newborn infant is considered to be sterile. Bacteria from the environment, mainly from the mother, colonize the infant gut immediately following birth. Dominant members of anaerobic Firmicutes and Bacteroidetes do not appear to grow outside the gut and hence need to be transmitted between human hosts [1]. To what extent the transmission occurs from mother to offspring is not clear, but differences in microbiota composition depending on delivery mode indicate a mother-child transmission during vaginal delivery. A recent study based on pyrosequencing of 16S rRNA genes demonstrated that the microbiota of vaginally delivered (VD) neonates (<24 hours post delivery) resembled the vaginal microbiota of their own mother and was similar across multiple body habitats (skin, oral, nasopharynx and feces), while in neonates born by caesarean section (CS), it resembled the mother's skin microbiota [2]. While this study provided evidence that microbiota from the birth channel is transferred from mother to child, providing an inoculum for the initial microbiota, it remains to be shown that specific gut microbes are successfully transmitted during vaginal delivery.

The incidence of caesarean delivery has increased from 5% in the 1970s to more than 60% in some hospitals in China according to recent reports [3]. The early colonization pattern differs between vaginally delivered infants and those delivered by caesarean section, including a delayed colonization of e.g. *Bacteroides* and *Bifidobacterium* spp. in CS infants [4, 5]. The influence on delivery mode on gut microbiota development has not previously been longitudinally characterized using powerful cultivation-independent microbiologic methods, however. The intestinal microbiota is important for the development of the immune system and immunological tolerance [6]. Differences in the postnatal microbial colonization may explain the higher incidence of immune mediated diseases such as allergy in children born by

CS as compared to those born vaginally [7, 8]. Indeed, allergic disease has been associated with low prevalence of *Bacteroides* and *Bifidobacterium* [9, 10], and a low intestinal microbiota biodiversity in early infancy appears to have an impact on the development of allergic disease later in life [11, 12, 13]. A failure of Th2-silencing during maturation of the immune system may underlie development of Th2-mediated allergic disease [14]. Appropriate microbial stimulation may be required to avoid this pathophysiological process, as early differences in the gut microbiota may shape later immune development [6, 15]. The influence of CS on immune development is largely unknown, however [16]. The aim of the present study was to monitor the development of the infant intestinal microbiota in babies born vaginally and through caesarian section, and to relate the findings to the maternal microbiota and to Th1- and Th2-associated chemokine levels during infancy.

MATERIALS AND METHODS

Ethics

The human ethic committee at Linköping University, Linköping, Sweden, approved the study. Informed consent was obtained from both parents before inclusion.

Subjects and sample collection

The study group comprised 24 healthy women and their infants. Nine of the infants were born by caesarean delivery (CS) and 15 by vaginal delivery (VD) (c.f. Supplementary Table 1 regarding, sex, delivery, birth weight, the use of antimicrobials, and length of breast-feeding for the different infants). Seven out of nine mothers, who gave birth through CS were given antibiotics prophylactically during the surgery (Supplementary Table 2). This was done after the delivery, however, and thus the infants were not exposed to antibiotics via the placenta.

No children were treated with antibiotics during the neonatal period. Twenty of the infants (83%) were partly breast fed until at least six months of age. The women and children included in this study were part of a larger study assessing the prevention of allergic disease by probiotics [17] (ClinicalTrials.gov ID NCT01285830) and they all received placebo. Stool samples were collected from the mothers one week after delivery and from the children at one week, one, three, six, twelve, and 24 months. The fecal samples were immediately frozen at -20°C following collections and later stored at -70°C. Samples were collected in 2002-2005 and stored in -70°C until DNA extractions were conducted in 2009. No systematic differences in storage times existed between the VD and CS samples (Mean months: VD: 80±7, CS: 78±4, Student's t-test, P = 0.42).

DNA extraction and 16S rRNA gene amplification

The DNA extraction and 16S rRNA gene amplification were performed as described previously [18] with the following modifications; the primer pair used, targeting the variable regions 3 and 4 of the 16S rRNA gene, was 341f 5´CCTACGGGNGGCWGCAG with adaptor B and 805r 5´GACTACHVGGGTATCTAATCC with adaptor A and a sample-specific barcode sequence consisting of five nucleotides [19]. The barcodes contained no homopolymers and a pair of barcodes differed in at least two positions. The 341f-805r primer pair was shown to be the least biased among 512 primer pairs evaluated *in silico* for bacterial amplification and was experimentally shown to give a taxonomic composition similar to shotgun metagenomics [20]. The primer pair has good coverage of bacterial groups typically found in the human lower intestine. For phylum Bacteroidetes, 121,862 of 132,120, for phylum Firmicutes 376,912 of 406,649, for phylum Proteobacteria 336,471 of 368,375 and for family Bifidobacteriaceae 1112 of the 1239 sequences are matched, when considering Ribosomal Database Project (RDP) sequences that span the region. A negative PCR reaction

without template was also included for all primer pairs in each run. The PCR-products with approximate lengths of 450 bp were purified with AMPure beads (Becton Dickinson, Franklin Lakes, NJ, USA) using a Magnet Particle Separator (Invitrogen, Carlsbad, Calif.). The concentrations of the purified products were measured by Qubit fluorometer (Invitrogen, CA), the quality was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), and the samples were amplified in PCR-mixture-in-oil emulsions and sequenced from the 805r primer on different lanes of a 2-lane PicoTiterPlate on a Genome Sequencer FLX system (Roche, Basel, Switzerland) at the Swedish Institute for Infectious Disease Control (Solna, Sweden). Sequence processing was carried out with the AmpliconNoise software package [21] correcting for errors introduced in the PCR and pyrosequencing as well as removing chimeric sequences. Also, reads lacking a correct primer and/or having less than 360 successful pyrosequencing flows were excluded [21]. Denoised sequences were trimmed to 198 bp after primer and barcode removal and clustered by complete linkage clustering into Operational Taxonomic Units (OTUs) at the 97% similarity level using AmpliconNoise.

Taxonomic classification

Each denoised sequence, as well as the most abundant sequence for each OTU, was BLAST searched with default parameters against a local BLAST database comprising 836,814 near full-length bacterial 16S rRNA gene sequences from the Ribosomal Database Project (RDP) v. 10.10 [22]. The sequences inherited the taxonomic annotation (down to genus level) of the best scoring RDP hit fulfilling the criteria of ≥ 95% identity over an alignment of length ≥ 180 bp. If no such hit was found the sequence was classified as “no match”. If multiple best hits (of same score) were found, the taxonomy was set to the most-detailed level of taxonomy shared by the best hits. The majority of reads had an RDP relative within 95% sequence similarity and were hence of bacterial origin. After removal of pyrosequencing noise and

chimeric sequences using the AmpliconNoise package [21], 357,685 high quality, typically 198 bp long, sequence reads remained, with 828 to 4395 reads per sample (mean = 2129). These corresponded to 3048 unique sequences and 1818 OTUs, clustered at 97% similarity level using complete linkage clustering.

Sample clustering

The online version of Fast Unifrac (http://bmf2.colorado.edu/fastunifrac/) [23] was used to calculate weighted sample distances by mapping our OTU sequences with BLAST onto the Greengenes reference sequences (downloaded from the Fast Unifrac web page, May 2009) and using the corresponding Greengenes tree. A Principal Coordinates Analysis (PCoA) plot based on all pair-wise sample distance was created on the Fast Unifrac web page. Our OTU sequences were mapped onto 154 Greengenes sequences.

Statistical testing for over or under-representation of bacterial lineages

Statistical tests of over or under-representation of bacterial lineages among sample groups were made at the phylum and genus levels using Wilcoxon rank-sum test. To correct for multiple testing, the P-values were converted to False Discovery Rate values (Q-values).

Diversity estimations

Shannon diversity index was calculated as $H = -\Sigma \log(p_i)p_i$, where p_i denotes the relative frequency of OTU i [24], Pielou's eveness index as $-\Sigma \log(p_i)p_i / \log(S_{obs})$, where S_{obs} denotes the number of observed OTUs in the sample [25], and Chao1 richness estimate as $S_{obs}+n_1(n_1-1)/n_2(n_2-1)$, where n_1 and n_2 are the number of observed singleton and doubleton OTUs, respectively [26]. Since these parameters are influenced by sequencing depth and the sequencing depth differed between samples, we subsampled (with replacement) 1400 reads

from each sample, counted the occurrences of the corresponding OTUs, and performed the diversity calculations on these counts. This was repeated ten times and averages of the diversity parameters calculated and used for further analysis. Four (out of 168) samples had fewer than 1400 reads and were excluded from this part of the analysis (three VD infants; one at one week, one at three months and one at twelve months, and one CS infant at one month). Diversity calculations and statistics were done with the *R* software (http://www.r-project.org/) and the *R* package vegan (http://cran.r-project.org/web/packages/vegan/). Repeated measures ANOVA were employed, using the Shannon diversity index at the different time points (one week, one, three, six, twelve and 24 months) as the repeated measures.

Statistical testing of mother-child overlap in sequence types

For each time point and for each infant we calculated the number of specific sequences (sequence types) shared with its mother/number of sequence types observed in the infant (R_{own}). Likewise we calculated the average number of sequence types shared with other mothers/number of sequence types observed in the infant (R_{other}). We then compared the R_{own} and R_{other} values pairwise for all infants within each group (VD or CS) with the Wilcoxon signed rank test.

Chemokine analyses in venous blood and association with mode of delivery

Venous blood was collected at six (n=24), twelve (n=24) and 24 months (n=24) and stored as plasma or serum in -20°C pending analysis. The Th1-associated chemokines CXCL10, and CXCL11 and the Th2-associated chemokines CCL17 and CCL22 were analyzed with an in-house multiplexed Luminex assay [27, 28]. The limit of detection was 6 pg/ml for CXCL10 and CXCL11 and 2 pg/ml for CCL17 and for CCL22. All samples were analysed in duplicates and the sample was re-analysed if the coefficient of variance (CV) was >15%. The

chemokine levels were compared between infants being born vaginally or by caesarean section by repeated measures ANOVA using log transformed chemokine levels at the different time points (six, twelve and 24 months) as the repeated measures.

RESULTS

Microbiota succession in infants

At the phylum level, the microbiota developed in a similar fashion in infants delivered vaginally and by CS, with a gradual decline in Proteobacteria from one week to 24 months, a peak of Actinobacteria at three months, an expansion of Firmicutes from three months and onward, and the emergence of Verrucomicrobia at around six months of age (Figure 1, Supplementary Table 3). However, a notable difference between the VD and CS infants was the higher proportion of Bacteroidetes in VD infants during the first twelve months (significantly higher at one week, three months and twelve months; Supplementary Table 3).

The maternal microbiota resembled the typical adult flora as demonstrated in several previous studies [18, 29, 30] and was independent of delivery mode. The Firmicutes was the dominating phylum, representing 74% and 71% in mean relative abundance for the 15 VD and nine CS mothers respectively, followed by Bacteroidetes (16% and 13% respectively), Actinobacteria (7% and 12% respectively), Proteobacteria (3% and 2% respectively) and Verrucomicrobia (1% and 2% respectively) (Figure 1, Supplementary Table 3).

The relative abundance of the major genera found in the infants and mothers are illustrated in Figure 2A-B (see also Supplementary Table 4). Infants in the VD group were colonized by *Bacteroides* to a greater extent than in the CS group (significantly more at one week [11/15 in

VD vs. 1/9 in CS; Fischer's exact test $P = 0.005$], three months [11/15 vs. 1/9; $P = 0.005$] and twelve months [14/15 vs. 4/9; $P = 0.015$]). At one month of age *Bifidobacterium* dominated the microbiota in both groups. The *Enterococcus* genus was found in significantly higher relative abundance in the CS compared to the VD infants at one month ($P < 0.0001$; Supplementary Table 4). Following six months of age there was a gradual increase in previous low abundant genera in both VD and CS infants. At 24 months of age, *Bacteroides* and several genera belonging to the Clostridia class, for example *Ruminococcus*, a dominant member of the adult microbiota also dominated the infant microbiota.

The gradual shift in community composition was accompanied by an increase in α-diversity over time, with a significant increase in Shannon diversity index between each pair of succeeding time points from three months of age and onward (Figure 3). Similar results were obtained for evenness (Pielou's index; Supplementary figure 1A) and estimated richness (Chao1; Supplementary figure 1B). The low increase in diversity during the first three months may be related to that most (83%) infants were exclusively breast fed up to this age (Supplementary Table 1). CS delivery was associated with significantly lower total microbiota diversity when considering all time points in the infants ($P = 0.047$ with repeated measures ANOVA; Supplementary table 5). At individual time points, the total microbiota diversity was significantly lower in the CS delivered infants at twelve months (Supplementary Table 5; Figure 3). The total microbiota diversity did not differ significantly between the VD and CS mothers (Supplementary Table 6). Narrowing the analysis to the phylum level, CS delivery was associated with a lower diversity of the Bacteroidetes phylum when considering all time points ($P = 0.002$; Supplementary table 5). For individual time points the Bacteroidetes diversity was significantly lower in the CS infants than VD infants at one, three, twelve and 24 months (Figure 4; Supplementary Table 6). The other phyla,

Firmicutes, Proteobacteria, and Actinobacteria did not display any consistent differences in Shannon diversity between the groups, although diversity of Firmicutes was significantly lower at twelve months and Proteobacteria at 24 months in the CS infants (Supplementary Table 6).

A Principal Coordinates Analysis (PCoA) plot based on pair-wise sample community differences calculated with UniFrac [23] is illustrated in Figure 5. As shown in a previous study [31] the microbial communities became more uniform across infants over time, which is also evident when comparing the distributions of pair-wise community differences at each time point (Supplementary figure 2). By 24 months of age, the communities closely resembled those of the mothers (Figure 5). Although community composition converged to an adult-type microbiota, diversity estimates were still significantly lower at 24 months than in the mothers (Figure 3; Supplementary figure 1). Birth weight, antibiotic intake during the time course, sex, and breast-feeding had no apparent impact on the microbiota composition at any time point (Supplementary figures 3-6).

In order to investigate possible mother-to-child transmission of bacteria, the presence of specific unique sequences (sequence types) was compared between infants and their mothers, as well as between infants and other mothers. For each mother-child pair, we calculated the fraction of the sequence types found in the child that were also found in the mother (number of shared sequence types/number of child sequence types). The VD infants shared a significantly higher proportion of sequence types with their own mother than with the other mothers at one week and 24 months when considering all bacterial taxa (Supplementary Table 7). Considering one phylum at a time, Bacteroidetes displayed a significantly higher

overlap at one and six months, and Firmicutes at six and 24 months. No significant overlap was observed for the CS delivered infants at any time point.

Caesarean section was associated with moderately lower levels of the Th1-associated chemokines CXCL10 and CXCL11 (Table 1). The levels of the Th2-chemokines CCL17 and CCL22 did not differ significantly between the birth modes.

Table 1. Repeated measures ANOVAs to test whether there were any significant differences in CXCL10 and CXCL11 levels during the first two years of life between caesarean (CS) and vaginally (VD) delivered infants. The ANOVAs where calculated on log chemokine levels. n = number of infants.

		CXCL10 (mean, pg/ml)							CXCL11 (mean, pg/ml)						
Birth mode		6 m	n	12 m	n	24 m	n	p*	6 m**	n	12 m	n	24 m	n	p*
VD	n=14	97	9	116	13	112	13	0.05	529	9	500	13	527	13	0.008
CS	n=7	37	4	166	3	71	4		49	4	347	3	518	4	

*= Repeated measures ANOVA including all time points (six, twelve, and 24 months). Because the non-normal distribution, the values were log transformed before. One VD infant and two CS infants where not measured at any time point and were hence excluded from the analysis. For the remaining subjects, when a sample was missing at a specific age, the value corresponding to the median value for the specific chemokine at that age group was given before repeated measures ANOVA was performed.
**= $p<0.001$ with student t-test after log transformation at that specific time point.

DISCUSSION

Microbial colonization of the infant gut gastrointestinal tract is important for the postnatal development of the immune system. In this study, caesarean section delivered infants who are not entering the birth canal of the mother, either lacked or displayed a delayed colonization of one of the major gut phylum, the Bacteroidetes. The colonization of this phylum was delayed by up to one year for some infants. The total microbiota diversity was also lower in the CS infants, probably largely as a consequence of the lack of this phylum. This was not a consequence of antimicrobial treatment, as none of the CS mothers were given antibiotics before surgery and the microbial diversity did not differ between mothers who were given antibiotics prophylactically and those who did not receive antibiotics. Comparisons of

intestinal microbiota have not conclusively confirmed bacterial transmission [31, 32], although the genus *Bacteroides* has been proposed to be transmitted from the maternal gut [33, 34]. Our study corroborates earlier studies reporting a delayed colonization of *Bacteroides* in babies delivered by CS [4, 5]. In addition our study provides evidence that specific lineages of the intestinal microbiota, as defined by 16S rRNA gene sequences, are transmitted from mother to child during vaginal delivery.

It is important to note that bacterial composition changes as a consequence of freezing the fecal samples [35, 36] and that PCR amplification can induce taxon-specific biases. However, there were no significant differences in storage times between the VD and CS samples and since the same primer pair and PCR conditions were used for all samples, these effects should not contribute to the observed differences in microbiota composition and alpha-diversity between the sample groups.

The genus *Enterococcus*, which is a typical fecal bacterium, is usually acquired during the first week of life [37]. Colonization has previously not been shown to depend on delivery mode, suggesting other sources in addition to the maternal intestinal microbiota [5], such as the environment [38] and breast milk [39]. We found that CS infants had a higher relative abundance of *Enterococcus* at one month of age, suggesting that the lack of bacteria transmitted through vaginal delivery favors the growth and colonization of enterococci.

Appropriate microbial stimulation during infancy is required for the development of a more balanced immune phenotype, including maturation of Th1-like responses and appropriate development of regulatory T cell responses [6, 40, 41]. It is well known that early life events occurring during critical windows of immune development can have long-term impact on

immune-mediated diseases such as allergy [15, 42, 43], diabetes, and inflammatory bowel disease. We hypothesized that early differences in the gut microbiota could shape later immune responsiveness, influencing the Th1 maturation trajectory. Our findings of a lower microbial diversity in the CS infants, and lower circulating levels of the Th1-related chemokines CXCL10 and CXCL11, support this view. Previous studies have shown that *Bacteroides fragilis* exert strong effects on the immune system. This is mediated by the capsular polysaccharide (PSA), which enhances T-cell mediated immune responses and affects the Th1/Th2 balance [44, 45]. Furthermore, *B. thetaiotamicron* is also known to affect the immune system [46]. Thus, the lower abundance of *Bacteroides* among the CS infants may be a contributing factor to the observed differences in Th1-associated chemokines. Future studies with larger sample sizes will be able to address the effects of individual microbes on chemokine levels.

With few exceptions [31], previous studies have reported *Bifidobacterium* to be one of the dominant genera of the early infant intestinal microbiota [4, 33, 47, 48, 49], and changes in relative abundance of this genus have been related to delivery mode [4, 16, 34]. Also in the present study, *Bifidobacterium* was the dominant genus from one to twelve months of age with a gradual decline following weaning. The abundance was not affected by delivery mode, however, and we could not detect any significant overlap in the mothers' and babies' rRNA sequences. Hence, *Bifidobacterium* could primarily be transmitted from the breast milk, and to a lesser extent from the intestinal micobiota, as suggested but not confirmed previously [50].

In accordance with recent studies [31, 47, 48], our results demonstrate considerable individual differences in the microbial succession during the first year of life. This is

probably a result of differences in time of weaning, and incidental exposure of bacteria from the environment. Community composition converges to an adult-like state within two years. However, even at two years the microbiota appears not to be fully developed, since the diversity was significantly lower than in mothers. This was also evident in a recent study reporting a lower microbial diversity in a 2.5 years old child than in its mother [51].

An association between CS delivery and the development of allergic disease has been observed in several studies [52, 53] and a lower microbial diversity has been observed in allergic infants before onset of disease [13]. We conclude that CS is associated with a lower bacterial diversity during the first two years of life, a lower abundance and diversity of the phylum Bacteroidetes, and lower circulating levels of Th1-associated chemokines during infancy.

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COMPETING INTERESTS

None.

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Figure legends

Figure 1. Phylum level microbiota composition in mothers and their infants and at one week, one, three, six, twelve, and 24 months. The mean relative abundances (%) of the most abundant bacterial phyla in the 15 VD infants (A) and nine CS infants (B), as well as in their mothers are shown.

Figure 2. Genus level microbiota composition in mothers and their infants and at one week, one, three, six, twelve, and 24 months. The mean relative abundances (%) of the most abundant bacterial genera in the 15 VD infants (A) and nine CS infants (B), as well as in their mothers are shown. Only genera comprising ≥ 1% of the total community were included. Abbreviations: Pr, Proteobacteria; Fi, Firmicutes; Ba, Bacteroides; Ac, Actinobacteria.

Figure 3. Increase in fecal microbiota alpha-diversity over time. Distributions of Shannon diversity indices displayed for the 15 VD infants and 9 CS infants at one week, one, three, six, twelve, and 24 months, and for their mothers. Fifty percent of the data points reside within boxes, 75% within whiskers, and median values are indicated by horizontal lines within boxes (circles indicate individual values). Wilcoxon signed rank tests were conducted to compare Shannon diversity between adjacent time points, and Wilcoxon rank-sum tests to compare diversity between delivery modes within time points; *** indicates $P < 0.001$, ** indicates $P < 0.01$ and * indicates $P < 0.05$.

Figure 4. Increase in Bacteroidetes alpha-diversity over time. Distributions of Shannon diversity indices displayed for the 15 VD infants and 9 CS infants at one week, one, three, six, twelve, and 24 months, and for their mothers. *** indicates $P < 0.001$, ** indicates $P < 0.01$ and * indicates $P < 0.05$. Fifty percent of the data points reside within boxes, 75% within whiskers, and median values are indicated by horizontal lines within boxes (circles indicate individual values).

Figure 5. Individuality and convergence of infant microbiota. Principal Co-ordinates Analysis (PCoA) was performed on all pair-wise community differences (calculated with UniFrac (Hamady M, 2009)), and samples from infants at one week, one, three, six, twelve, and 24 months, and from the mothers are highlighted in the different boxes. VD and CS infants/mothers are displayed in red and blue, respectively.

Figure 1.

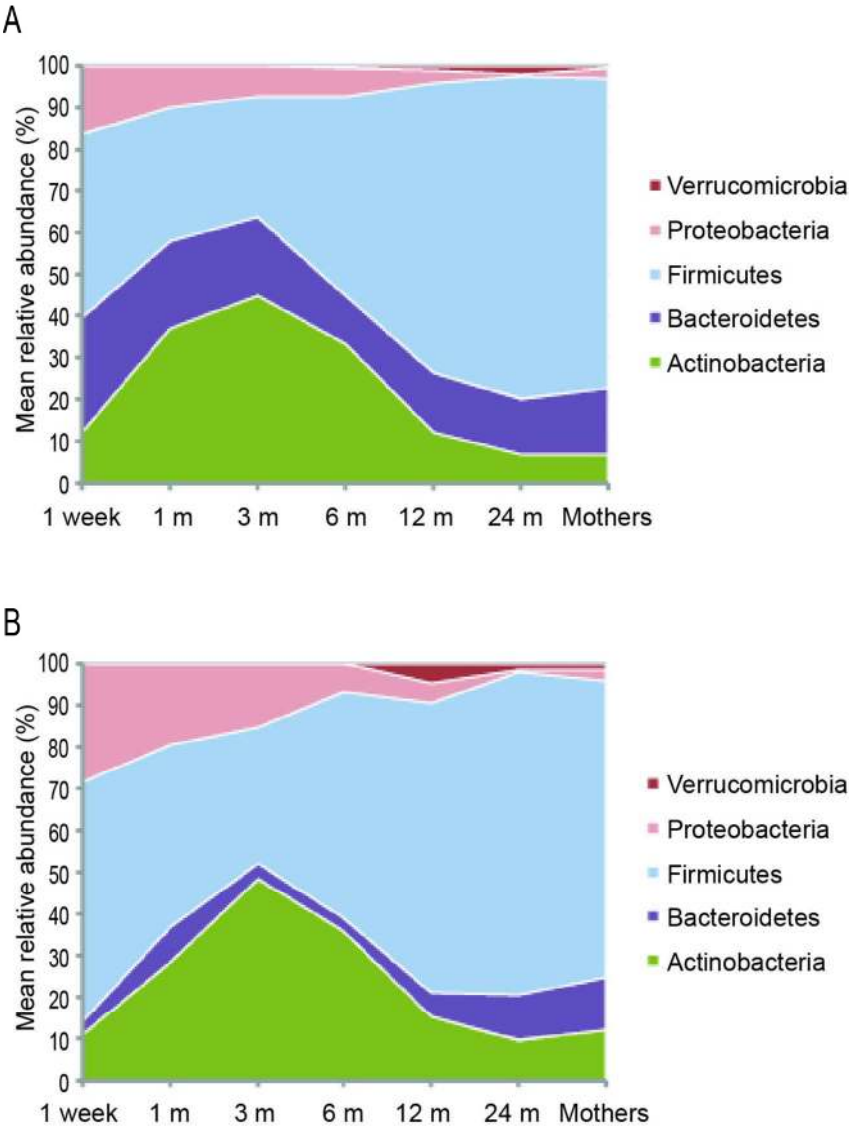

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Figure 2.

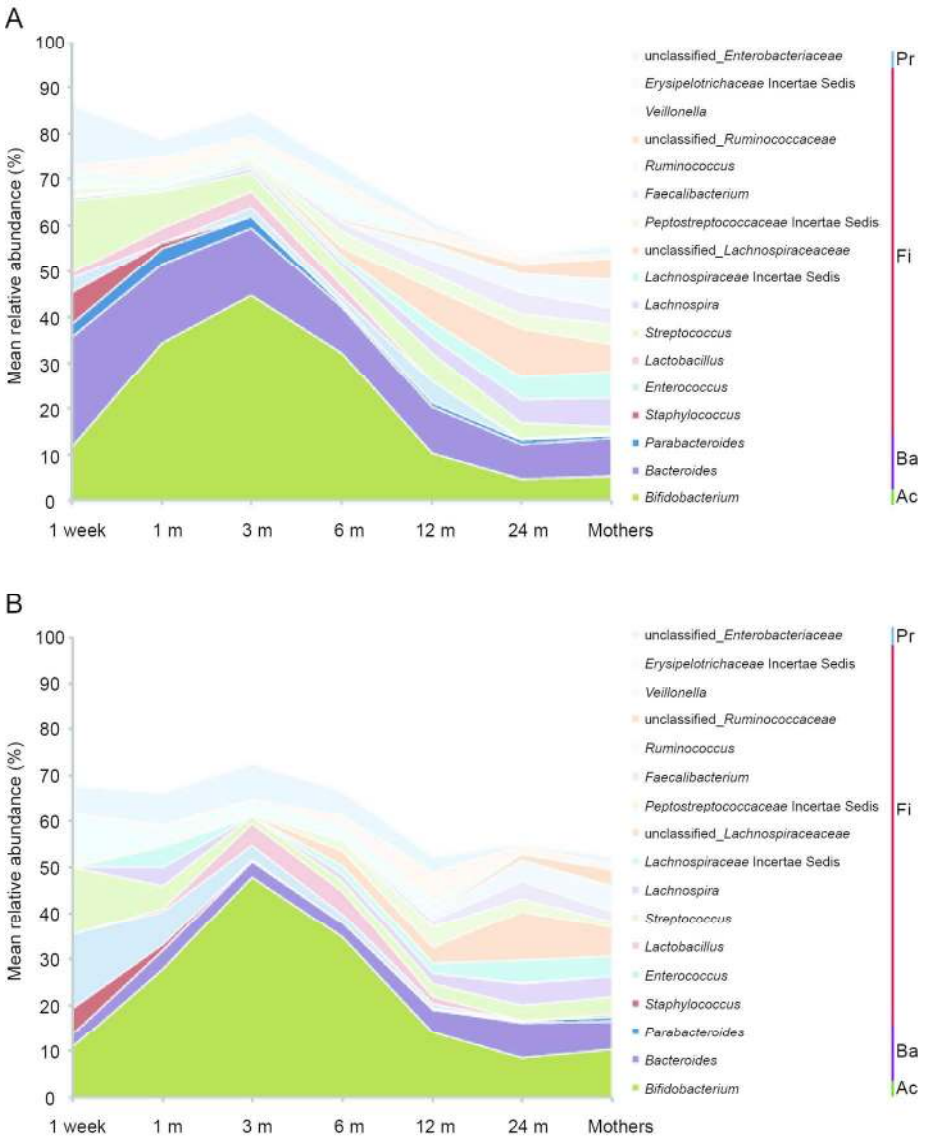

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Figure 3.

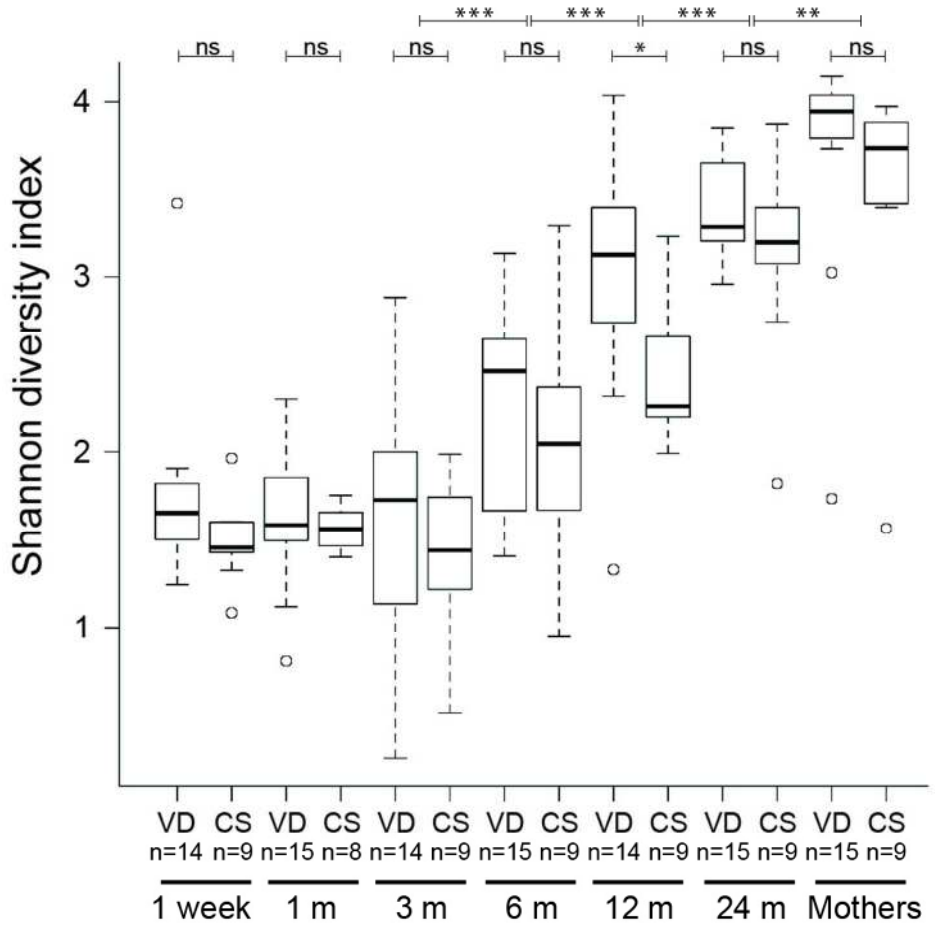

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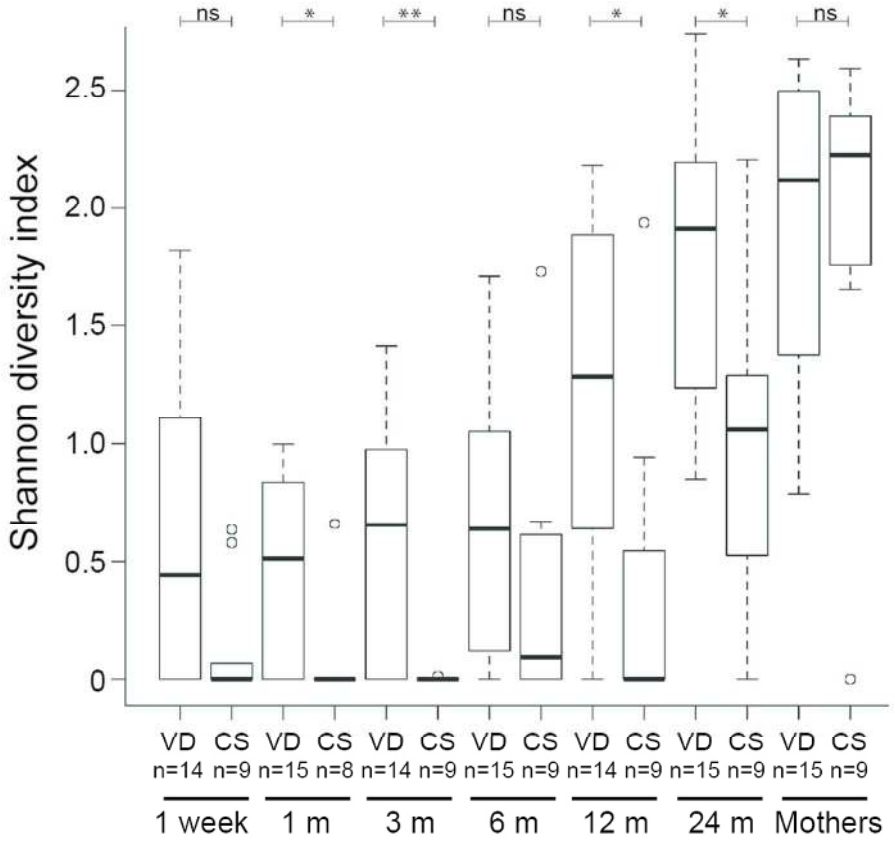
Figure 4.



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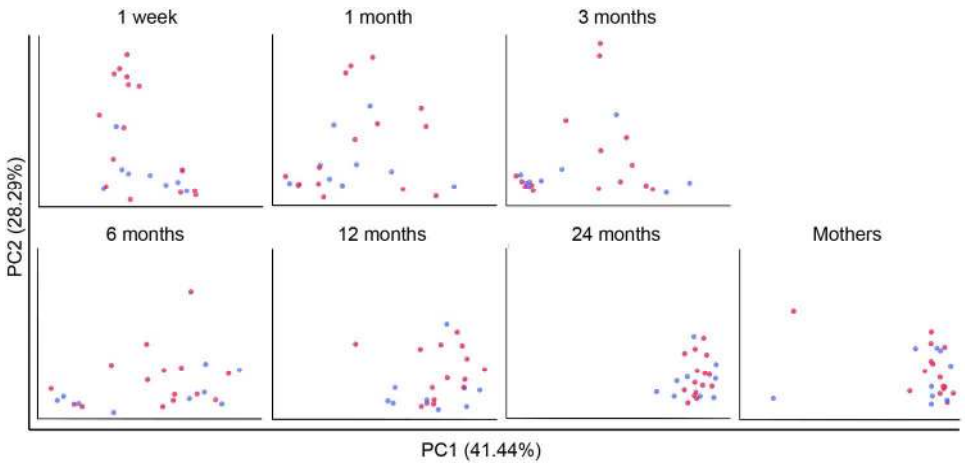

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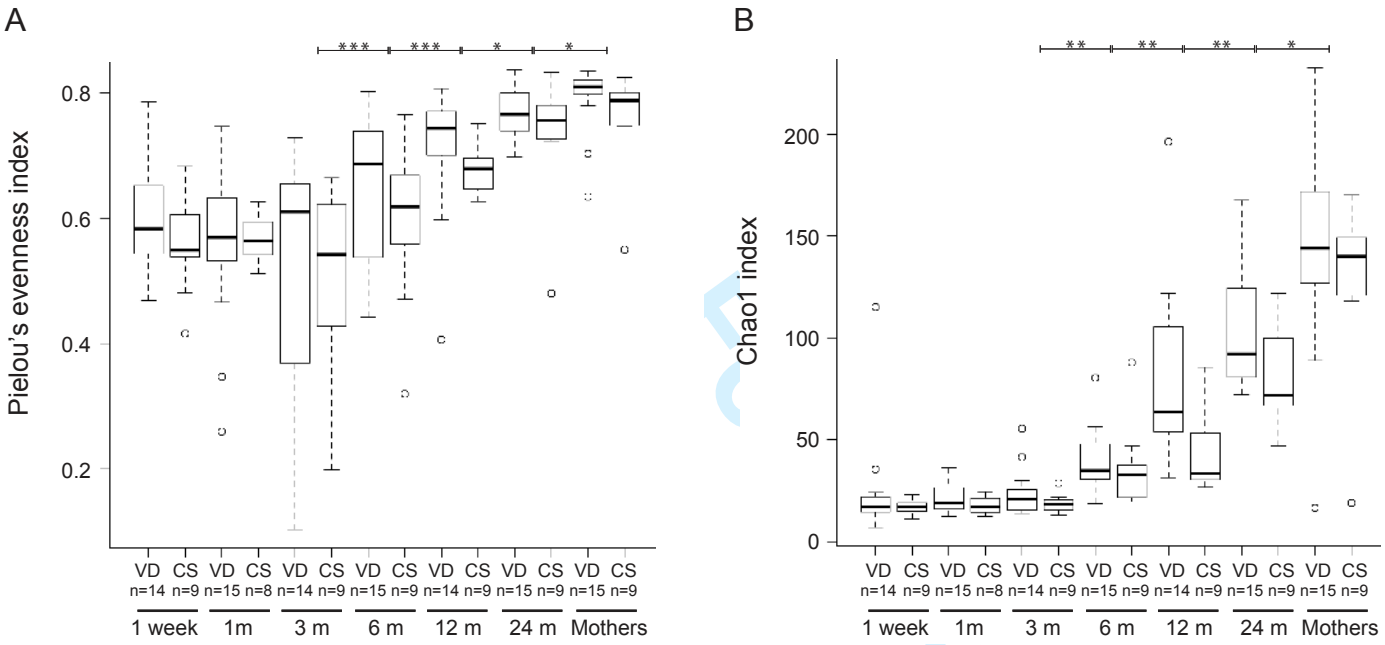
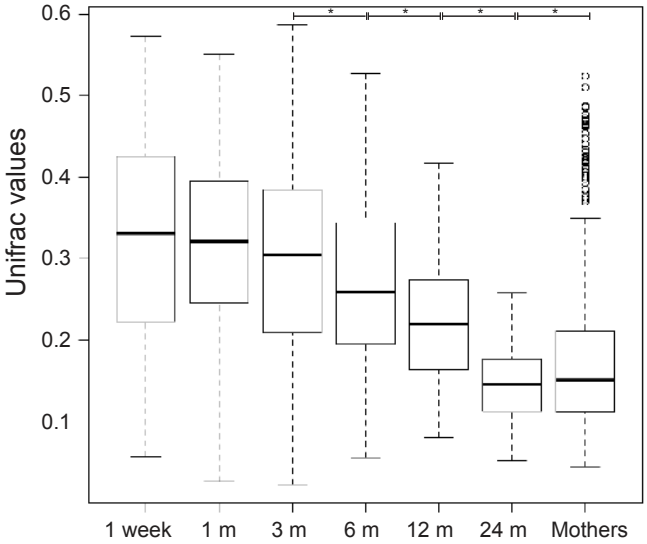
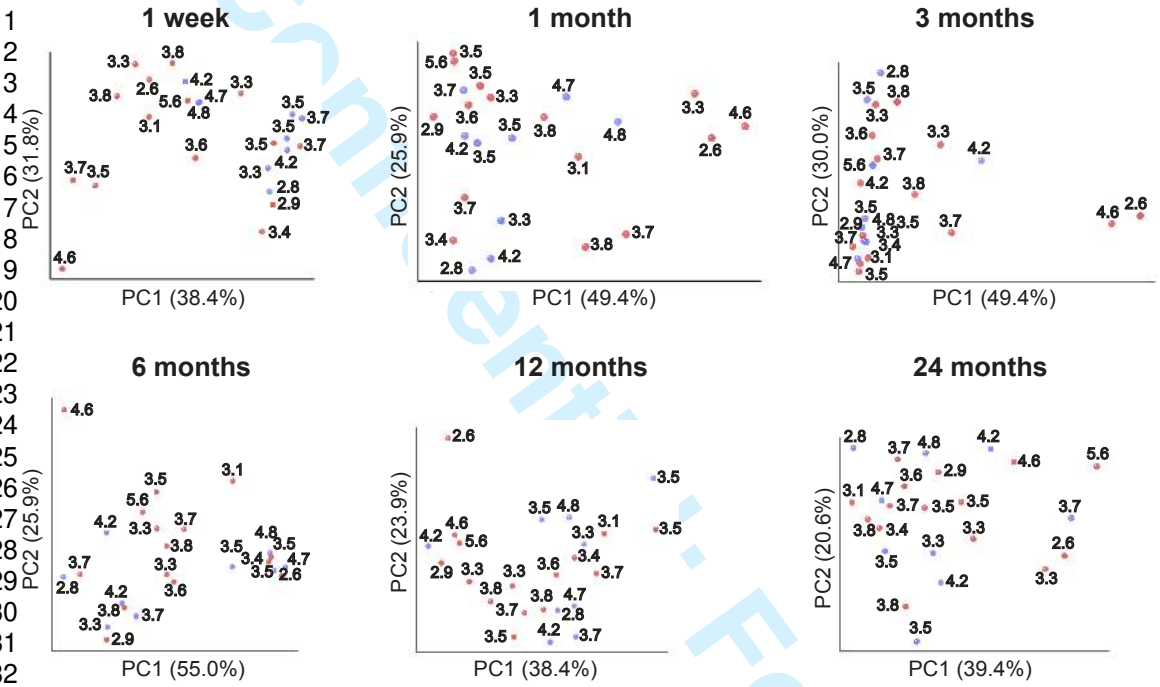


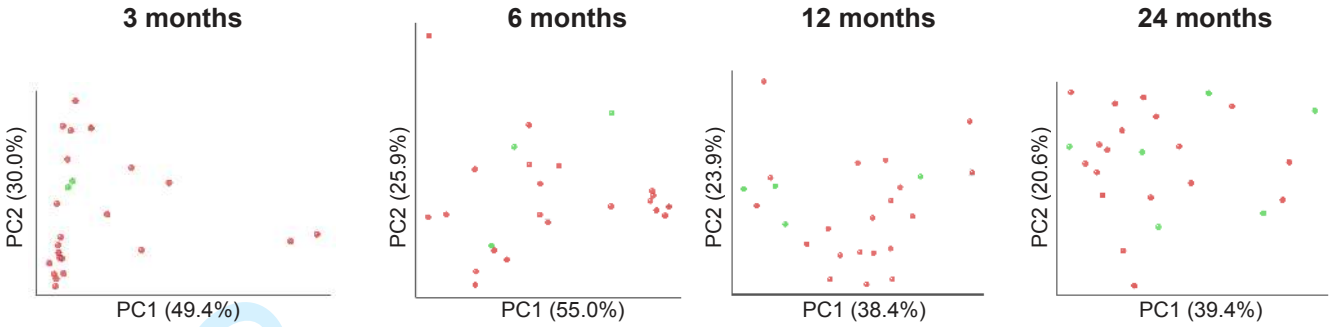
Supplementary figure 1. Pielou's evenness index (A) and Chao1 index (B) values for all 24 infants as well as for the 24 mothers. Statistical significance was measured using Wilcoxon signed rank test and * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.



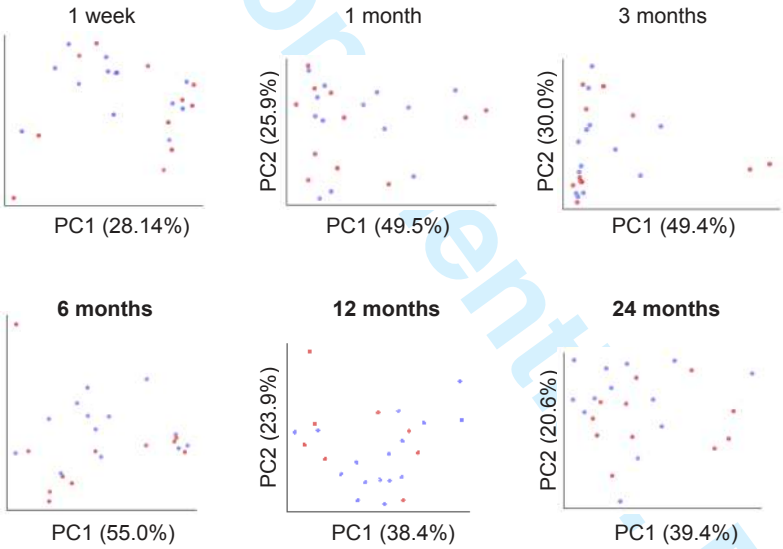
Supplementary figure 2. Pair-wise community differences. Distributions of Unifrac distance values for all 24 infants as well as for the 24 mothers. Overall, inter-subject differences declined significantly over time (Spearman rank order correlation r = -0.45, P < 2.2e-16). There was also significant differences between adjacent time points from three months and onward; stars indicate Wilcoxon signed rank test P < 10-4.



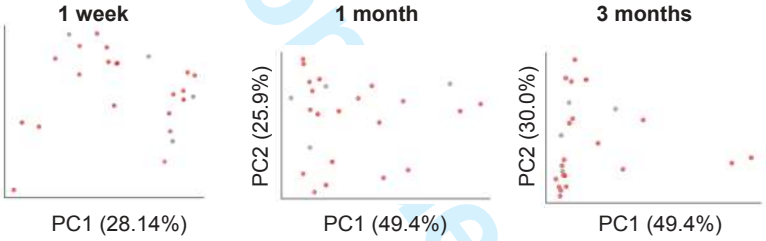
Supplementary figure 3. Principal Coordinates Analysis (PCoA) was performed on pair-wise community differences at the different time points (calculated with UniFrac (Hamady M, 2009)), and samples from all 24 infants are highlighted in the different boxes. Vaginal delivered infants are highlighted in red and CS infants are displayed in blue. Birth weights (kg) are indicated in the figure.



Supplementary figure 4. Principal Coordinates Analysis (PCoA) was performed on pair-wise community differences at the different time points (calculated with UniFrac (Hamady M, 2009)), and samples from all infants are highlighted in the different boxes. All VD and CS are displayed in red except those infants that received antibiotics that are displayed in green.



Supplementary figure 5. Principal Coordinates Analysis (PCoA) was performed on pair-wise community differences at the different time points (calculated with UniFrac (Hamady M, 2009)), and samples from all 24 infants are highlighted in the different boxes. Males are displayed in blue and females are displayed in red.



Supplementary figure 6. Principal Coordinates Analysis (PCoA) was performed on pair-wise community differences at one week, one month, and three months (calculated with UniFrac (Hamady M, 2009)), and samples from all 24 infants are highlighted in the different boxes. All VD and CS that were exlusively breast-fed are displayed in red except those infants that were partially breast-fed from birth that are displayed in grey.

Supplementary Table 1. Descriptive data of the infants included in the study. Abbreviations: VD, vaginal delivery; CS, caesarean delivery; PcV, Penicillis V; Amp, Ampicillin derivative; Her, Heracillin.

Baby	Sex	Delivery	Birth weights (grams)	Antimicrobials	Length of exclusive and partial breast-feeding (months)
Infant 1 VD	Male	Vaginal	3740		3, 8
Infant 2 VD	Female	Vaginal	2590		3, 12
Infant 3 VD	Female	Vaginal	2970		0, 5
Infant 4 VD	Female	Vaginal	4620		3, 12
Infant 5 VD	Female	Vaginal	3710		0, 5
Infant 6 VD	Male	Vaginal	3800		3, 8
Infant 7 VD	Male	Vaginal	5590	3 mo PcV, Amp, 10 mo Amp, 13 mo Amp, 15 mo Her, Amp, 19 mo Amp, 24 mo Amp.	3, 12
Infant 8 VD	Male	Vaginal	3110	5 mo PcV, 12 mo PcV.	3, 12
Infant 9 VD	Female	Vaginal	3810		3, 8
Infant 10 VD	Female	Vaginal	3460	20 mo PcV.	3, 12
Infant 11 VD	Male	Vaginal	3550		3, 10
Infant 12 VD	Female	Vaginal	3400		3, 12
Infant 13 VD	Male	Vaginal	3250		0, 3
Infant 14 VD	Male	Vaginal	3450		3, 8
Infant 15 VD	Female	Vaginal	3260	8 mo PcV, 20 mo PcV, 21 mo PcV,	0, 8
Infant 1 CS	Female	C-section	3480		3, 6
Infant 2 CS	Female	C-section	3340		3, 5
Infant 3 CS	Male	C-section	3515		3, 12
Infant 4 CS	Male	C-section	4160	22 mo PcV.	3, 6
Infant 5 CS	Male	C-section	4240	2 mo PcV, 3 mo Amp, 13 mo PcV, Amp.	3, 12
Infant 6 CS	Male	C-section	2800		3, 8
Infant 7 CS	Female	C-section	3690		3, 12
Infant 8 CS	Male	C-section	4785		3, 24
Infant 9 CS	Male	C-section	4775		3, 18

Supplementary Table 2. Descriptive data of the mothers included in the study. All antibiotic treatments were only with one dose (prophylactically). Abbreviations: VD, vaginal delivery; CS, caesarean section.

Mother	Delivery	Reason for caesarean section	Antimicrobials at delivery time
Mother 1 VD	Vaginal		
Mother 2 VD	Vaginal		
Mother 3 VD	Vaginal		
Mother 4 VD	Vaginal		
Mother 5 VD	Vaginal		
Mother 6 VD	Vaginal		
Mother 7 VD	Vaginal		
Mother 8 VD	Vaginal		
Mother 9 VD	Vaginal		
Mother 10 VD	Vaginal		
Mother 11 VD	Vaginal		
Mother 12 VD	Vaginal		Bensyl-penicillin*
Mother 13 VD	Vaginal		Bensyl-penicillin*
Mother 14 VD	Vaginal		
Mother 15 VD	Vaginal		
Mother 1 CS	C-section	Elective, humanitarian indication	Single dose of Cephalosporin**
Mother 2 CS	C-section	Elective, humanitarian indication	Single dose of Clindamycin**
Mother 3 CS	C-section	Perinatal distress	Single dose of Cephalosporin**
Mother 4 CS	C-section	Perinatal distress	Single dose of Cephalosporin**
Mother 5 CS	C-section	Elective, humanitarian indication	
Mother 6 CS	C-section	Elective, breech delivery	Single dose of Cephalosporin**
Mother 7 CS	C-section	Elective, breech delivery	
Mother 8 CS	C-section	Elective, breech delivery	Single dose of Cephalosporin**
Mother 9 CS	C-section	Perinatal distress	Single dose of Cephalosporin**

* Prophylactic single dose of Bensyl-penicillin before delivery because of Group B *Streptococcus* colonization in the mother during pregnancy.

**The routine was to give prophylactic single dose of antibiotics intravenously during the surgery after the child had been delivered. Thus, the child did not get any antibiotics via the placenta.

Supplementary Table 3. Mean relative abundance (%) and standard deviation (SD) of the most abundant phyla found at different time points in the infants born by vaginal delivery (15;VD) and caesarean section (9;CS) and in the mothers. Statistical analysis of phylum abundances between the VD and CS infants at the various time points and between the mothers was performed using Wilcoxon rank-sum test. To correct for multiple testing, the P-values were converted to False Discovery Rate values (Q-values).

Time	**Delivery mode**	**Actinobacteria** Mean % (SD)	**Bacteroidetes** Mean % (SD)	**Firmicutes** Mean % (SD)	**Proteobacteria** Mean % (SD)	**Verrucomicrobia** Mean % (SD)
1 week	VD	12 (16)	*27 (28)	44 (32)	16 (18)	0 (0)
	CS	11 (19)	*3 (8)	57 (21)	29 (16)	0 (0)
1 month	VD	37 (29)	21 (25)	32 (23)	10 (8)	0 (0)
	CS	29 (23)	8 (15)	44 (22)	19 (15)	0 (0)
3 months	VD	45 (31)	**^19 (29)	29 (25)	*7 (8)	0 (0)
	CS	48 (30)	**^4 (10)	33 (28)	*15 (14)	0 (0)
6 months	VD	33 (24)	11 (16)	48 (23)	7 (9)	0 (0)
	CS	36 (33)	3 (6)	54 (30)	7 (5)	0 (0)
12 months	VD	12 (11)	*14 (11)	69 (15)	3 (6)	1 (1)
	CS	15 (14)	*6 (12)	69 (16)	5 (7)	5 (9)
24 months	VD	7 (4)	13 (7)	77 (7)	0 (0)	2 (3)
	CS	10 (9)	11 (8)	77 (11)	1 (1)	1 (2)
Mothers	VD	7 (7)	16 (11)	74 (21)	3 (7)	1 (1)
	CS	12 (15)	13 (10)	71 (17)	2 (5)	2 (4)

* = P < 0.05, ** = P < 0.01, ^ = Q < 0.05.

Supplementary Table 4. Mean relative abundance (%) and standard deviation (SD) of the most abundant genera found at different time points in the infants born by vaginal delivery (15;VD) and caesarean section (9;CS) and in the mothers. Only genera displaying >1% average abundance were included. Statistical analysis of genera abundances between the VD and CS infants at the various time points and between the mothers was performed using Wilcoxon rank-sum test. To correct for multiple testing, the P-values were converted to False Discovery Rate values (Q-values).

Vaginal delivery	1 week Mean %(SD) n=15	1 month Mean %(SD) n=15	3 months Mean %(SD) n=15	6 months Mean %(SD) n=15	12 months Mean %(SD) n=15	24 months Mean %(SD) n=15	Mothers Mean %(SD) n=15
Bifidobacterium	11.6 (16.2)	34.4 (30)	44.7 (32.2)	32.1 (23.7)	10.5 (10.6)	4.9 (3.3)	5.3 (7)
Bacteroides	*24.1 (26.3)	17.3 (25.2)	*14.6 (28.6)	10.2 (16.1)	10 (9.3)	7.4 (6.2)	8.2 (9.2)
Parabacteroides	2.8 (6.6)	*3.3 (5.3)	2.5 (7.2)	0.7 (1.5)	*0.9 (2.5)	1 (2.4)	0.8 (1)
Staphylococcus	6.9 (7.4)	1.3 (1.7)	0.2 (0.4)	0.1 (0.5)	0 (0)	0 (0)	0.1 (0.4)
Enterococcus	3.3 (9.4)	**^0 (0.1)	1.8 (3.8)	1.2 (3.1)	4.5 (16.5)	0 (0.1)	0.1 (0.2)
Lactobacillus	1 (2.1)	3.2 (5.7)	3.6 (7)	2.4 (6.5)	*0 (0)	0 (0.2)	0.3 (0.6)
Streptococcus	15.8 (17.2)	7.8 (7.2)	4.1 (9.8)	4.2 (4.6)	6.5 (10.4)	3.7 (4.9)	1.4 (1.6)
Lachnospira	0.4 (1.1)	0.4 (1.2)	0.9 (1.6)	1.3 (1.5)	3.3 (2.2)	4.9 (2.2)	6.1 (2.6)
Lachnospiraceae Incertae Sedis	0.4 (1.1)	0.4 (1.2)	0.9 (1.6)	1.3 (1.5)	3.3 (2.2)	4.9 (2.2)	6.1 (2.6)
Unclassified_Lachnospiraceae	0.3 (1.2)	0 (0.1)	0.4 (1.6)	1.7 (2.8)	7.3 (6.5)	10.9 (6.3)	5.9 (3.2)
Peptostreptococcaceae Incertae Sedis	1.4 (4.1)	0.4 (1)	0.7 (1.2)	3.6 (6.9)	3.1 (4)	3 (3)	4.5 (5.2)
Faecalibacterium	0.1 (0.4)	0 (0)	0 (0.1)	1.7 (2.8)	3.1 (3.5)	4.6 (4)	3.5 (3)
Ruminococcus	0.3 (1)	0 (0)	0 (0.1)	0.2 (0.5)	3.4 (4)	4.2 (3.5)	6.1 (5.2)
Unclassified_Ruminococcaceae	0.3 (0.8)	0 (0.1)	0.4 (1.2)	0.4 (0.8)	*1 (1.3)	2.1 (1.6)	4.6 (3.7)
Veillonella	*3.3 (4.3)	1.5 (1.6)	2.4 (3.3)	6.2 (5.8)	**0.4 (0.8)	0.1 (0.2)	0 (0.1)
Erysipelotrichaceae Incertae Sedis	1.1 (2.7)	4.6 (10.9)	2.5 (5.5)	1.7 (4.1)	2.2 (2.5)	1.5 (2.2)	0.9 (1.1)
Unclassified_Enterobacteriaceae	13.1 (19.3)	4 (7)	5 (7.9)	3.6 (9)	*1.4 (4.4)	0.2 (0.3)	2 (7.6)

Caesarean section delivery	1 week Mean %(SD) n=9	1 month Mean %(SD) n=9	3 months Mean %(SD) n=9	6 months Mean %(SD) n=9	12 months Mean %(SD) n=9	24 months Mean %(SD) n=9	Mothers Mean %(SD) n=9
Bifidobacterium	11 (20.5)	27.8 (24.5)	47.8 (31.4)	34.8 (33.6)	14.2 (14.6)	8.5 (9)	10.4 (16.8)
Bacteroides	*2.8 (8.4)	4.1 (12.1)	*3.6 (10.9)	3.1 (5.8)	5.1 (11.3)	7.9 (7.1)	6.4 (5.5)
Parabacteroides	0 (0)	*0 (0)	0 (0)	0 (0)	*0.1 (0.3)	0.4 (0.4)	0.7 (0.9)
Staphylococcus	5.8 (7.9)	1.4 (1.7)	0.1 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)
Enterococcus	15.7 (20)	**^7.2 (9.5)	3.1 (3.6)	2 (2.8)	1.2 (2.2)	0 (0)	0 (0)
Lactobacillus	0 (0)	0.5 (1.5)	4.9 (3.6)	4.8 (8.6)	*1.6 (4.4)	0 (0)	0.8 (1.5)
Streptococcus	14.6 (17.6)	4.9 (4.1)	1.5 (1)	2.2 (2.1)	2.5 (3.1)	3.2 (5.5)	3.7 (6)
Lachnospira	0 (0)	4.2 (8.5)	0 (0.1)	1.8 (2)	2.3 (2.7)	4.9 (2.6)	4.4 (2.4)
Lachnospiraceae Incertae Sedis	0 (0)	4.2 (8.5)	0 (0.1)	1.8 (2)	2.3 (2.7)	4.9 (2.6)	4.4 (2.4)
Unclassified_Lachnospiraceae	0 (0)	0 (0)	0.1 (0.2)	3.1 (6.2)	3.4 (5.2)	10.6 (4.9)	6.2 (4.8)
Peptostreptococcaceae Incertae Sedis	0.2 (0.6)	0.6 (1.6)	0.4 (0.9)	2.2 (2.7)	4.5 (5.2)	3 (4.2)	1.4 (1.6)
Faecalibacterium	0 (0)	0 (0)	0 (0)	0.2 (0.6)	1.4 (2.6)	3.5 (2.7)	2 (1.8)
Ruminococcus	0 (0)	0 (0)	0 (0)	0 (0)	1.6 (2.7)	4.7 (2.6)	5.7 (3.5)
Unclassified_Ruminococcaceae	0 (0)	0.2 (0.5)	0 (0)	0.1 (0.1)	*0.3 (0.7)	1.4 (1.7)	3.4 (3)
Veillonella	*11.2 (8.5)	3.4 (7.4)	3.1 (3.9)	3.2 (2.4)	**2.4 (2.3)	0.3 (0.3)	0.1 (0.3)
Erysipelotrichaceae Incertae Sedis	0.1 (0.2)	0.6 (1.3)	0.2 (0.7)	2.3 (3.4)	6.2 (7.5)	1.5 (1.9)	1.2 (2.7)
Unclassified_Enterobacteriaceae	6.2 (14.4)	7 (14.6)	7.6 (8.1)	5.2 (5.7)	*3.4 (5.3)	0.4 (0.7)	1.5 (4.3)

* = P < 0.05, ** = P < 0.01, ^ = Q < 0.05.

Supplementary Table 5. Repeated measures ANOVAs to test whether there were any significant differences in Shannon diversity between delivery mode (VD and CS) when including the diversity indices from all time points (one week, one, three, six, twelve and 24 months). The ANOVAs where calculated on non-log Shannon diversity indicies from 15 vaginal delivered and nine caesarean section delivered infants, except for a few time points where the data point was missing due to subsampling (14 VD and 9 CS at one week; 15 VD and 8 CS at one month, 14 VD and 9 CS at twelve months).

Taxa	P-value
Total microbiota	**0.047**
Firmicutes	0.062
Proteobacteria	0.903
Actinobacteria	0.132
Bacteroidetes	**0.002**

Supplementary Table 6. The Shannon diversity index of the total microbiota and most abundant phyla in stool samples obtained at various ages from vaginal (VD) or caesarean section (CS) delivered infants as well as from the mothers.

	Vaginal delivery		Caesarean section		P-value*
	median	iqr**	median	iqr**	
1 week	**n =14#**		**n = 9**		
Total microbiota	1.66	1.49-1.83	1.46	1.38-1.61	0.096
Firmicutes	1.49	1.20-1.70	1.18	0.78-1.49	0.072
Proteobacteria	0.09	0.00-0.22	0.12	0.07-0.38	0.393
Actinobacteria	0.02	0.00-0.29	0.05	0.00-0.52	0.511
Bacteroidetes	0.30	0.00-1.11	0.00	0.00-0.03	0.077
1 month	**n =15**		**n = 8#**		
Total microbiota	1.59	1.50-1.97	1.57	1.45-1.67	0.681
Firmicutes	1.27	1.02-1.68	1.31	0.97-1.68	0.925
Proteobacteria	0.16	0.00-0.44	0.00	0.00-0.04	0.058
Actinobacteria	0.13	0.03-0.75	0.23	0.05-1.01	0.457
Bacteroidetes	0.42	0.00-0.81	0.00	0.00-0.00	**0.022**
3 months	**n = 15**		**n = 9**		
Total microbiota	1.73	1.09-2.01	1.45	1.21-1.77	0.369
Firmicutes	1.43	1.16-1.71	1.45	1.03-1.77	0.975
Proteobacteria	0.19	0.07-0.45	0.26	0.17-0.41	0.395
Actinobacteria	0.45	0.05-0.67	0.14	0.05-0.61	0.682
Bacteroidetes	0.64	0.00-0.87	0.00	0.00-0.00	**0.004**
6 months	**n = 15**		**n = 9**		
Total microbiota	2.48	1.55-2.68	2.06	1.53-2.52	0.29
Firmicutes	2.25	1.65-2.44	1.81	1.66-2.30	0.482
Proteobacteria	0.67	0.23-0.78	0.27	0.09-0.59	0.238
Actinobacteria	0.51	0.38-0.63	0.64	0.09-0.74	0.976
Bacteroidetes	0.62	0.00-1.09	0.09	0.00-0.22	0.202
12 months	**n =14#**		**n = 9**		
Total microbiota	3.15	2.67-3.44	2.28	2.18-2.82	**0.013**
Firmicutes	2.90	2.09-3.16	2.04	1.91-2.86	**0.046**
Proteobacteria	0.70	0.00-0.96	0.61	0.23-0.77	0.506
Actinobacteria	0.51	0.25-0.75	0.59	0.11-0.72	0.801
Bacteroidetes	1.23	0.50-1.82	0.00	0.00-0.74	**0.023**
24 months	**n =15**		**n = 9**		
Total microbiota	3.33	3.23-3.71	3.23	2.93-3.46	0.194
Firmicutes	3.10	2.91-3.36	3.19	2.64-3.36	0.682
Proteobacteria	0.50	0.34-0.61	0.13	0.00-0.39	**0.035**
Actinobacteria	0.72	0.48-1.23	0.49	0.29-0.82	0.123

Bacteroidetes	1.83	1.19-2.21	1.03	0.49-1.37	**0.018**
Mothers	**n =15**		**n = 9**		
Total microbiota	3.99	1.35-3.79	3.78	3.45-3.97	0.084
Firmicutes	3.65	3.48-3.88	3.38	3.21-3.68	0.073
Proteobacteria	0.63	0.07-1.08	0.35	0.12-0.87	0.309
Actinobacteria	0.86	0.62-1.35	1.0	0.64-1.21	0.64
Bacteroidetes	1.97	1.31-2.45	2.20	1.63-2.37	0.907

*Wilcoxon rank-sum test.
**interquartile range.
Missing data points due to the subsampling of the data and exclusion of samples with less than 1400 reads per sample.

Supplementary Table 7. Statistical testing of mother-child overlap in microbial community composition. For each time point and for each infant we calculated the number of sequence types shared with its mother/number of sequence types observed in the infant (R_{own}). Likewise we calculated the average number of sequence types shared with other mothers/number of sequence types observed in the infant (R_{other}). We then compared the R_{own} and R_{other} values pairwise for all infants within each group (VD or CS) with the Wilcoxon signed rank test. P-values for these tests are reported below, considering either sequences belonging to specific phyla or all sequences.

	Vaginal delivery					
	1 week	1 month	3 months	6 months	12 months	24 months
Firmicutes	0.421	0.804	0.191	**0.041**	0.073	**0.048**
Proteobacteria	0.724	1	0.932	0.088	0.49	0.834
Actinobacteria	0.308	0.53	0.551	0.916	0.802	0.187
Bacteroidetes	0.147	**0.042**	0.224	**0.018**	0.802	0.095
All taxa	**0.03**	0.121	0.074	0.064	0.055	**0.022**
	Caesarean section					
	1 week	1 month	3 months	6 months	12 months	24 months
Firmicutes	0.834	0.183	0.359	0.496	0.652	0.57
Proteobacteria	0.284	0.905	1	0.812	0.858	0.933
Actinobacteria	1	0.722	0.678	0.945	0.098	0.652
Bacteroidetes	0.461	0.371	1	0.786	0.352	1
All taxa	0.813	0.301	0.363	0.359	0.652	0.734