Mode of delivery and weight shape the intestinal microbiome composition progression in preterm infants: results of a prospective study

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Abstract

Background: In preterm infants the intestinal microbiome differs markedly from term infants. However, it is unclear whether the microbiome follows infant specific maturation patterns or whether it is mainly characterized by varying states of dysbiosis. We investigated the development of the intestinal microbiome in extremely preterm infants over time by 16S rRNA amplicon sequencing. We analysed the first meconium and faecal samples from the 2 nd , 3 rd and 4 th weeks, and (clinical) metadata to identify the main factors influencing the microbiota composition development.

Results: The study included 41 extremely preterm infants (gestational age 25-30 weeks; birth weight (BW) 430-990g). Birth via Caesarean section (CS) was associated with placental insufficiency during pregnancy and lower BW. In meconium and in weeks 2 and 3 an increased combined abundance of Escherichia and Bacteroides (maternal aerotolerant fecal bacteria) was associated with vaginal delivery (p=0.039, p=0.0002, p=0.034, respectively) while Staphylococcus epidermidis (skin bacterium) was associated with CS (p=0.001, p=0.0003 p=0.048, respectively). Secondly, a switch was observed from a microbiome dominated by S. epidermidis (Bacilli) towards a microbiome dominated by Enterobacteriaceae (Gammaproteobacteria, mainly represented by Klebsiella and Escherichia), in which the stage of progression appeared to be dependent upon the current weight of the infant, irrespective of the week of sampling or the mode of birth.

Conclusions: Our data shows that the mode of delivery does affect the meconium microbiome composition. It also suggests that the weight of the infant at the time of sampling is a better predictor for the stage of progression of the intestinal microbiome development/maturation than gestational/postnatal age. We hypothesize that impaired growth, for example due the effects of diminished placental function during pregnancy, is a key factor in the maturation of the intestinal microbiome in extreme premature infants.

Introduction

Extreme prematurity involves high mortality and morbidity [1]. Diseases such as necrotizing enterocolitis (NEC) are related to prematurity with aberrant gut microbiome colonization patterns [2–4]. For example, NEC has been associated with a particular group of clostridial species closely related to *Clostridium perfringens* in many studies, while staphylococci appear to be associated with a decreased risk for NEC development [5–13].

Previous studies on the intestinal microbiome of preterm infants, using 16S rRNA-based sequencing technologies, have revealed remarkable differences with the microbiome of term infants, including higher abundances of bacilli and Gammaproteobacteria [14–26]. The process of microbiota maturation, a pattern where microbiome maturation is mirrored by the maturation of the infant, is important in understanding differences between the microbiome of preterm- and term infants. In preterm infants, the

prolonged absence of bacteria that usually colonize and protectterm infants (bifidobacteria) is a clear indicator that the intestinal maturation process is either severely disturbed or altered [5,6,21,26–29].

Besides host biology, such as low birth weight (BW) and immaturity of the multiple organs including the gastrointestinal tract as a result of low gestational age (GA), there are multiple other exogenous factors that could affect the intestinal microbiome development/maturation of preterm infants (i.e. mode of delivery, neonatal feeding regime, the neonatal intensive care unit (NICU) environment and peri- and postnatal antibiotic exposure) [5,6,14,16,19,29].

While interest in this subject is growing, the focus of research is typically, with a few exceptions [15–19, 23], on the relation between the intestinal microbiome of the preterm infant and disease instead of on microbiome composition development itself. We aimed to determine whether the early microbiome development in extremely premature infants born between 25 and 30 weeks of gestation is dependant by infant maturity/maturation or whether it is mainly characterized by varying states of dysbiosis. Secondly, we aimed to analyse whether exogenous factors were intricately linked with microbiome maturation.

Results

Patient characteristics

In this study 41 preterm born infants were included. The GA ranged between 25 and 30 weeks (median 27.6 weeks IQR 26.0–28.1). Table 1 summarizes the baseline characteristics. Detailed patient characteristics per week are shown in Table 2. Important for understanding this cohort, the mode of delivery was strongly associated with the birth weight Z-score for GA and gender (Figure 1a). Infants delivered by CS had a higher GA on average than their vaginally born counterparts (median 28.1 IQR 26.7–28.2) vs 26.0 IQR 25.3–26.9 weeks, p = 0.002), yet were of lower BW (median 778 IQR 641- 923 vs 835 IQR 800–980 grams, p = 0.03). Underlying causes of prematurity for all vaginally delivered infants (13/13) were intra-uterine infection, (prolonged) premature rupture of membranes and/or cervix insufficiency while most infants delivered by C-section were born preterm because of placental insufficiency (22/27) (Table 1). Placental insufficiency was negatively associated with the Z-score for birthweight (p = 0.0005, Figure 1b).

Intestinal microbiome development over time

We analyzed 142 samples (3.5 samples per patient on average, range 2–4), including the first meconium. An overview of the abundances (% of reads per sample) of the most important bacterial groups (species/genera) in these 4 timepoints is shown in Figure 2. Within meconium samples *Staphylococcus epidermidis* was most frequently the most abundant bacterial species (11/37 samples, median 14.6% IQR 1.0%–39.8%), followed by *Klebsiella oxytoca* (10/37 samples, median 11.4% IQR 1.0%–31.0%). On the family level *Enterobacteriaceae,* mainly represented by *Klebsiella oxytoca, Klebsiella pneumonia and Escherichia coli* were the most abundant (20/37 samples, median 37.2% IQR 3.5%–58.0%) in meconium.

In week 2 a large increase in the abundance of staphylococci was observed in this cohort with *S. epidermidis* becoming the dominant species in the majority of samples (20/37 samples, median 55.9% IQR 13.8%–82.7%). A decline however of *S. epidermidis* is observed in weeks 3 and 4 (median 5.2% IQR 0.2%–26.5% and median 2.3% IQR 0.2–11.0%, respectively) with *Enterobacteriaceae* becoming dominant again in most samples (median 53.3% IQR 1.5%–74.6% and median 63.2% IQR 0.4–92.5%, respectively). Bifidobacteria and lactobacilli were groups of minor importance within this cohort.

Mode of delivery and intestinal microbiota development

One of the most striking patterns in the data, alluded to in Figure 2, is that the mode of delivery has a significant influence on the microbiome composition. This was most evident within the first three weeks after birth (Figure 3). Abundance of *S. epidermidis,* a typical skin bacterium, was significantly associated with CS delivery in samples from the first 3 weeks of life ($p = 0.001$, $p = 0.0003$ $p = 0.048$ and $p = 0.22$ for weeks 1–4, respectively). The combined prevalence the facultative anaerobe *E. coli* and members of the aero-tolerantanaerobic *Bacteroides* genus (*B. fragilis, B. vulgatus, B. dorei, B. thetaiotaomicron, B. uniformis & B. ovatus)*„ typical maternal fecal representatives, were significantly associated with vaginal delivery during these first 3 weeks ($p = 0.039$, $p = 0.0002$, $p = 0.034$ and $p = 0.95$ for weeks 1–4, respectively).

Mode of delivery and infant weight development

In this cohort the mode of delivery was significantly associated with BW and GA (Figure 1). The association between the infant's current weight at sampling time and the mode of delivery remains largely unchanged during the first four weeks ($p = 0.04$, $p = 0.03$, $p = 0.02$ and $p = 0.04$ for weeks 1–4, respectively), as no difference was present in weight gain in g/week of infants delivered vaginally and by CS ($p = 0.9$; $p = 0.8$; $p = 0.6$ for weeks 2–4, respectively). Z-scores from BW were strongly correlated with absolute weight in all 4 weeks (Spearman correlation coefficients of 0.70, 0.67, 0.66 and 0.52, respectively).

Infant weight and intestinal microbiome development

While the mode of delivery was the most important determinant for the infants' initial microbiome (Figures 2 and 3), the increase of *Enterobacteriaceae* in weeks 3 and 4 appeared associated with absolute weight at sampling time (Figure 4). When comparing infants with an above-median weight (835g) with their lighter counterparts, little difference was observed in the microbiome composition of the meconium (week 1). In week 2, infants with an above median weight (860g) contained significantly less *S. epidermidis* ($p = 0.013$). This particular association was however partially indirect, as infants delivered by CS had a lower median BW and were more frequently colonized during delivery by *S. epidermidis (*Figure 3a). The weight of infants increased from a median of 860g in week 2 to 969g in week 3 to 1095g in

week 4, respectively. In week 3 *Enterobacteriaceae* became more dominant again at the expense of *S. epidermidis,* as the influence of the mode of delivery declined. In week 4 the correlation coefficient between abundance of *Enterobacteriaceae* and current weight was significant (= 0.4, p = 0.04). More specifically, at a body weight of >1100g nearly all samples were dominated by *Enterobacteriaceae.* In general, the absolute weight of infants, irrespective of the week of sample collection, appeared to be (independently) associated with the shift from a *S. epidermidis* dominated gut towards one dominated by *Enterobacteriaceae* (Figure 5). When combining all samples from all 4 timepoints, *S. epidermidis* was negatively associated with absolute weight measured at sampling time (= 0.39, p = 0.000001) while the abundance of *Enterobacteriaceae* correlated positively with absolute weight measured at sampling time (= 0.25, p = 0.003).

Intestinal microbiota development and health

While staphylococci were associated with low absolute weight in this cohort, they did not appear detrimental to health as 1) the amount of weight gain during any single week did not correlate with the gut microbiome composition (or any of the individual species) at the start of that week and 2) their previously reported negative association with necrotizing enterocolitis development was similarly found within this cohort in meconium samples (p = 0.034). Overall infant mortality in this cohort, in part caused by necrotizing enterocolitis (n = 8 in total; 3 caused by NEC), was not significantly associated with the gut microbiome composition or with BW (p = 0.36) but it was negatively associated with GA at birth (p = 0.005) and positively but not significantly (p = 0.08) with prolonged premature rupture of membranes (PPROM).

Exogenous factors and microbiota development

Exogenous factors such as antibiotics use and/or feeding regime were found to be of ancillary importance in comparison with patterns associated with the mode of delivery or with absolute body weight. Associations of bacterial groups with antibiotics use were either found to be non-significant or disappeared when adjusting the analyses for mode of delivery. The current number of subjects was insufficient to unravel significant associations between microbiome development with antibiotics and feeding regimes.

Discussion

This study, which prospectively investigated the development of the microbiome of extremely preterm infants during the first four weeks of life, has three main findings. First, confirming current data [30], differences caused by mode of delivery have a strong but transient influence on intestinal microbiome composition. Importantly, this effect can already be observed in the meconium, in contrast to findings of others [31] who perhaps do not properly correct for reagent contamination in low biomass samples [32]. Second, our data suggest that there are weight thresholds, which determine the stage of the progression

in maturation and development of the colonization process. There is a transition from a microbiome with a high abundance of {1} staphylococci (bacilli) in extremely preterm infants (<1000 g) towards one dominated by {2} *Enterobacteriaceae* as they gain weight. Third, this study reveals that current absolute weight is a better marker for the maturation of the infants' intestinal microbiome than postconceptional age as it less confounded by various infant-specific factors.

Recently, La Rosa et al [26] and Korpela et al [33] both described the hypothesis that the gut microbiome of the preterm infant appears to follow a patterned progression linked with postconceptional age as a key marker for host biology / maturity. La Rosa, et al. [26] describes a progression from {1} bacilli to {2} γ-Proteobacteria (*Enterobacteriaceae)* to {3} clostridia (and Negativicutes). In this hypothesis, the place where preterm infants step into this progression is mainly dependent on their GA at birth [26]. A longer follow-up study would have seen a progression into {4} *Bifidobacterium* (and/or *Bacteroides)*.. Our study, which focused on the first 4 weeks of life of extremely preterm infants, confirmed the first part of this transition, namely the progression of a staphylococci (bacilli) dominance tot an *Enterobacteriaceae* dominated microbiome in extremely preterm infants. We however found that gestational/postnatal age was an inaccurate descriptor of maturity/host biology with regard to the development of the gut microbiome. A reason for this inaccuracy is highlighted by our comparison of infants delivered vaginally or by CS. In our cohort CS delivered infants had on average a higher GA at birth but a lower BW (Z-score) than their vaginally delivered counterparts (Figure 1). In this dataset the type of pregnancy complication (placental insufficiency vs intra-uterine infection/spontaneous preterm birth) was not only significantly associated with the mode of delivery but also with Z-scores for BW, representing fetal growth restriction.

In the cohort observed by Ho et al. [16] they similarly found that bacilli and *Enterobacteriaceae* formed the dominant groups but they ascribed their findings to a dichotomous development of the gut microbiome. The dichotomous development of the gut includes one cluster (I) of samples starting off with a high abundance of staphylococci which in time gave way to an increased abundance of *Enterobacteriaceae* and the other cluster (II) starting off with a high abundance of *Enterobacteriaceae* that declined slowly as clostridia became more prominent [16]. The developments in these two clusters fit perfectly into the aforementioned patterned progression if weight is used as a marker of intestinal microbiome maturity instead of gestational/postnatal age. Instead of a threshold for GA, which La Rosa [26] suspected, there might be a weight-determined threshold, which influences the gut microbiome maturation. In the cohort of Ho et al., infants from cluster I and II had a similar GA at birth on average (28.0 vs. 27.9) but infants assigned to cluster I had a BW of 1053 g while those assigned to cluster II had a BW of 1176 g [16]. In our study we found that the switch (threshold) between staphylococci and *Enterobacteriaceae* was particularly evident around 1100 g, consistent with the difference between these 2 clusters.

The main driver for this difference is the cause of prematurity; placental insufficiency is typically accompanied by C-section and is logically negatively associated with lower BW Z-scores due to fetal growth restriction of the infant. Furthermore, some infants thrive and concomitantly gain weight after birth while others do not, although both increase equally in gestational/postnatal age. We observed that absolute weight, as a logical key marker for host biology, does not suffer from these confounders and

could be used to clearly visualize the patterned progression of {1} staphylococci to {2} *Enterobacteriaceae* (Figure 5) in this dataset. The underlying mechanism why weight influences the gut microbiome maturation is not understood. We hypothesize that fetal growth restriction / low (birth) weight influences immune response modulation and altered intestinal development (i.e. influencing maturation of paneth cells, mucus production) that could cause weight dependent microbiome maturation differences [34].

Exogenous factors other than the mode of delivery such as antibiotics, neonatal feeding regime were also analyzed with regard to their association with the intestinal microbiome but no significant correlations were found in this cohort. While such external factors have been found to be associated with certain aspects of the gut microbiome development, they do not appear to represent the main drivers of gut maturation in preterm infants. More samples would be required to study these factors. Maternal data, such as the results of vaginal swabs and/or the use of maternal antibiotics, were not available for this study but could be of possible relevance for initial colonization.

This study highlights that the intestinal gut microbiome development in the extremely preterm infants is mainly driven by weight but is initially strongly affected by the mode of delivery. It also stresses the importance of pregnancy complications with diminished placental function as it directly affects the actual physical maturity level of the infant in which in turn directly affects the stage of progression [26; 33] from which the gut microbiome development starts. Underlying mechanism behind weight thresholds is not yet understood. There is a need for more knowledge on the affect of (birth) weight on immunological responses and organ maturation.

Before interventions are implemented, such as targeted antibiotic therapies or the use of pre- and probiotics, it is critical to understand which organisms are to be considered normal (part of the maturation process), in regards to the level of gut development of the infant at a certain weight, and which ones are indicative of potential dysbiosis/disease. For example, *K. oxytoca* or *K. pneumonia,* which belong to the family *Enterobacteriaceae,* have been frequently associated with, amongst other things, sepsis in newborns, but they appear to be a ‘normal’ part of the patterned progression of the bacteria composition as the infant gut matures [16, 26]. Trials with i.a. strain resolved metagenomic analyses of a larger number of samples might show that only particular *Klebsiella* strains are to be associated with disease directly or indirectly. Colonization with *Klebsiella* appears to be normal whereas sepsis with *Klebsiella* might merely be a symptom of other things going wrong in the preterm infants’ gut [35, 36]. Our results underline the importance of larger multi-center observational studies to reveal the exact intestinal microbiome maturation of the extremely preterm infant and its underlying driving factors, such as immunomodulation.

Conclusion

During the first four weeks of life the gut microbiome of extremely preterm infants (birth weight < 1000) undergoes a transition from a gut microbiome with a high abundance of staphylococci towards one dominated by *Enterobacteriaceae.* This study improves the patterned progression hypothesis [26] by

stressing the importance of the infants' absolute weight at sampling time above gestational/postnatal age as an accurate marker of host biology and maturation. These findings were independent of pregnancy complications with diminished placental function. We hypothesize that weight thresholds determine the stage of the progression in maturation and development of the colonization process. Exogenous factors were in this cohort, apart from the strong yet transient effect of the mode of delivery, of minor importance.

Methods

This study is part of a prospective observational trial (CALIFORNIA trial; registered as NTR4153 in the Dutch trial registry, and approved by the Medical Ethics Committee of the University Medical Center Groningen), which studied infants for developing NEC [5,6,37–40]. The CALIFORNIA trial included 100 infants admitted to the Neonatal Intensive Care Unit of the University Medical Center Groningen between October 2012 and December 2014, after informed consent of their parents was given. Infants born at a GA of ≤ 30 weeks and born with a BW of ≤ 1000 grams were applicable for the study. Patients with other abdominal diseases (abdominal wall defects or congenital intestinal atresia) were excluded from this trial.

Patients

We selected infants from the CALIFORNIA trial who were born at a GA of ≤ 30 weeks and / or who had a BW of ≤ 1000 grams, from whom more than two samples during the first 4 weeks were available. Patient characteristics and demographic variables were derived from the CALIFORNIA database.

Demographic and clinical variables

Data from each sample day were used. Variables consisted of mode of delivery, BW, GA at birth, z-score BW (which represents the standard deviation in SD units from the Dutch reference growth curves) [41], bodyweight on the sample day, the administration of mothers' milk and / or of formula milk in milliliters/kg on the sample day, the antibiotic use on the sample day and if antibiotics were administered for more than 48 hours after birth. Complications during pregnancy were classified as placental insufficiency (pre-eclampsia/HELLP, fetal growth restriction and fetal distress) and intra-uterine infection/spontaneous preterm birth (chorioamnionitis, PPROM, cervical insufficiency and premature contractions).

Faecal samples

We intended to analyze one fecal sample per week, starting with the first meconium, and afterwards the first fecal sample of every week during the first four weeks after birth. Faecal samples were stored at—

80°C prior to the start of this study. The first sample of each week was used for analyses of the current sub-study.

DNA extraction and sequence library preparation

Faecal DNA was extracted from a 0.25g faecal sample by double bead beating in combination with the QIAamp DNA Mini kit (Qiagen; Hilden, Germany), as described by a study that used the same technique [42]. Polymerase chain reaction (PCR) amplification targeted the V3 and V4 region of the 16S rRNA gene by using modified 341F and 806R primers [43–44]. The 806R primers contained a 6-nucleotide barcode. An detailed description of the PCR reaction, DNA cleanup, and MiSeq library preparation is found in the Appendix file 1.

Analyses of sequence reads

The software used to analyze the data received from Illumina paired-end sequencing included PANDAseq [45] and ARB [46]. Readouts with a quality score lower than PANDAseq as standard practice to increase the quality of the sequence readouts discarded 0.9. ARB was used to identify sequences to the species level. As particularly meconium samples, but also many later samples have low bacterial biomasses, reagent contamination needs to be accounted for. Reagent contamination recognition analyses were subsequently performed as described by de Goffau et al [42], using the Spearman's rank correlation coefficients method. The consistency of the ratio of reagent-derived species within samples allows for their rapid identification. As a result, all reads identified as *Undibacterium oligocarboniphilum, Variovorax paradoxus, Sphingomonas oligophenolica, Ralstonia pickettii, Curvibacter lanceolatus, Ralstonia insidiosa, Erythrobacter aquimaris, Afipia* genosp., *Ochrobactrum pseudintermedium, Sphingomonas mucosissima, Brevundimonas vesicularis, Arthrobacter russicus* and *Pelomonas saccharophila* were removed before further analysis.

Statistics

Statistical analyses were conducted with IBM SPSS Statistics 21.0. Combinations of principal component analysis (PCA), regression and paired analyses were performed to examine the relationship between the microbiota and the following factors: mode of delivery, the birth weight, gestational age at birth, z-score birth weight, bodyweight on the sample day, the administration of mothers' milk and / or of formula milk in milliliters/kg on the sample day, the antibiotic use on the sample day and antibiotic use for more than 48h after birth. Two sided P-values less than 0.05 were considered statistically significant. Unless otherwise indicated, the Mann-Whitney-U test or Chi-square test were used to test differences between groups. Testing of the correlation between parameters was done with the Spearman's correlation test, while one-way-ANOVA was used to assess individual parameters development in time.

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Declarations

Clinical trial informative: see attachment

Ethical approval: This study is part of a prospective observational trial (CALIFORNIA trial; registered as NTR4153 in the Dutch trial registry, and approved by the Medical Ethics Committee of the University Medical Center Groningen),

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Conflict of Interest: No conflicts of interest have to be disclosed.

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Availability of data and materials:

The datasets during and/or analyzed during the current study are available on reasonable request.

Author consent and contribution:

F.H. Heida: Dr. Heida conceptualized and designed the microbiome study, performed data analysis, drafted the initial manuscript, and approved the final manuscript as submitted.

E.M.W. Kooi: Dr. Kooi, performed data analysis, reviewed and revised the manuscript and approved the final manuscript as submitted.

T.Y. Nguyen: BSc Nguyen performed the DNA extraction of the fecal samples, reviewed and revised the manuscript and approved the final manuscript as submitted.

J.B.F. Hulscher: Dr. Hulscher conceptualized and designed the study, supervised the study, reviewed and revised the manuscript and approved the final manuscript as submitted.

G.J.F. van Zoonen: Dr. van Zoonen designed and conceptualized the CALIFORNIA study, performed sample collection, reviewed the manuscript and approved the final manuscript as submitted.

A.F. Bos: Prof. dr. Bos conceptualized and designed the study, reviewed and revised the manuscript and approved the final manuscript as submitted.

H.J.M. Harmsen: Dr. Harmsen supervised the study (laboratory), reviewed and revised the manuscript and approved the final manuscript as submitted.

M. C. de Goffau: Dr. de Goffau performed data analysis (including statistical analysis), supervised the microbiome study, reviewed and revised the manuscript and approved the final manuscript as submitted.

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Tables

Due to technical limitations, Table 1-2 are provided in the Supplementary Files section.

Figures

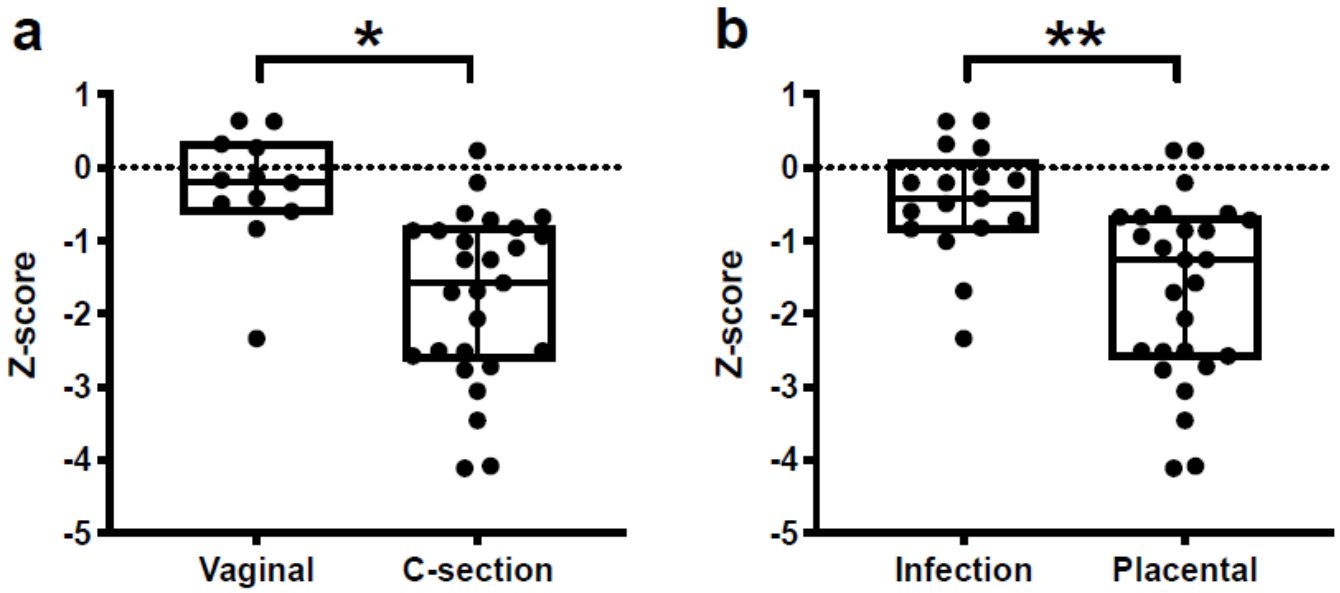

Figure 1

Boxplot of the relation between birthweight z-scores and the a) mode of delivery and b) the underlying cause of prematurity

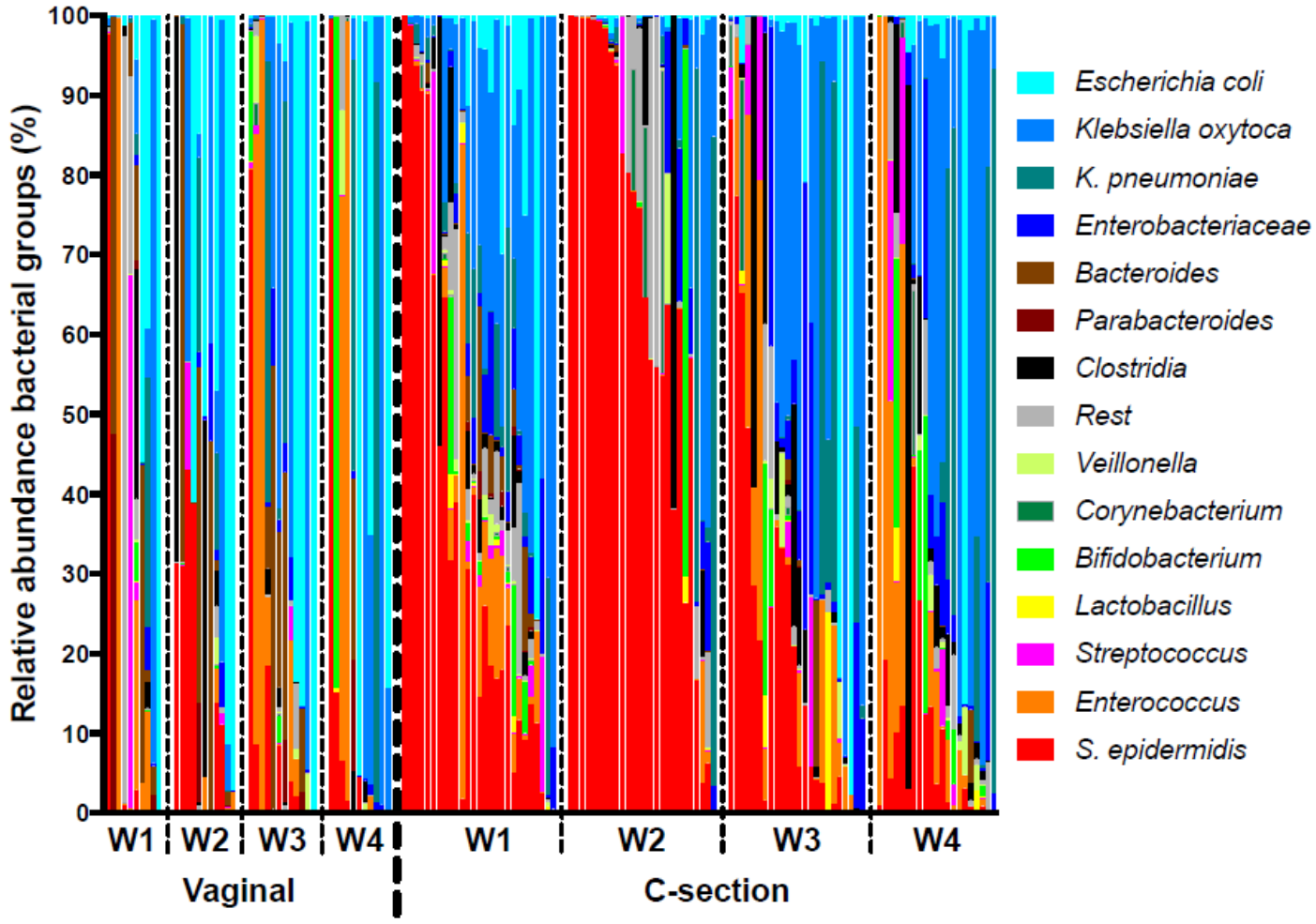

Figure 2

Overview relative abundance main bacterial groups per week (W1-4) in preterm infants delivered vaginally or by CS

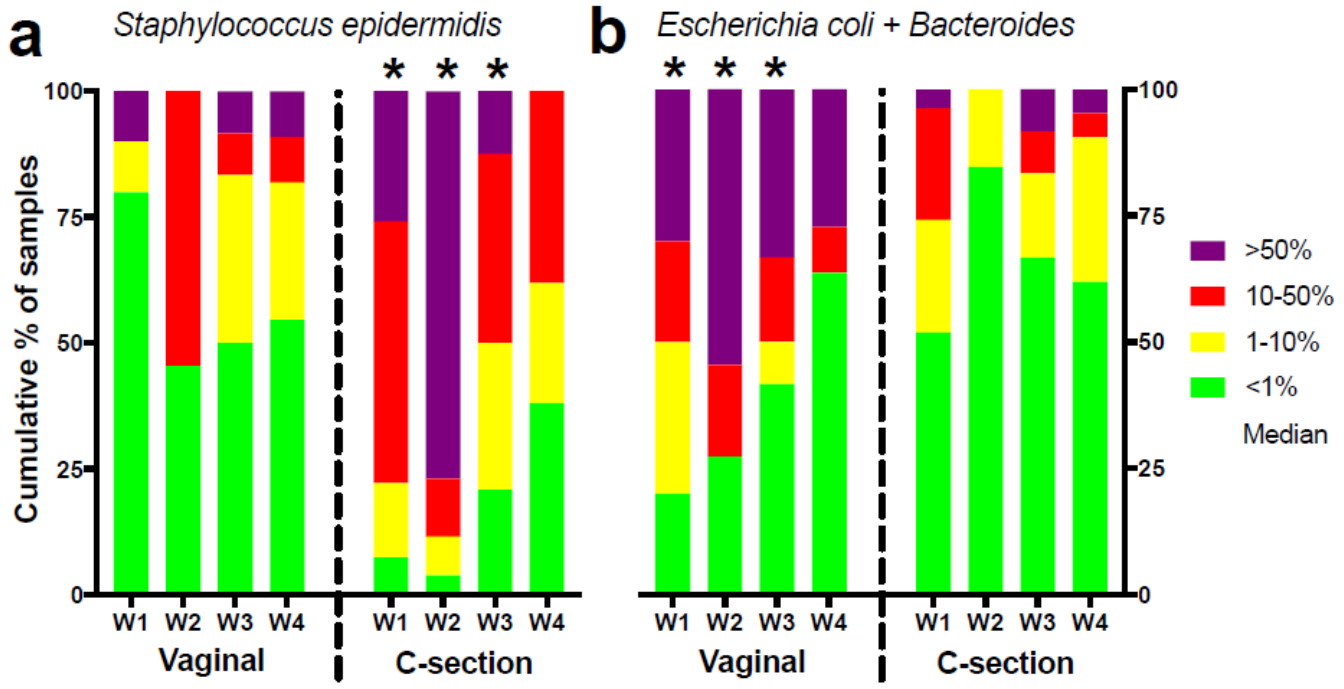

Figure 3

Prolonged effect of the mode of delivery on the faecal microbiota composition during the first weeks of life of preterm infants

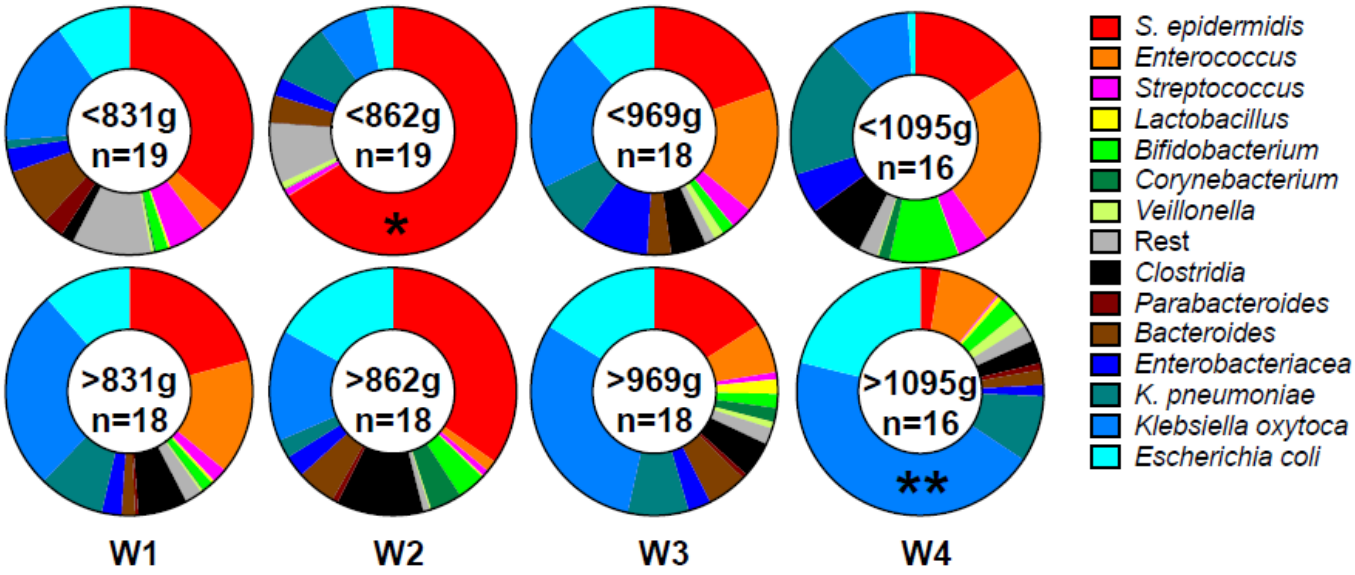

Figure 4

Average abundance of bacterial groups in samples below or above the median weight of each week

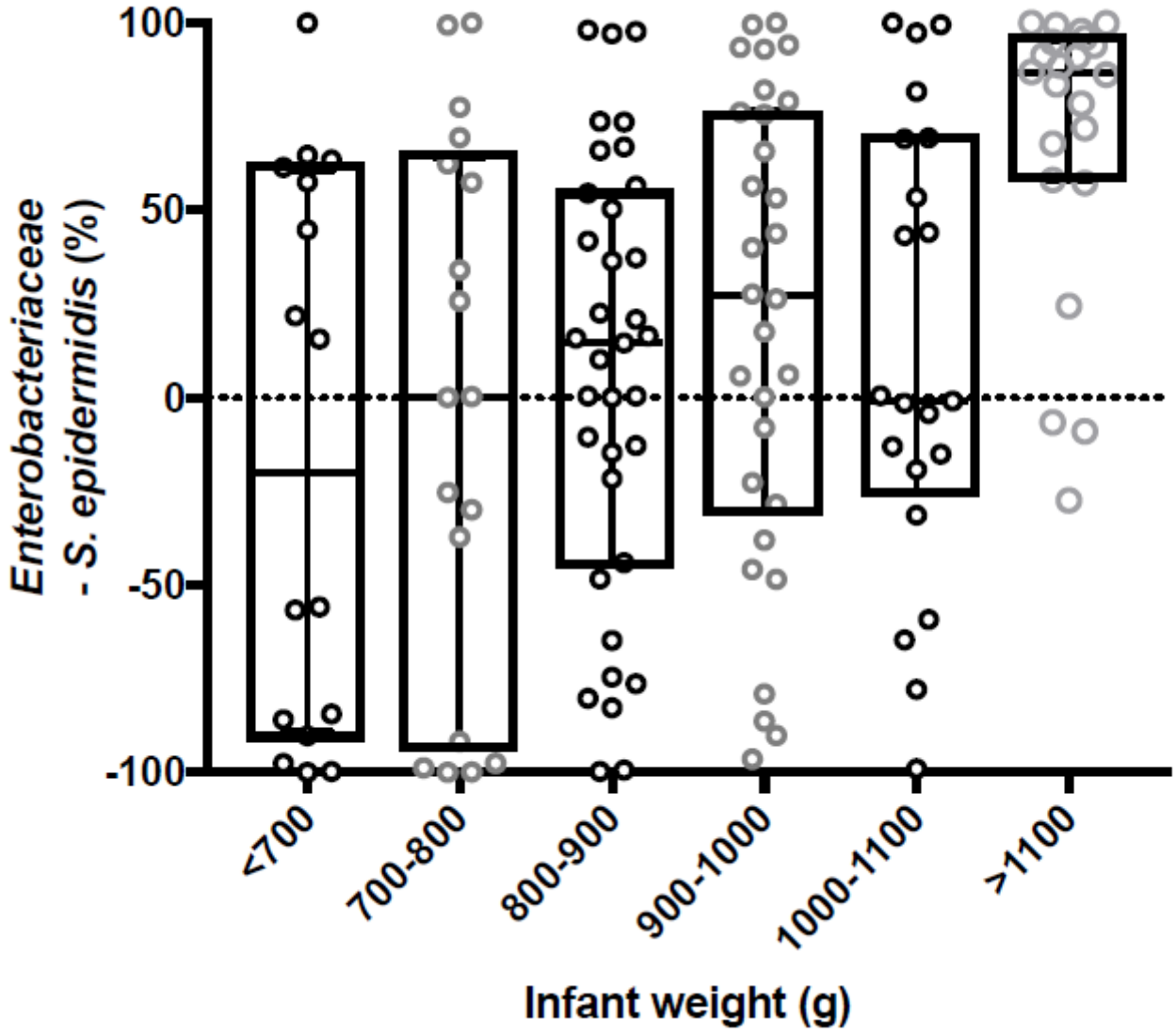

Figure 5

Boxplot of infant microbiota composition in relationship to infant weight

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table2.pdf
- SupplementalTable1.pdf
- SupplementalDatafile1.docx