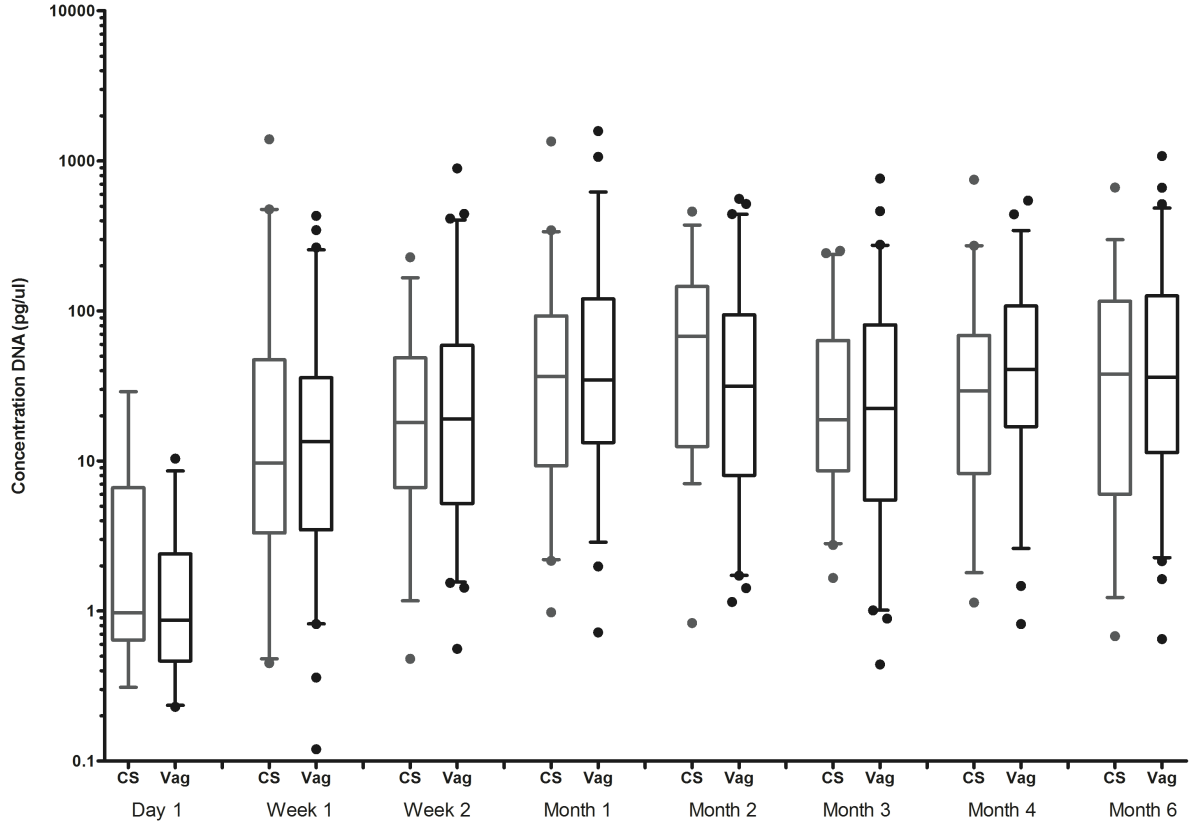
Figure S1 DNA concentration of samples per time point stratified for mode of delivery

Boxplot, box extends from the 25th to 75th percentiles of the median with whiskers from 5th to 95th percentiles. The dots represent samples that are either below the 5th or above the 95th percentile; measured by universal 16S quantitive polymerase chain reaction (PCR)).



CS; C-section born children, vag; vaginally born children

Figure S2 The number of oligotypes and diversity increased during the first months of life

For every time point, (A) the number of oligotypes and (B) the diversity of the microbial community are depicted (boxplot, box extends from the 25th to 75th percentiles of the median with whiskers from 5th to 95th percentiles). Kruskal-Wallis, post hoc analyses with Dunn's FDR correction was used to calculate the differences in respectively the number of oligotypes and diversity between each boxplot compared to week one.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure a

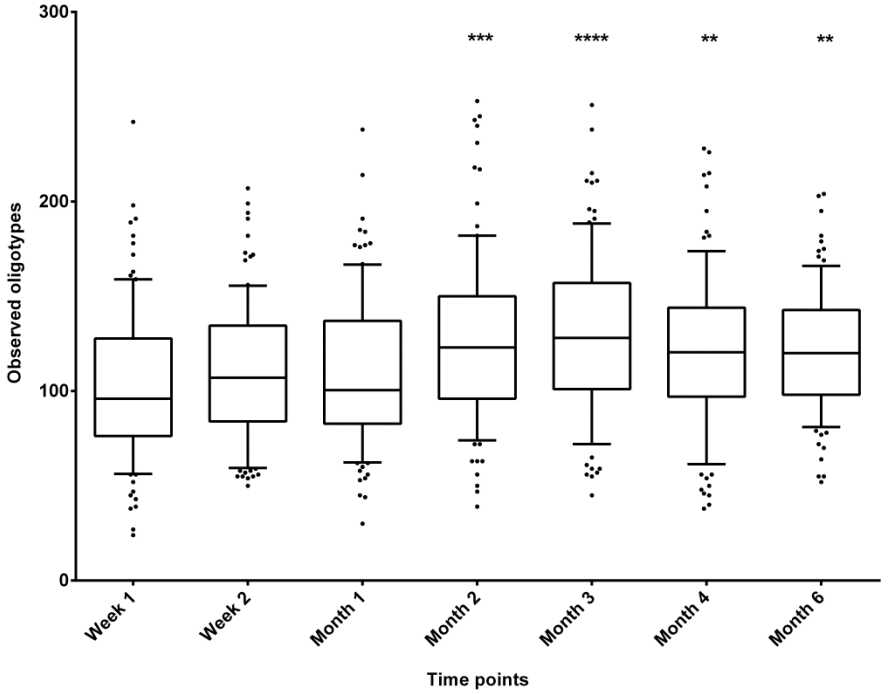


Figure b

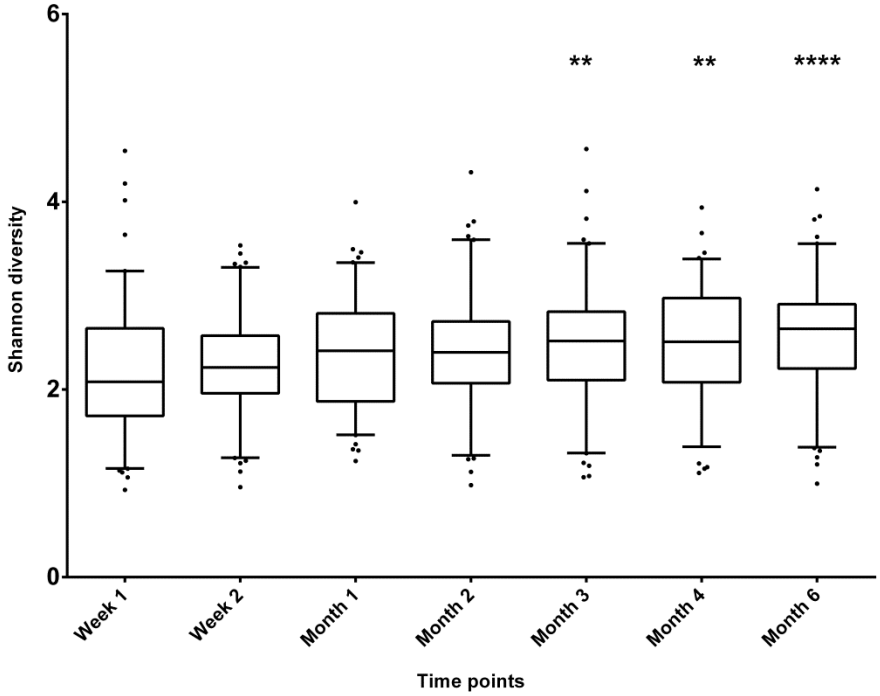
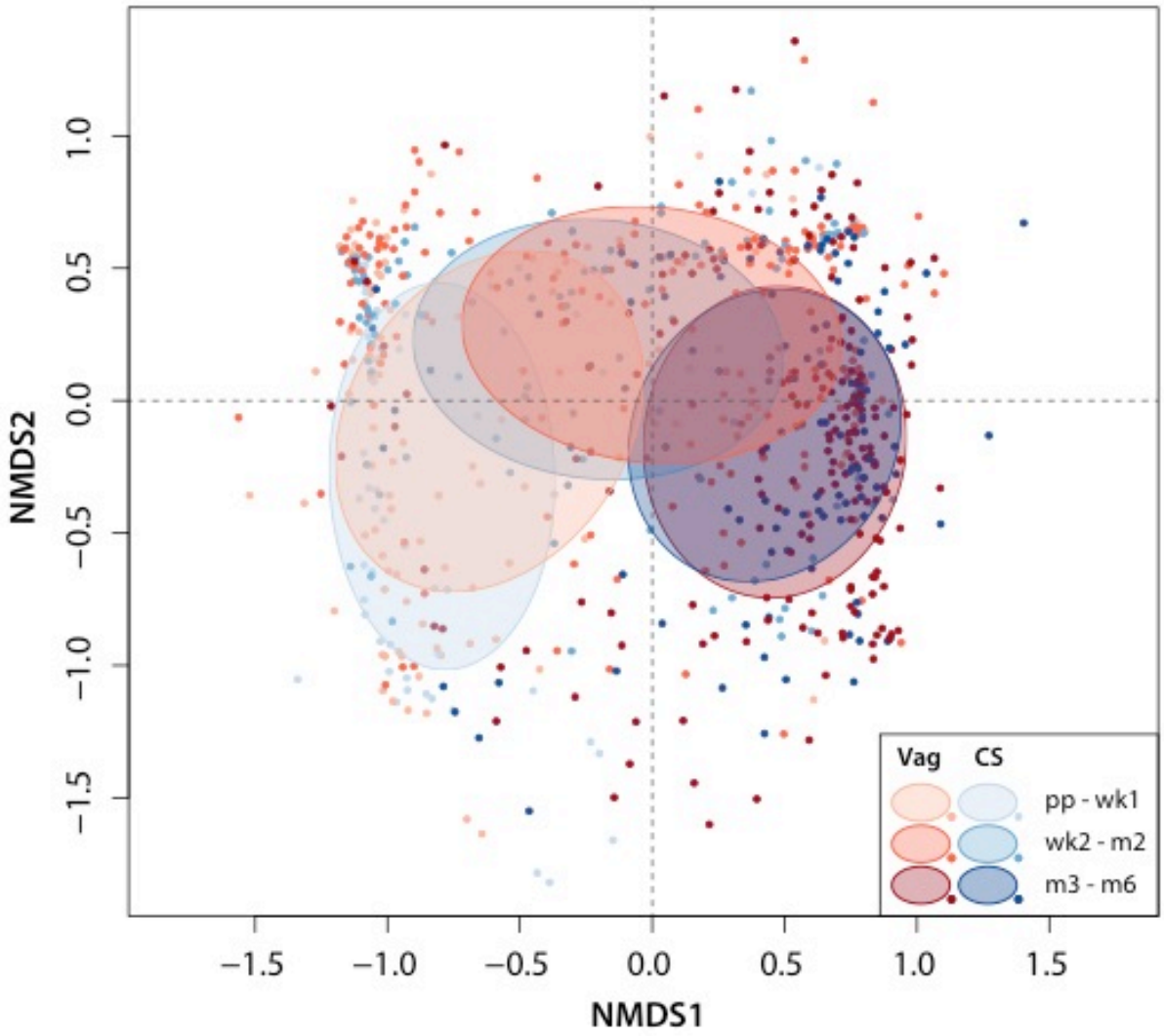


Figure S3 Development of microbial community composition stratified for mode of delivery



The figure represents a two-dimensional nonmetric multidimensional scaling (nMDS) plot of the nasopharyngeal microbiota composition of vaginally delivered children (red colors) compared to C-section born children (blue colors) overtime. By using Bray-curtis distance measure, the distance between the total microbial composition of a child on a certain time point is depicted. Light colors represent the early samples where darker colors depict the samples collected during further follow-up. The ellipse represents the standard deviations of samples per time point from

light colors in the first week of life, to dark colors in the follow-up (see legend). We observed significant differences in the overall microbiota composition between vaginally delivered children and children born by C-section (PERMANOVA unadjusted R2 =0.004, p-value=0.007).

SD; Standard deviation, CS; Caesarian section, vag; vaginal birth, pp; postpartum, wk1; week 1, wk2; week 2, m2; month 2, m3; month 3, m6; month 6

Figure S4 Clustering of highly similar children by co-occurrence spectral clustering

By co-occurrence spectral clustering, we could define 11 clusters of samples of children with highly similar microbial profiles. The figure depicts the consensus matrix, ranging from red (highly similar microbial profile) to blue (completely different microbial profile)(Biesbroek et al., 2014b; Cornelisse et al., 2012). Detailed information on the presence of clusters per time point can be found in supplemental figure 5. Names were assigned to the clusters based on the biomarker analyses and based on the biomarkers determined in supplemental figure 6.

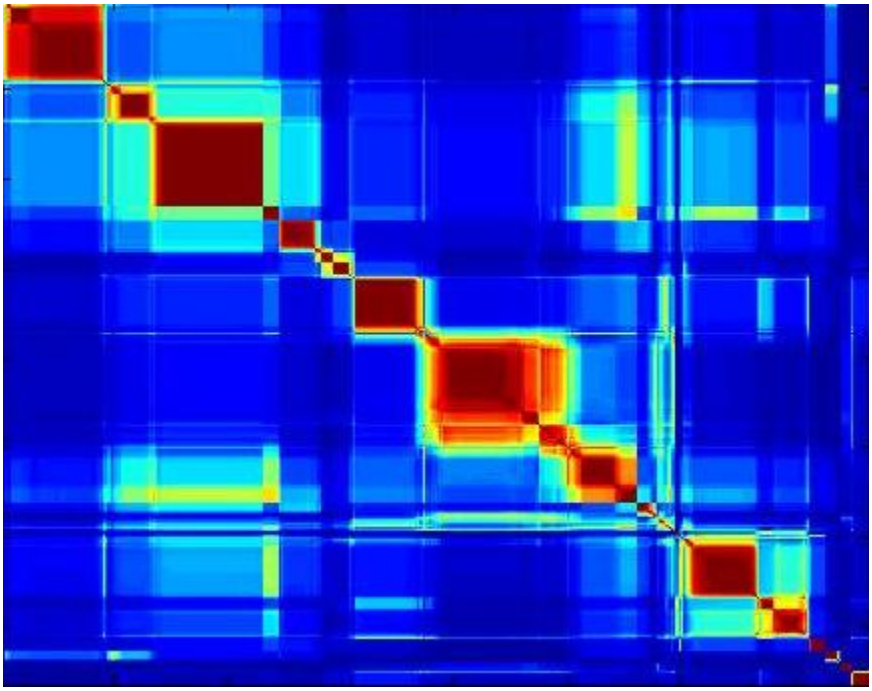
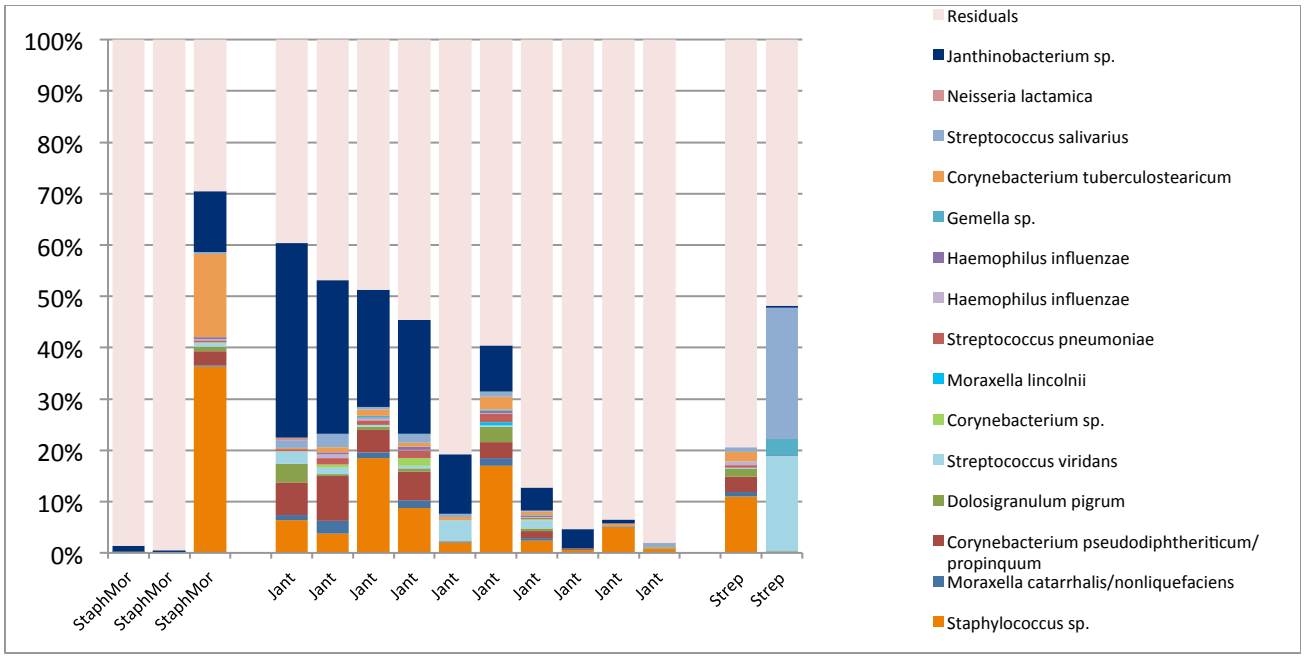


Figure S5 Microbiota profiles according to clusters

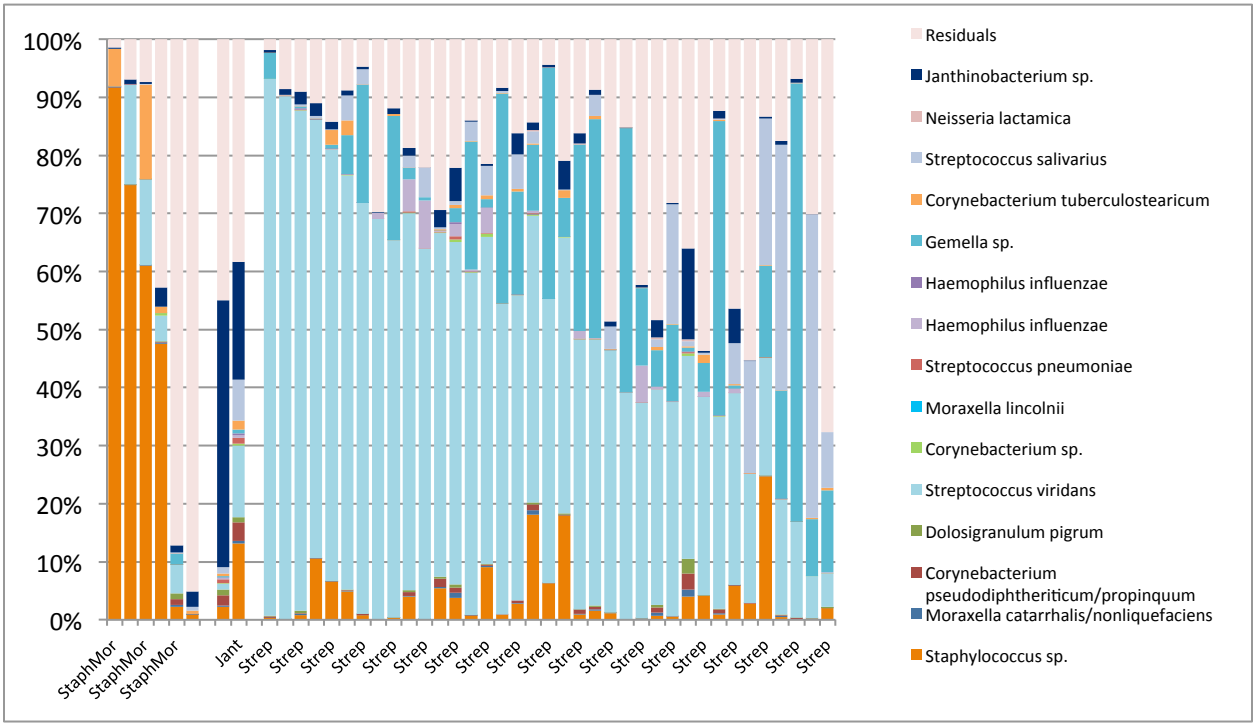
For each child the relative abundances of the top 15 oligotypes (with an abundance > 0.4% in the total dataset) are depicted for each time point. The sample profiles are depicted according to the in supplemental figure 4 defined clusters. The clustering technique takes into account the total microbial community, including low-abundant species. The most abundant biomarker-species seemed to be the major drivers of a cluster (shown in supplemental figure 6), except for the cluster STAPH-MOR, where low-abundant species seem to drive clustering as well. This cluster is predominated by *Staphylococcus aureus* at the early age and gradually switches to predominance of *Moraxella catarrhalis/nonliquefaciens* later on. Additional analyses on both the total set of samples (with adjusted parameters) and further zoom-in analyses of only this cluster showed similar results, suggesting that either minor species are the major driver of this cluster or that this cluster is a transition cluster.

Relative abundance and the cluster assignment are depicted for each time point. Presence and size of the clusters seemed to be strongly age-dependent.

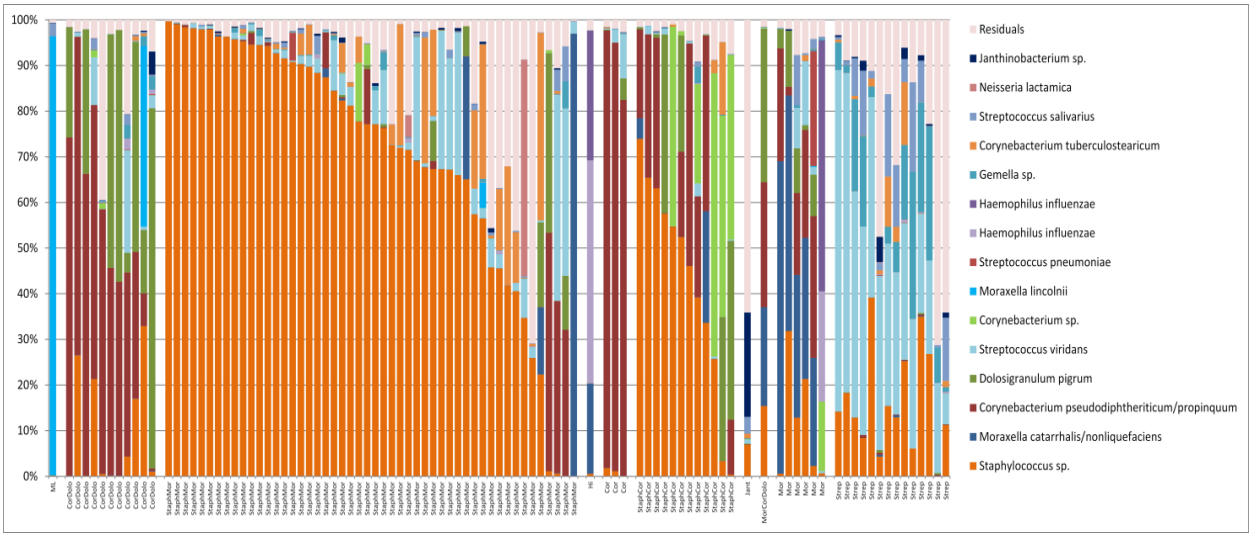
(a) Shortly after birth


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
StaphMor
StaphMor
StaphMor
Jant
Jant
Jant
Jant
Jant
Jant
Jant
Jant
Jant
Jant
Strep
Strep
Residuals
Janthinobacterium sp.
Neisseria lactamica
Streptococcus salivarius
Corynebacterium tuberculostearicum
Gemella sp.
Haemophilus influenzae
Haemophilus influenzae
Streptococcus pneumoniae
Moraxella lincolnii
Corynebacterium sp.
Streptococcus viridans
Dolosigranulum pigrum
Corynebacterium pseudodiphtheriticum/ propinquum
Moraxella catarrhalis/nonliquefaciens
Staphylococcus sp.

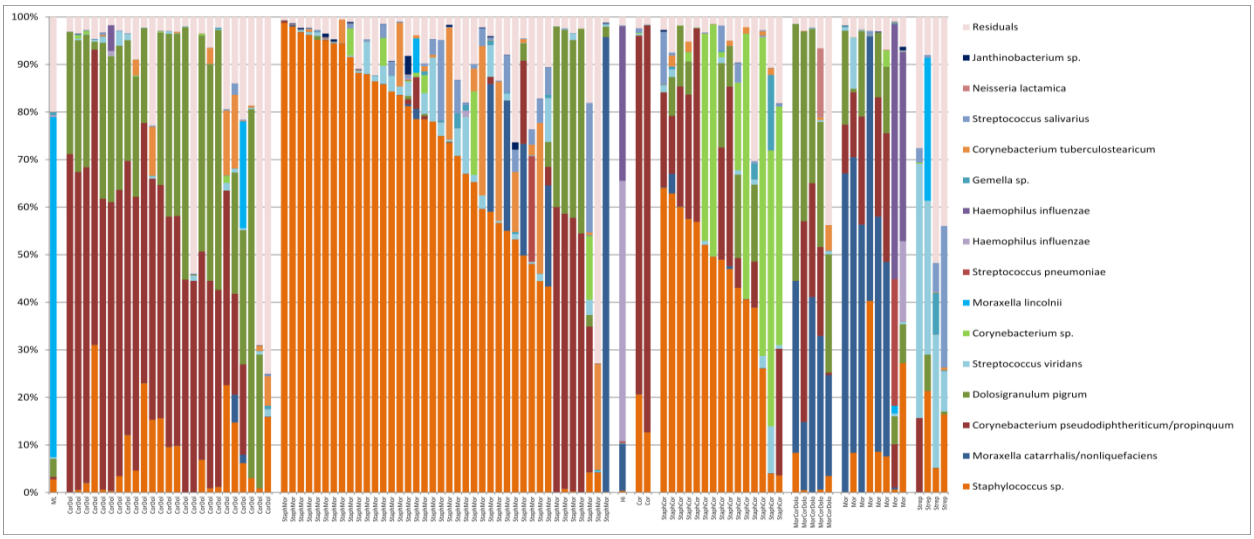
(b) Day 1



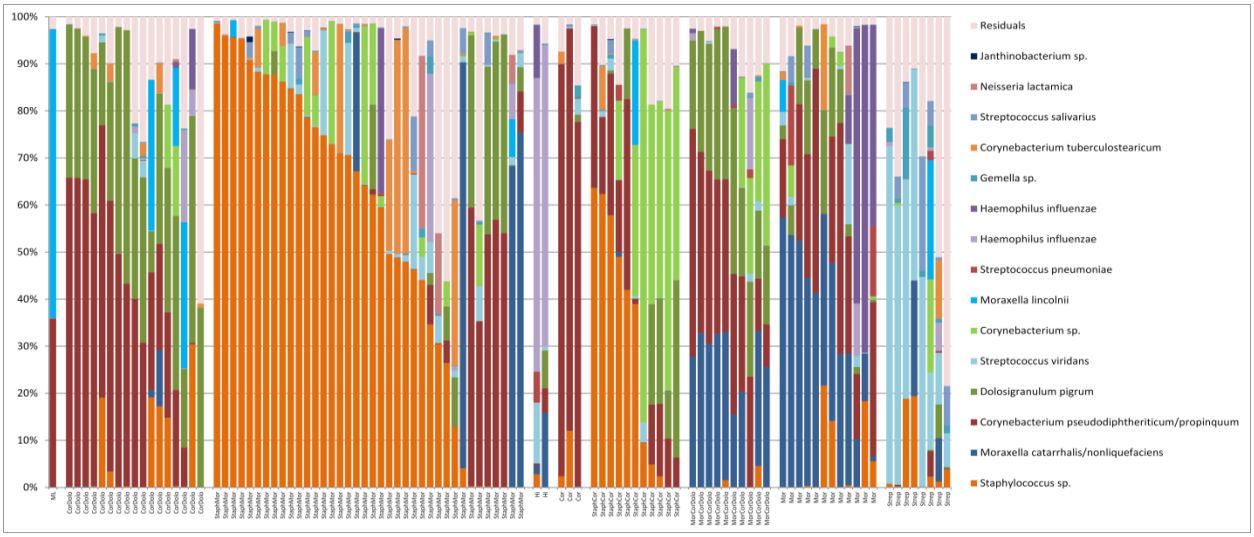
(c) Week 1



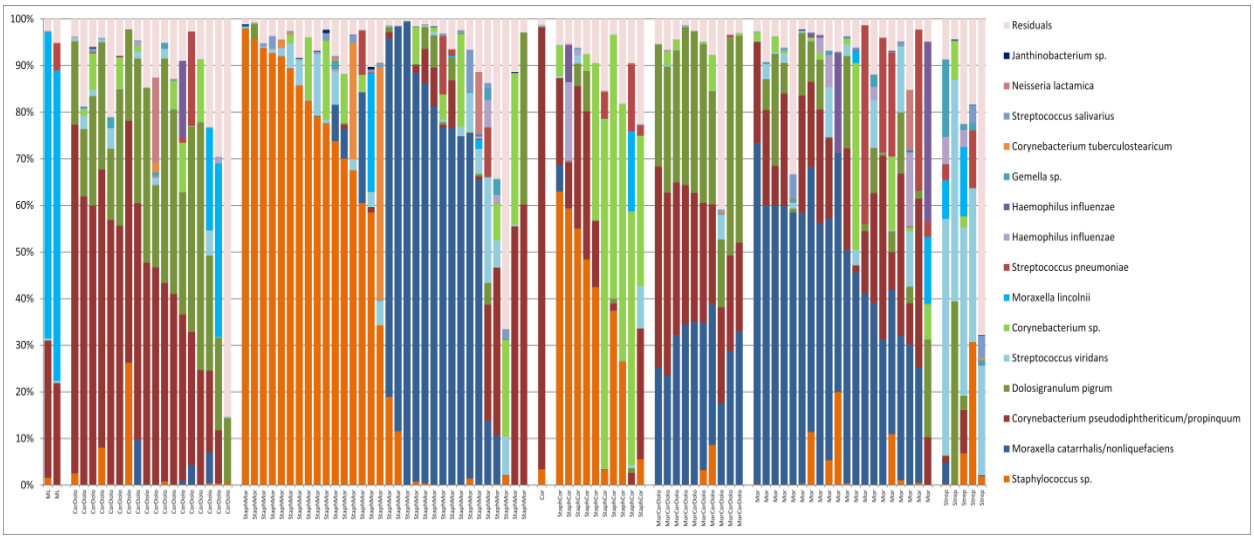
(d) Week 2



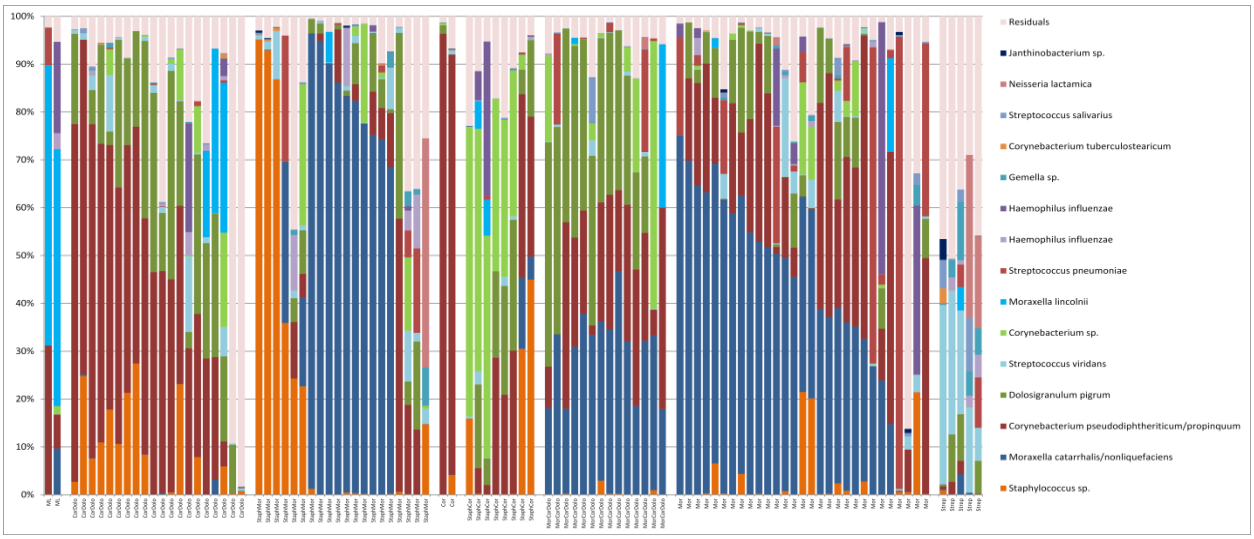
(e) Month 1



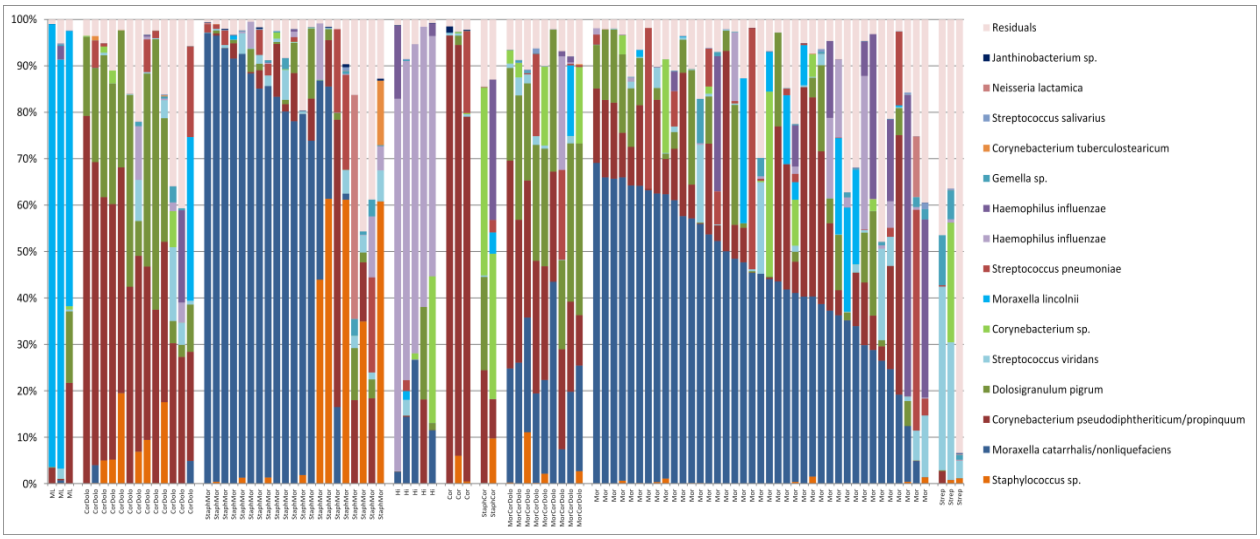
(a) Month 2



(b) Month 3



(c) Month 4



(d) Month 6

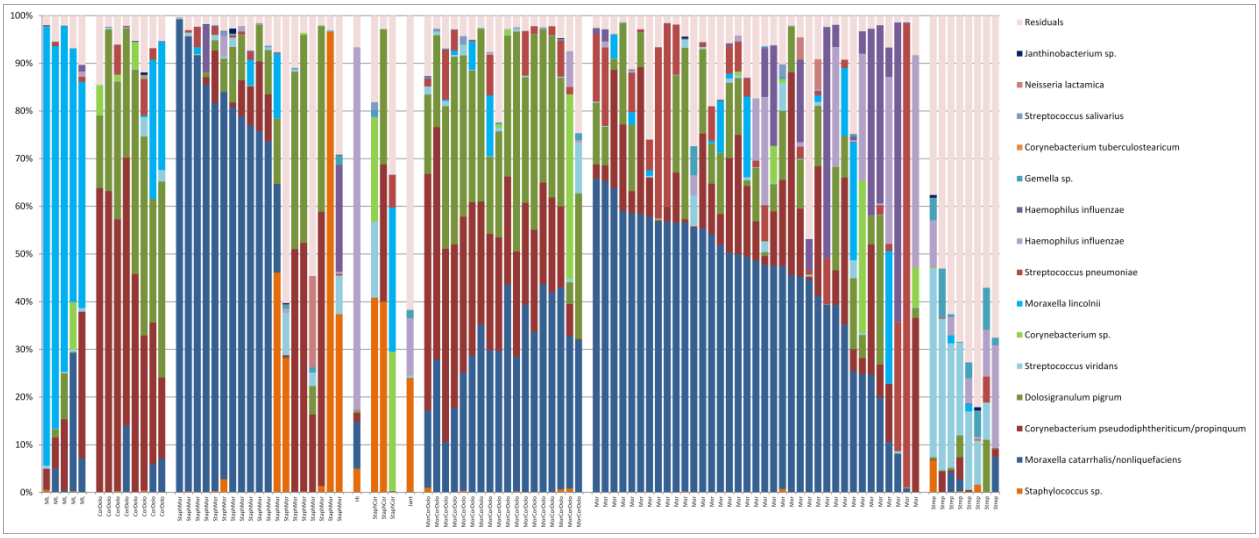


Figure S6 Identification of biomarker species that characterize the clusters

We used an unsupervised feature selection approach to determine which oligotype was the major driver of the clusters found by unbiased machine learning techniques. Based on the presence of the most abundant oligotype we named the cluster. The biomarkers of each cluster are depicted; their abundance is represented by their size.

Abbreviations; Staph, *Staphylococcus*; Jant, *Janthinobacterium*; Strep, *Streptococcus viridans*; ML, *Moraxella lincolnii*; Cor, *Corynebacterium*; Dolo, *Dolosigranulum*; Mor, *Moraxella*; INT, Intermediate cluster; HI, *Haemophilus influenzae*.

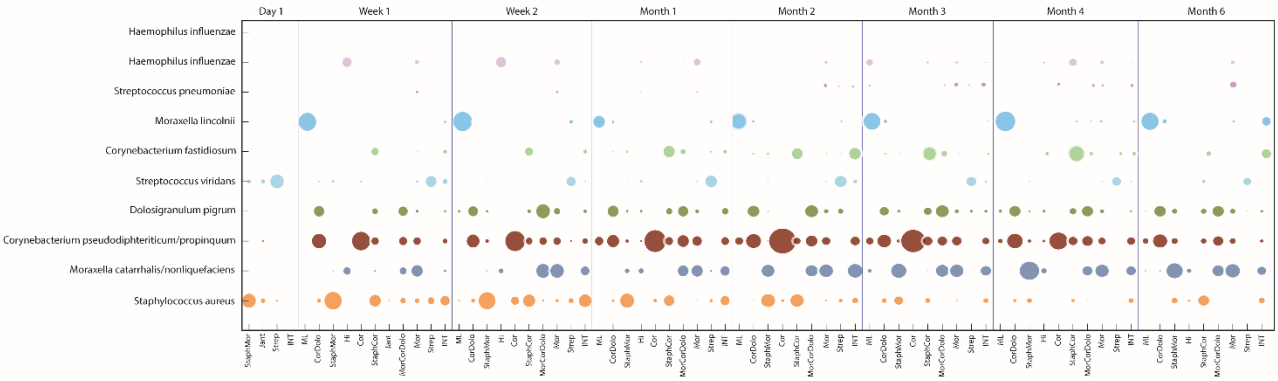


Figure S7 Magnitude of change in relation to clusters

For every time frame of two months (0-2 months, 2-4 months, and 4-6 months), we calculated the magnitude of change of microbial profiles by computing the norm value (L2 norm vector analyses(Biesbroek et al., 2014b; Harville, 1997)). Afterwards, we related the amount of relative change towards the cluster a child was going to (upper panels, e.g. the cluster at 2 months of age in the 0-2 months interval) or from which cluster a child was deviating from (lower panels, e.g. the cluster at 0 months of age in the 0-2 months interval). The higher the norm value, the higher the magnitude of change. Most noticeable is the cluster HI characterized by *Haemophilus influenzae* abundance, which is associated with a high amount of change both from and towards the HI cluster at all three time frames.

Abbreviations; Staph, *Staphylococcus*; Jant, *Janthinobacterium*; Strep, *Streptococcus viridans*; ML, *Moraxella lincolnii*; Cor, *Corynebacterium*; Dolo, *Dolosigranulum*; Mor, *Moraxella*; INT, Intermediate cluster; HI, *Haemophilus influenzae*.

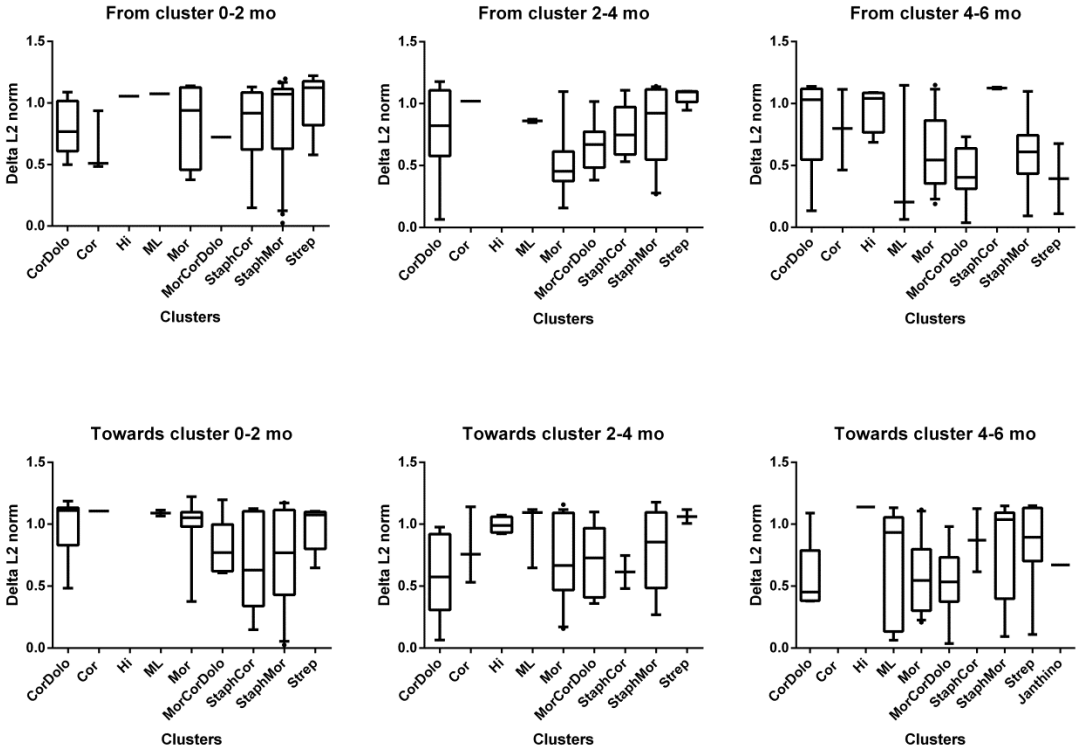

From cluster 0-2 mo
From cluster 2-4 mo
From cluster 4-6 mo
Towards cluster 0-2 mo
Towards cluster 2-4 mo
Towards cluster 4-6 mo
Delta L2 norm
1.5
1.0
0.5
0.0
CorDolo
Cor
Hi
ML
Mor
MorCorDolo
StaphCor
StaphMor
Strep
Janthino
Clusters

Table S1 Population description of the 102 children, subdivided by the mode of delivery (vaginal birth, n=62; C-section birth, n=40)

Of the 40 children born by C-section, 30 were born after a planned C-section and the other 10 were born following an emergency C-section because of fetal distress (n=5) and failure to progress/prolonged labor (n=5). The median duration of ruptured membranes was 5 hours in vaginally delivered children (range 0-44 hours), 9 hours for children born after an emergency C-section (range 0-48 hours), and 0 hours in case of an elective C-section.

	Children born by vaginal birth (n=62)	Children born by C-section (n=40)	P value
Boys (n,%)	32 (51.6)	19 (47.5)	NS
Birth weight (grams)	3522.9 (SD 481)	3624.5 (SD 476)	NS
Gestational age (weeks)	39.7 (SD 1.1)	39.1 (SD 0.9)	**P= 0.005**
Age mother by birth child (years)	33.7 (SD 4.5)	35.1 (SD 3.7)	NS
Breastfed > 3 months (n, %)	37 (59.7)	13 (32.5)	**P=0.009**
Season of birth (n, %)			NS
Spring	17 (27.4)	12 (30.0)	
Summer	21 (33.9)	9 (22.5)	
Autumn	11 (17.7)	9 (22.5)	
Winter	13 (21.0)	10 (25.0)	
Smoke exposure (n, %)	0	1 (2.5)	NA
Antibiotics use in children in first week of life (n, %)	2 (3.2)	2 (5.0)	NS
in first six months of life (n, %)	5 (8.1)	7 (17.5)	NS
Siblings under 5 years of age (n, %)	32 (51.6)	24 (60.0)	NS

Daycare from 4 months of age (n, %)	29 (46.8)	19 (47.5)	NS

Data depicted as number (percentage) or mean +/- SD.

Abbreviations; SD, standard deviation; NS, not significant; NA: not applicable.

Table S2 Sample description

Samples of 102 children were collected shortly after birth, 24 hours after birth and at the age of one and two weeks, and month one, two, three, four, and six. Due to logistic or personal reasons, samples were missing postpartum (n=4, 3 vaginally delivered children, 1 child born by C-section), day one (n=1, born by C-section), week two (n=1, born by C-section), month two (n=1, born by C-section), and month four (n=1, born by C-section). Of 93 (91%) of the children samples were collected at each time point, of eight (8%) children one sample was missing and only one child had two missing samples (postpartum and day one). We included only the samples with a bacterial DNA concentration with a sufficient amount of DNA and/or a significant different microbiota profile as compared with the blanks. In total, 84% of the collected samples were analyzed (297/355 samples of C-section born children and 464/555 samples of vaginally delivered children), nearly all of the excluded samples were collected postpartum and at day one.

Time point	Post-partum	Day 1	Week 1	Week 2	Month 1	Month 2	Month 3	Month 4	Month 6
Total number of samples	98	101	102	101	102	101	102	101	102
No of samples of sufficient quality	15 (15%)	45 (45%)	100 (98%)	101 (100%)	102 (100%)	99 (98%)	101 (99%)	98 (97%)	100 (98%)

Supplemental methods

Study population

Upper respiratory (nasopharyngeal) samples were obtained from healthy children that participated in an ongoing prospective birth cohort study with the primary aim to study the development of the respiratory microbiome in children born vaginally compared to children born by C-section. The cohort is a population-based study in the Netherlands, which is a small country in Europe (approximately 16.8 million inhabitants) with a high socio-economic status, educational level, and life expectancy comparable to other North-European countries like the UK, Germany, and Scandinavia. Inclusion criteria at baseline were term birth (gestational age >37 weeks). Exclusion criteria were major congenital anomalies, severe maternal or neonatal complications during birth, language barrier, intention to move outside the research area, or parents under 18 years of age. Since generally 15% of the children in the Netherlands are born by C-section (Delnord et al., 2014), we decided to enrich the enrollment for children born by C-section with the goal to enroll approximately 50%/50% children born by C-section and vaginal delivery respectively. In this study, we included the first 102 children that completed the six months follow-up (40 were born by C-section, 62 by vaginal birth). All children were born between December 19th, 2012 and June 16th, 2014. Written informed consent was obtained from both parents before birth of the child. Participants did not receive any financial compensation. An acknowledged national ethics committee in the Netherlands (METC Noord-Holland, committee on research involving human subjects) approved the study (M012-015, NH012.394, NTR3986). The study was conducted in accordance with the European Statements for Good Clinical Practice.

Data collection

At baseline, data were collected on prenatal and perinatal characteristics. Follow-up of participants in the current study included hospital visits directly post-partum and if necessary 24-36h after delivery, and home visits at day one, week one, week two, and at one, two, three, four, and six months of age. Deep nasopharyngeal swabs (Copan eSwab, 484CE) were collected in a semi-sterile setting and according to the World Health Organization guidelines (O'Brien et al., 2003). In short, nasopharyngeal swabs were introduced transnasally and passed backwards, parallel to the nasopharyngeal floor until the posterior pharynx was reached. In this position, the swab was rotated two times before it was slowly removed. After collection, samples were immediately snap frozen, transported on dry ice, and stored at -80°C in a sterile, filtered solution of 10% Glycerol (VWR international BV 1.04093.1000) in 0.1% DEPC water (SERVA Electrophoresis, 39798.03) until further analyses. Nasopharyngeal swab were collected during each visit by a trained research team of doctors and research nurses. At maximum, samples were collected within a time frame of two hours for the postpartum samples, 12 hours for day 1, two days for the other sample moments. During each home visit the research team completed an extensive survey on the health status of the child and potential environmental drivers of microbiota including breastfeeding. Patient records were consulted to extract perinatal data. In general, the antibiotics use in the Netherlands is considered to be low. In case of a C-section, one dose of prophylactic intravenous antibiotics was administered to mothers after the child was born according to the guidelines of the participating hospitals. Maternal antibiotic use for reasons other than prophylactic antibiotics in case of a C-section within the first week of the neonate's life was low (4/102 mothers, of which two gave breastfeeding at that time).

Construction of the phylogenetic library

Nasopharyngeal samples were further processed in a medical microbiological lab, within a laminary flow cabinet that was thoroughly cleaned before and after each DNA isolation. Gloves were worn during all handlings to minimize potential contamination with environmental DNA (Biesbroek et al., 2014a, 2014b, 2012; Wyllie et al., 2014). Bacterial DNA of the nasopharyngeal samples was isolated using a mechanic disruption method as described previously (Biesbroek et al., 2012; Wyllie et al., 2014). Only samples with a bacterial density at least 0.3 pg/µl above the background (negative control) as measured with Real-Time PCR were analyzed to avoid interference of background DNA. Since the samples collected postpartum and at day one generally had very low DNA density, we further analysed 12 samples collected postpartum and at day one that did not meet our primary criteria (< 0.3 pg/µl more DNA than paired negative controls) but that generated amplicon by our 16S-qPCR method. We used complete linkage hierarchical clustering based on the Bray-Curtis dissimilarity matrix (log10+1 transformed relative abundance) to analyse these samples in the context of a broad set of negative controls that had also generated >200 reads in our diverse Miseq runs (n=16). By this method, we were able to identify nasopharyngeal samples that had significantly different microbiota profiles compared to the negative controls (i.e. blanks) (n=9) and samples that did not (n=3). Based on these results, we included the first group in and excluded the latter samples from further analyses. After amplification of the hypervariable V4 region of the 16S-rRNA gene, samples were sequenced by Illumina MiSeq (Illumina Inc., San Diego, CA, USA) and processed using modules implemented in the Mothur software platform, version 1.31.2 (Schloss et al., 2009) and Btrim (Kong, 2011). First, reads were checked quality trimmed (quality threshold 30) by using the ‘Btrim’ command. Next, read pairs were merged (‘make.contigs’), and merged reads with a length of 240-260 base pairs were aligned (‘align.seqs’) to a SILVA reference database.

Following, a region from the alignment was selected in such a fashion that at least 90% of all merged reads completely covered this region. The length of the reads was trimmed to the boundaries of this region. Subsequently, chimeric sequences ('chimera.uchime') and sequences that were present less than ten times were removed (Schloss et al., 2009). The unsupervised sequence clustering method Minimum Entropy Decomposition (MED) was used to assemble the sequences into high-resolution oligotypes ((Eren et al., 2014) and oligotyping.org; default settings except for minimal substantive abundance=100). MED is based on the assumption that phylogenetically important nucleotide variations most likely occur at specific positions, whereas sequencing errors are more likely to be randomly introduced. Therefore MED allows to concatenate on high-information nucleotide positions and is able to discriminate phylogenetically highly similar, but ecologically and biologically different taxa (Eren et al., 2014, 2013). Oligotypes occurring less than four times over all samples were removed. Taxonomic classification of oligotype node representatives was performed using a naive Bayesian RDP classifier and classification reliability was assessed by bootstrapping (60% confidence threshold, 1000 bootstraps). We calculated the relative abundance of oligotypes per sample and determined the Shannon diversity index to describe the microbial diversity.

Analyses

For all analyses, we used normalized microbial abundance. Differences in baseline characteristics and metadata were statistically tested using the 2-sided Chi-square or Fisher's exact test for dichotomous data and Students T-test for continuous data (SPSS version 21). P-values <0.05 were considered significant. Calculations on Shannon diversity and observed oligotypes were performed per time point using Kruskal-Wallis and corrected for multiple testing (Dunn's multiple comparison test) in GraphPad (version 6).

Nonmetric multidimensional scaling plots based on Bray-Curtis dissimilarity of log-transformed relative abundances were used to visualize the differences between mode of delivery and time-dependent microbiota development. Statistical significance of the difference in the overall microbiota composition driven by mode of delivery was assessed using PERMANOVA.

To analyze the trajectory of the microbiota composition of individuals over time, an unsupervised co-regularized spectral clustering algorithm was applied on the data of all 102 children. A detailed description of the clustering method can be found elsewhere (Biesbroek et al., 2014b; Imangaliyev et al., 2015; Tsivtsivadze et al., 2013). In short, this multiview clustering algorithm allows for 1) identification of clusters comprised of individuals with similar microbial profiles in an unbiased and robust manner and 2) detection of bio-marker species by using unsupervised feature selection approach (Tsivtsivadze et al., 2014). We can further derive probabilistic assignment to the cluster using methodology as described by Cornelisse et al. (Cornelisse et al., 2012). In short, the probabilistic assignment to a particular cluster varies between zero and one. The higher the probabilistic score, the higher the assignment of a child to a cluster. First, we used a probabilistic likelihood of 0.5 as cut-off value to define clusters. Next, we used a probability of 0.8 to assign an individual to a corresponding cluster to identifying more stringent clusters. Cluster names were assigned based on the biomarkers identified (Figure S6). The most abundant biomarker-species appeared to be the major drivers of the clusters. The only exception was the cluster STAPH-MOR, this cluster is at the early age dominated by *Staphylococcus aureus* from where a gradual switch to *Moraxella catarrhalis/nonliquefaciens* was observed over time (Figure S5). Additional analyses on both the total set of samples (with adjusted parameters) and zoom-in analyses of only this cluster showed similar results, suggesting

that either minor species are the major driver of this cluster or that this cluster is a transition cluster.

To describe intra-individual changes in relative abundance and presence of microbial species over time, we calculated a relative change matrix (Harville, 1997) per oligotype for two-month timeframes (i.e. time point month week one and time point month two; time point month two and time point month four; time point month four and time point month six). From this relative change matrix per timeframe, we calculated the magnitude of microbiota change (norm value) using L2 norm (Harville, 1997). In short, all the number were lifted by +1 to prevent division by zero, then we calculated the current change between a time point minus the previous time point, divided by the latter one for every time frame (so called relative change in percentages). The higher this norm value, the higher the magnitude of change in that time frame. We chose to use a two-month-interval to compare timeframes, since this technique is based on the relative change between time points and the uneven distribution of the time points (more time points at the begin, fairly larger intervals at the end of the follow-up) would make interpretation more difficult To assess the intra-individual changes between timeframes, we used Friedman's test with post-hoc Dunn's multiple comparison test.

To investigate whether there is statistically significant correlation between colonization trajectory (e.g. the change of the clustering profile in time) and the mode of delivery, we have conducted a randomization test as described previously (Biesbroek et al., 2014b).

To identify biomarker species that are associated with modus partus, we evaluated oligotypes that varied in relative abundance over time between vaginally and C-section born children independently of feeding type using natural spline regression models (R version 3.2.0, edge R-

package, (Storey et al., 2005) and tutorial "Extraction of Differential Gene Expression Version 2.0.0" March 2015). In short, we curated our dataset, excluding samples taken at the first two time points (at birth and at day 1) and only selecting oligotypes with a mean relative abundance of >0.5% over all individuals at all time points. For each oligotype that passed the abundance filter, a null model including birth mode, feeding type and age (natural spline fit with three degrees of freedom) and a full model, additionally including the interaction between age and birth mode were fitted and the statistical significance of the difference between these models was assessed using a likelihood ratio test (test statistic derived from null distribution based on bootstrapping techniques). We conducted the analysis described above for the total dataset (n=102 children) and for a subset of children who were exclusively breastfed or exclusively formula-fed during the study period (respectively n=19 and n=28 children). Resulting p-values were corrected for multiple testing using the Benjamini-Hochberg method. Q-values <0.05 were considered significant for the whole dataset and q-values <0.10 for the subset analyses. Significant results suggest there is a difference in relative abundance of a given oligotype driven by birth mode as time goes on.

To visualize results of the relative abundance and clustering, we used Excel (version 2011), Python (version 2.7.8, packages Numpy, Scipy, Scikits Learn, Matplotlib), R (version 3.1.2) package 'rChart' (Sankey plot), and Adobe Illustrator CS6. Time-course data and estimates of spline fits were visualised using the ggplot2 R-package (smoothing method 'loess').

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