Microbial community assembly and metabolite profile of the gut microbiome in extremely low birthweight infants

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Key-words: Preterm infant, intestinal microbiome, metabolome

Abstract

Background: The assembly of the intestinal microbiota of extremely low birthweight (ELBW) infants has an important impact on both immediate and long term health. ELBW infants are frequently given antibiotics which are likely to perturb the assembly of the microbiota. Health complications are not uncommon for ELBW infants; they face health crises including sepsis and necrotizing enterocolitis (NEC). Microbes are thought to be involved in the pathogenesis of NEC, but the mechanisms are unclear. New understanding of the importance of human milk oligosaccharides and the establishment of a *Bifidobacteria*-dominated gut microbiota early in infancy suggest that all preterm infants have abnormal microbial colonization. The initial assembly of intestinal microbial communities may have significant impact on immune development and lifelong health.

Results: We measured the bacterial composition and metabolite profile of 32 ELBW infants by 16S rRNA gene sequencing and untargeted gas chromatography mass spectrometry of fecal samples. Infants either remained healthy, developed late-onset sepsis, or developed necrotizing enterocolitis. The

bacterial compositions were similar to what has been observed in other studies of preterm infants. Fecal samples are dominated by aero-tolerant bacterial species, specifically *Enterococcus*, *Enterobacteriaceae*, and *Staphylococcus*. Only three ELBW infants were colonized by *Bifidobacteria*. Fecal samples from infants who developed NEC were not distinguishable from other infant samples based on bacterial compositions (Permanova $R^2 < 0.001$, $p = 0.99$) or metabolite profiles (Permanova $R^2 = 0.05$, $p = 0.24$). Instead the bacterial composition ($R^2 = 0.63$, $p < 0.001$) and metabolite profile ($R^2 = 0.43$, $p < 0.001$) were highly personalized for each infant. There were not significant correlations between the bacterial composition and metabolite profiles of fecal samples (Mantel test $r = 0.18$, $p < 0.001$).

Conclusions: Although antibiotics likely contribute to the instability of the ELBW infant intestinal microbiota, personalized signatures of bacteria and metabolites are still clearly present. Neither the bacterial composition or metabolite profile was unique in cases of disease. While bacteria certainly contribute to the profile of metabolites present in feces, in these ELBW infants, significant correlations between bacterial relative abundances as determined by 16S rRNA gene sequencing and untargeted GC-MS metabolite profiles were not detectable.

Background

The intestinal microbiota of infants initially assembles by exposure to the mother's microbiota as well as exposure to microbes in the environment [1]. In the first few days, the intestines are colonized by facultative anaerobes, which consume the oxygen in the intestines, creating an anaerobic environment that results in the growth of anaerobic *Bifidobacteria*, *Bacteroides*, *Lactobacillus* and *Clostridium* [2]. When infants are born preterm, they are exposed to environmental and human associated microbes earlier than normal. Moreover, preterm infants are almost uniformly treated with antibiotics, further

47 altering microbial exposure during a critical developmental window, with long-term metabolic
48 consequences even after the microbial community recovers from antibiotic treatment [3, 4].

49 While antibiotics have tremendously reduced infant mortality, their effect on microbiota assembly and
50 resulting health consequences is not fully understood. Prenatal and postnatal antibiotics have been
51 shown to reduce the diversity of the infant intestinal microbiota [5, 6]. Children under two years old are
52 prescribed antibiotics at a higher rate than any other age group, and 85% of ELBW infants are given at
53 least one course of antibiotics [7]. Even if an infant is not exposed to antibiotics after birth,
54 approximately 37% of pregnant women use antibiotics over the course of the pregnancy [8].

55 In healthy infants, breastfeeding is thought to select for strains of *Bifidobacteria* capable of digesting
56 human milk oligosaccharides [9, 10]. Throughout human history, healthy breastfed infant guts have
57 been dominated by *Bifidobacteria,* but modern sanitation and frequent use of antibiotics has led to
58 decreased colonization of infants with B*ifidobacteria* [10, 11]. Several studies profiling the intestinal
59 microbiota of preterm infants have shown *Bifidobacteria* to be largely absent [12, 13]. The lack of
60 *Bifidobacteria* in addition to frequent use of antibiotics on preterm infants may affect the microbiota in
61 a way that has both immediate and long-lasting health consequences.

62 In the short term, infants can be infected by pathogenic bacteria that results in sepsis, which is
63 categorized as early-onset or late-onset depending on the timing after birth. Preterm infants are also at
64 high risk to develop necrotizing enterocolitis (NEC), which is a devastating disease that causes portions
65 of the bowel to undergo necrosis. NEC is one of the leading causes of mortality in preterm infants, who
66 make up 90% of NEC cases. The incidence of NEC among ELBW preterm infants is approximately 7% and
67 causes death in about one third of cases [14]. NEC is thought to be caused by an excessive inflammatory
68 response to intestinal bacteria [15].

Many of the long-term consequences of microbial colonization are believed to be mediated by interactions between the intestinal microbiota and the immune system. The immune system extensively interacts with the intestinal microbiota by continuous sampling through the intestinal epithelium [16]. Evidence is building for associations between the microbiome and rising rates of obesity and type 2 diabetes [17-19]. Interactions between bacteria and the immune system are thought to contribute to autoimmune disorders including allergies, multiple sclerosis, irritable bowel syndrome, and Crohn's disease [20, 21]. Healthy microbial exposures could be important for reducing the incidence of these diseases, starting with a *Bifidobacterium longum* dominated gut in early infancy.

In addition to direct interactions, the microbiota interacts with the immune system through the production of metabolites that can be taken up directly by immune and epithelial cells. For example, bacterial production of short chain fatty acids can affect health and integrity of the intestinal epithelia and immune cells [22-25]. However, few studies use metabolites alongside bacterial community profiling.

In this retrospective study, we follow the changes in the gut microbiota over time in 32 ELBW infants born at Children's Hospital Orange County. We aim to simultaneously track the bacterial composition and metabolite profile to identify differences between healthy and sick infants. Almost all infants in the study received antibiotics. Infants were classified into three groups based on health outcomes: healthy controls, late-onset sepsis, and NEC. The composition of the intestinal microbiota was measured by 16S rRNA gene sequencing of fecal samples taken over time. Infant fecal communities were dominated by *Enterobacteriecae* and *Enterococcus*, while *Bifidobacteria* was almost completely absent. Untargeted metabolomics analysis of the fecal samples by gas chromatography mass spectrometry (GC-MS) was used to measure metabolite abundances to further characterize the activity of the intestinal microbiota. We found both the bacterial composition and metabolomics profile of each infant to be highly personalized rather than indicative of disease.

Methods

Sample Collection: Stool samples from preterm infants were collected by nurses at Children's Hospital Orange County (CHOC) for three years from 2011 to 2014 with approval from the CHOC Institutional Review Board (#100771). Samples were immediately stored at -20 °C then transferred to -80 °C no more than three days post-collection. Samples were kept at -80 °C and thawed once for DNA extraction and metabolomics. A total of 77 stool samples were collected from 32 preterm infants. Twenty-one infants remained healthy, three developed NEC, and nine developed late onset sepsis.

DNA extraction: DNA was extracted using a fecal DNA extraction kit (ZR Fecal DNA MiniPrep™ Kit, Zymo Research Corp, Inc.). Stool samples were partially thawed and between 10mg and 100mg of stool was scooped using a pipette tip. The extraction protocol was carried out as specified by the kit, with the exception of the vortexing step, which was done using a standard bench top vortex for 5 minutes. DNA was eluted in elution buffer and stored at -20 °C.

16S rRNA gene amplification and sequencing: The V3 to V4 sequence of the 16SrRNA gene was amplified with two-stage PCR to first attach Illumina adapters and then attach sample specific barcodes. The first PCR to amplify the V3 to V4 region of the 16S rRNA gene used forward primer (5'-CCTACGGGNGGCWGCAG-3') and reverse primer (5'- GACTACHVGGGTATCTAATCC -3'). These primers also contained Illumina adapters. The first PCR was done as follows: 30 cycles of 95 °C 30 seconds; 65 °C 40 seconds; 72 °C 1 minute. Immediately after completion of the first PCR, sample specific barcodes were added and a second PCR was performed as follows: 9 cycles 94 °C for 30 seconds; 55 °C 40 seconds; 72 °C 1 minute. PCR reactions were cleaned using Agencourt AMPure XP magnetic beads (A63880) using the recommended protocol. Amplicons were run on an agarose gel to confirm amplification and then pooled roughly by brightness of band. Pooled amplicons were run on an agarose

gel and the 500bp fragment was cut out and gel extracted using Millipore Gel Extraction Kit (LSKGEL050). The pooled library was quantified using Quant-iT Pico Green dsDNA Reagent and sent to Laragen Inc. for sequencing on the Illumina MiSeq platform with 250bp paired-end reads. We obtained 2.4 million paired-end reads.

*Sequence processing***:** Sequences were quality filtered using PrinSeq to remove adapters as well as any sequences less than 200 base-pairs, containing any ambiguous bases, or with a mean PHRED quality score of less than 23. These parameters resulted in discarding about 10% of reads from each sample. Overlapping forward and reverse reads were joined with PEAR. The requirements for joining reads were a minimum overlap of 20 base pairs and no more than 25% were mismatched. These parameters resulted in an additional 10-30% of reads being discarded. After quality filtering and merging of reads, 1.5 million reads remained. Quantitative Insights Into Microbial Ecology (QIIME) was used for closed reference OTUs picking at a 97% sequence similarity threshold with the UCLUST algorithm against the GreenGenes 13.5 database. An OTU table was constructed and used for downstream analysis. The OTU table was rarefied down to 2000 reads per sample, which was the largest number of reads that allowed us to keep most samples. The abundance counts were normalized by number of 16S copies in the genome of each species using PICRUSt.

Metabolite processing: When fecal samples were thawed for DNA extraction, approximately 50 mg was collected and refrozen at -80 ° for metabolomics. Samples were sent to the West Coast Metabolomics Center at UC Davis for untargeted metabolomics by gas chromatography time-of-flight mass spectrometry [26]. Metabolites were extracted from fecal samples with a 3:3:2 mixture of isopropanol, acetonitrile, and water respectively. Metabolite intensities were normalized by dividing each intensity by the average sum of all identified metabolites in all samples and multiplying by the sum of identified metabolites in the sample. Intensities were pareto scaled by metabolite.

Sequencing analysis: Bacterial composition plots based on the non-rarefied OTU tables were created using the ggplot2 package in R. The non-rarefied OTU table was used to make the composition plots since the samples were not being compared and rarefaction had little effect on the composition. QIIME was used to calculate Chao1 alpha diversity of each sample and UniFrac distances between each sample based on the rarefied OTU table. Unweighted UniFrac distances were visualized by PCoA using the vegan package for calculation and ggplot2 for visualization.

Metabolomics ordination: Bray-Curtis dissimilarity was calculated between each sample based on normalized, scaled metabolite intensities using the vegan package in R. Distances between samples were visualized by non-metric multidimensional scaling (NMDS).

PERMANOVA: Bacterial abundances and metabolite intensities were not normally distributed, so Permutational multivariate analysis of variance (PERMANOVA) was performed for statistical comparisons of samples. The inputs for PERMANOVA were UniFrac distances of the 16S data and Bray-Curtis distances of the metabolite abundances. Only infants with three or more longitudinal samples were included in PERMANOVAs. To determine if the bacterial composition of samples from infants with NEC were significantly different from samples from control or LOS infants, permutations assigned infants randomly to NEC of non-NEC, always assigning samples from an individual infant to the same group so that each permutation is equally biased by longitudinal sampling. To test if samples from an individual are personalized, every permutation randomly assigned samples to an individual with no restrictions. The adonis function in the vegan package in R was used to implement the PERMANOVAs.

Mantel test: To determine if fecal samples with similar bacterial compositions also have similar metabolite profiles, a mantel test was performed. To account for the effect of longitudinal sampling, each dataset was repeatedly subsampled down to one random sample per infant. A Bray-Curtis dissimilarity matrix was computed for both the metabolite abundances and bacterial abundances. The

mantel function in the vegan package of R was used to calculate the Mantel statistic for a Spearman correlation between the two dissimilarity matrices. Random selection of samples was repeated 100 times and a Mantel test computed for each resampling.

qPCR for bacterial load: The bacterial load of each fecal sample was measured with quantitative PCR of a conserved region of the 16S gene. The following primers were used: (5'- TCC TAC GGG AGG CAG CAG T-3') ; (5'- GGA CTA CCA GGG TAT CTA ATC CTG TT-3'). PerfeCTa SYBER Green SuperMix Reaction Mix (Quantabio #95054) was used on extracted DNA samples. Relative abundance of 16S rRNA genes relative to the mass of stool was compared for each sample. Total fecal DNA was measured with Quant-iT Pico Green dsDNA Assay Kit (ThermoFisher #P11496).

Results

Patient cohort : A total of 77 fecal samples were collected from 32 ELBW infants in the NICU at Children's Hospital Orange County (**Supp. Table 1**). Three infants developed NEC, eight developed late-onset sepsis, and 21 remained healthy. Three or more longitudinal samples were available from ten of the infants, while one or two samples were available from the remaining 22 infants. Nearly all infants received antibiotics around the time of sample collection, the most common being ampicillin and gentamycin. Twelve infants were delivered vaginally while the remaining 22 were delivered by cesarean section. All infants were fed by either breastmilk or a combination of breastmilk and formula.

Microbial Community Characterization : We sequenced the 16S rRNA gene content of each fecal sample to determine bacterial composition. The total bacterial load of each fecal sample was measured by qPCR of the 16S rRNA gene and scaled to the total weight of stool that DNA was extracted from. Among all infants, bacterial abundances vary over four orders of magnitude (**Supp. Figure 2**). Bacterial communities were composed of mostly *Firmicutes* and *Proteobacteria* (**Supp. Figure 1**). Most samples

were dominated by only one to three genus of bacteria (**Figure 1, Supp. Figure 3)**. The dominating taxa varied from infant to infant, but the most common were *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus*; all facultative anaerobes. The most common anaerobes detected were *Bacteroides* and *Viellonella*, although they were not present in all infants. Only three infants were colonized by *Bifidobacteria* with greater than 1% total relative abundance. Other typical anaerobic colonizers of infants seen in other infant studies such as *Clostridia*, and *Lactobacillus* were mostly absent. Longitudinal sampling revealed that over the course of days, the bacterial composition of these preterm infant's intestines could change dramatically, regardless of the overall health outcome of the infant.

The differences in overall bacteria composition of each sample were calculated by unweighted UniFrac distances and visualized by Principal Coordinates Analysis (PCoA) (**Figure 2**). Permutational Multivariate Analysis of Variance (PERMANOVA) shows that the bacterial composition of infants with NEC did not differ from that of healthy infants or infants that developed LOS ($R^2 < 0.001$, $p = 0.99$; **Table 1**). Alpha diversity as measured by Chao1 was not significantly different among sick and healthy infants ($p = 0.165$; **Supp. Figure 3**). While these infants' bacterial communities are prone to large-scale changes over time, they remain personalized. Samples taken from a single infant over time are more similar than samples taken from another infant ($R^2 = 0.63$, $p < .001$; **Table 1**).

Metabolomics : Metabolite profiles of infant fecal samples were also analyzed by gas chromatography mass spectrometry. Mainly small primary metabolites are detected by this method. Over 400 small molecules were detected from each fecal sample and 224 metabolites were confidently identified. Bray-Curtis dissimilarity between samples was computed and visualized by non-metric multidimensional scaling (NMDS)(**Figure 3**). PERMANOVA based on the dissimilarity matrix shows that the metabolite profiles of infants that develop NEC are not significantly different that other infants ($R^2 = 0.05$, $p = 0.24$; **Table 2**). Metabolite profiles were more variable over time in individual infants than bacterial abundances, but were still highly personalized ($R^2 = 0.43$,$p < 0.001$; **Table 2**).

209 Bacterial Composition is not correlated with metabolite profile : To test for a correlation between the
210 measured bacteria composition and metabolite profile, we used a mantel test with Spearman
211 correlations. The resulting Mantel statistic of r= 0.18 ($p < .001$) indicates that samples that are similar in
212 bacterial composition are not also similar based on metabolite profiles. To ensure results are not biased
213 by the particular random selection of infant samples, random selection and the Mantel test was
214 repeated 100 times. The 95 % confidence interval for the 100 Mantel correlations is r = 0.17 to 0.19. This
215 suggests the primary metabolite profile of fecal samples in preterm infants is not strongly driven by the
216 bacterial composition.

217

218 **Discussion**

219 Microbiota compositions in this cohort are consistent with an emerging picture that show preterm
220 infant intestines harbor unstable bacterial communities composed primarily of *Proteobacteria* and
221 *Firmicutes* [12, 27, 28]. Even though the bacterial compositions of infant guts varied over time, we saw
222 longitudinal samples from individual infants were significantly personalized over several weeks [29]. The
223 gastrointestinal tract of infants is not yet anaerobic like that of adults, and *Bifidobacteria* are limited due
224 to a combination of limited access, antibiotics and variable breastmilk consumption, so more aero-
225 tolerant taxa such as *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* dominated the infants in
226 this study as well as similar studies of the infant microbiota [2, 12, 27, 28]. Evidence is emerging that a
227 healthy infant gut microbiota is dominated by *Bifidobacteria* which are selected by their ability to digest
228 human milk oligosaccharides in breastmilk [9, 10]. The lack of a core *Bifidobacteria* community leaves
229 the microbiota open to colonization by facultative anaerobes [13].

230 Antibiotics impact the composition and abundances of the intestinal microbiota [30]. Even if microbiota
231 composition in adults largely returns to its previous state one month after antibiotic treatment, altering

the microbiota composition of infants in a critically important developmental period can have long lasting health consequences [3, 30-32]. Preterm infants are commonly given antibiotics, and all infants in this study received antibiotics around sampling time [33]. Antibiotic exposure likely contributed to the low bacterial diversities and variable bacterial loads observed in this study. Infants in this study that developed NEC or late-onset sepsis did not have a different bacterial load than healthy controls. Although the healthy control preterm infants did not become sick during this study, their developing microbiota will be shaped by exposure to antibiotics which could have profound effects on immune development and growth [3, 32, 34].

We did not find healthy preterm infants to have a different microbial composition or metabolite composition than infants that develop late-onset sepsis or NEC. Instead, findings from this cohort agree with the idea that initial colonizing bacteria of the preterm infant gut are largely unique to the individual [35]. Most of these initial colonizers are not species seen an adult microbiota, but the timing and composition of the early colonizers may have an effect on immune development that is independent of the stable, adult-like composition that emerges around age three [36]. Several studies have demonstrated a correlative relationship between strains of bacteria and NEC, but none have demonstrated a causative relationship [37-39]. Interactions between intestinal bacteria and the immune system are likely to be involved in pathogenesis, but the specific triggers are still unknown [15, 16, 40].

The metabolite profiles of fecal samples in this study were found to be highly personalized. Many factors influence the abundances of metabolites in fecal samples including: bacterial compositions, bacterial activity, host biology, and feeding. Among infants in this cohort, the global metabolite profile was not correlated with the composition of bacteria. However, bacteria have been shown to be important contributors to the fecal metabolome [41-43]. The primary metabolites measured by GC-MS in this study may be more difficult to correlate with specific microbe activity during antibiotic treatment than other forms of metabolite profiling, such as LC-MS targeting larger immune lipids. Metabolic signatures

256 of disease have great promise to complement microbiota profiling in human systems [13, 28]. However,
257 the transient and low abundance microbial populations in preterm infants may have a lesser effect on
258 the fecal metabolome than established microbial communities.

259

260 **Availability of data and materials**

261 The sequence data supporting the conclusions of this article are in the process of being posted to SRA
262 and will be made available upon publication.

263

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394 **Funding**

395 This project was supported by a UCI Single Investigator grant, the CORCCL SIIG-2014-2015-51, a pilot
396 grant from the UC Davis West Coast Metabolomics Core as part of NIH-DK097154, and start-up funds for
397 the Whiteson Lab in UC Irvine's Molecular Biology and Biochemistry Department.

398

399 **Acknowledgements**

400 We would like to acknowledge the contributions of Celine Mouginot, who shared protocols and primers,
401 and Ying (Lucy) Lu, who conducted some of the qPCR measurements and generally helped with the
402 sample preparation for 16S rRNA gene sequencing. Megan Showalter and Prof. Oliver Fiehn of the UC
403 Davis West Coast Metabolomics Core were very helpful in carrying out untargeted GC-MS profiling.
404 Thank you to those that discussed this project with me including Prof. Jen Martiny and Prof. Naomi
405 Morrissette.

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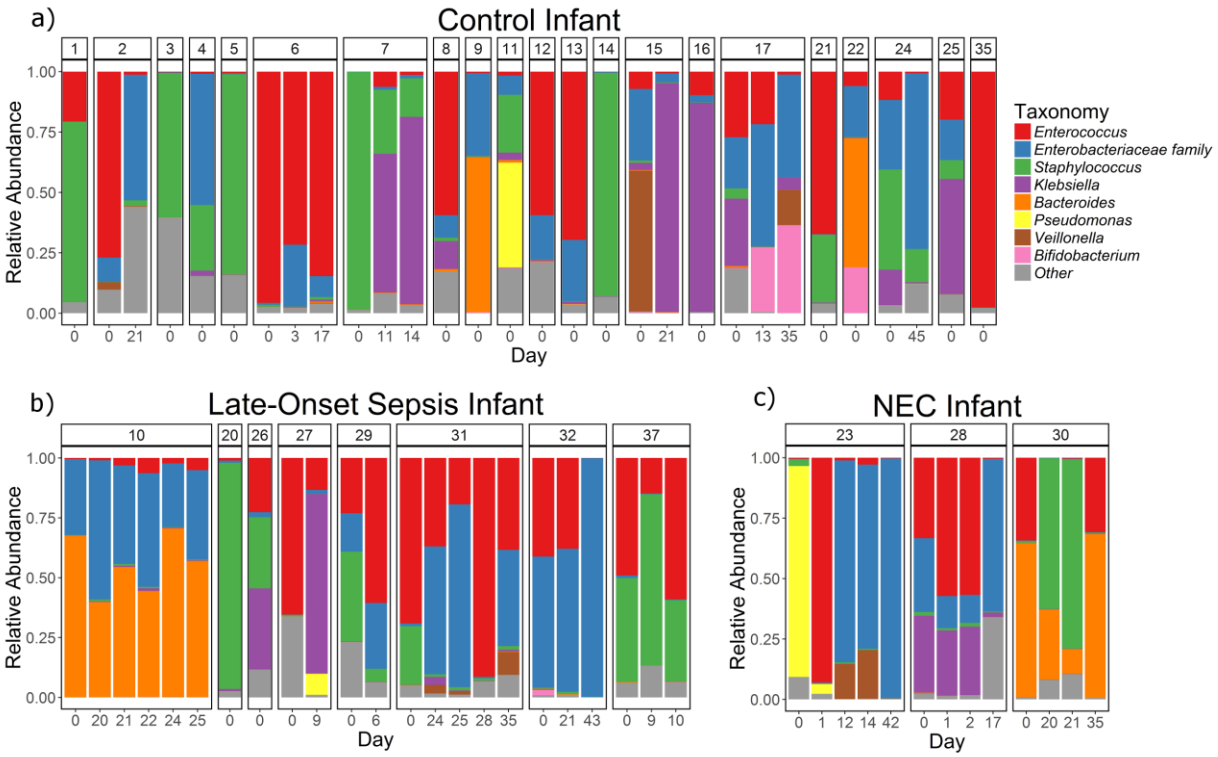


407

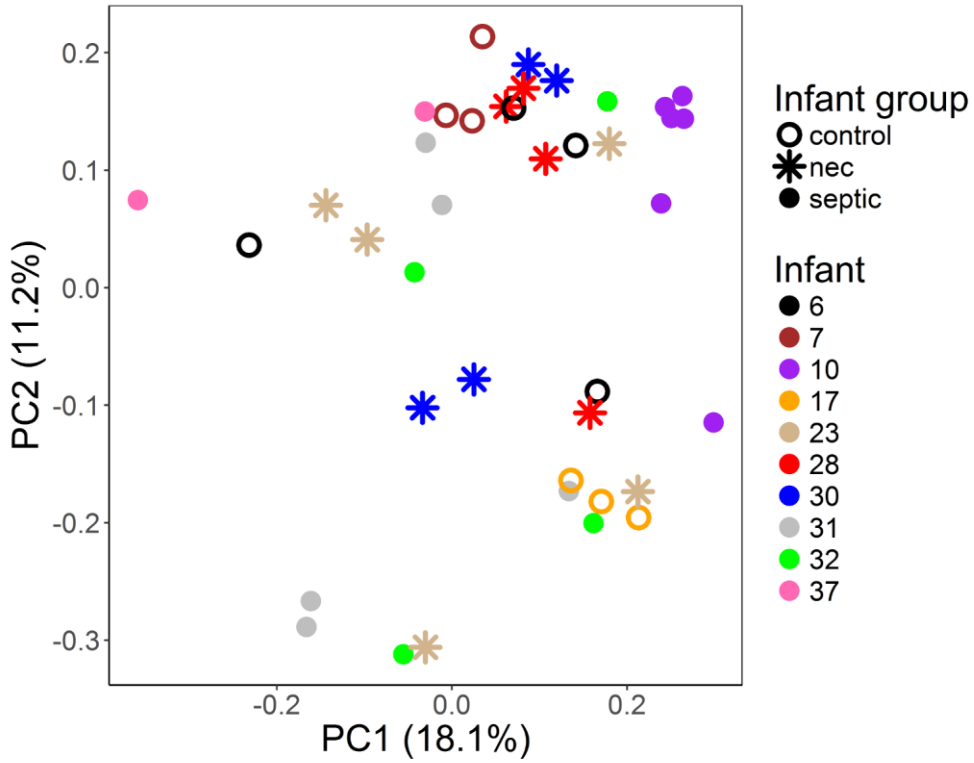
408 **Figure 1. The relative bacterial community composition of fecal samples level.** Relative bacterial

409 composition based on sequencing the V3-V4 region of the 16S rRNA gene. Infants are grouped by health

410 outcome as a) control, b) LOS, or c) NEC. Numbers at the top indicate identify individual infants. Days

411 are relative to the first sample from each infant, indicated as day 0. For cases in which multiple samples

412 were taken from one infant on the same day, only one is shown.



413

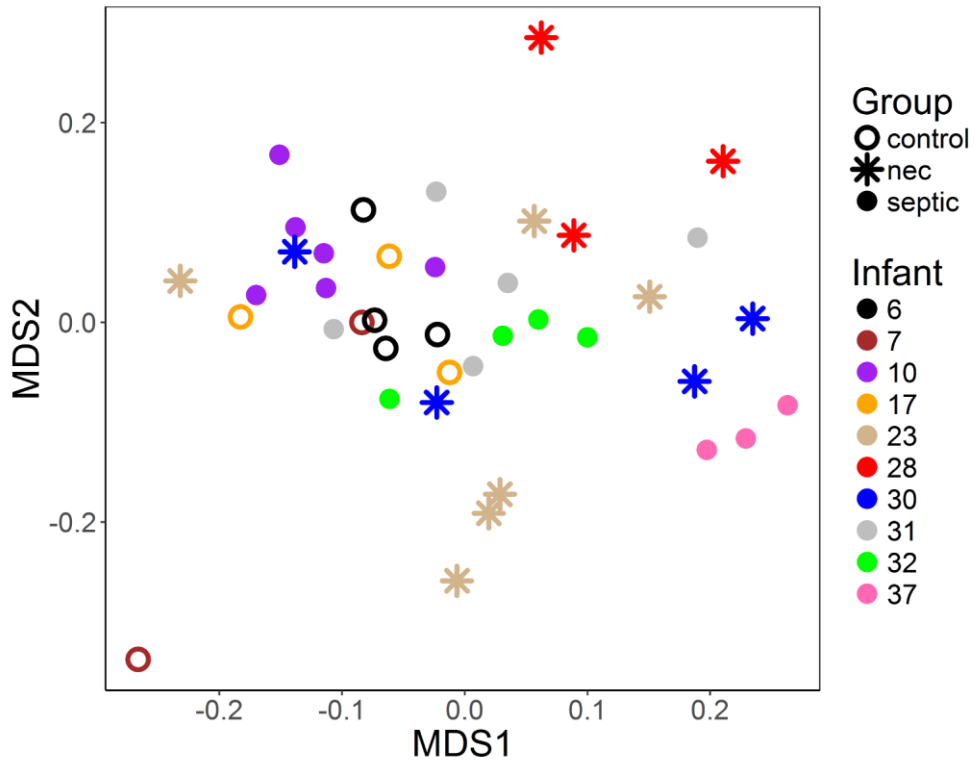
414 **Figure 2. Unweighted UniFrac distances between fecal bacterial communities were visualized by**

415 **Principal coordinates analysis (PCoA)**. Points are colored by infant from which the sample fecal sample

416 originated. The shape of each point indicates the infant health outcome. The first two principal

417 coordinates are plotted, which account for 17.9 % and 11.1 % of observed variation. Only samples in

418 which three longitudinal samples were available are shown.



419

420 **Fig. 3 Non-metric multidimensional scaling of Bray Curtis distances between fecal sample based on**

421 **metabolite abundances.** Distances calculated on metabolite abundances that were normalized by

422 sample and pareto scaled by metabolite. The stress associated with this ordination is 0.166. Points are

423 colored by infant from which the sample fecal sample originated. The shape of each point indicates the

424 infant health outcome. Only samples in which three longitudinal samples were available are shown.

	Df	Pseudo-F	Variance explained (R^2)	P-value
Health	1	0.03	0.001	0.99
Individual	9	3.22	0.62	< 0.001
Residuals	38		0.378	
Total	40		1.000	

Table 1. PERMANOVA based on unweighted UniFrac distances. PERMANOVA indicates that bacterial compositions of fecal samples are personalized to the induvial. Fecal samples from infants with NEC were not different from other infants based on bacterial compositions. Samples were permuted across health status (NEC versus control or LOS) or by individual (which infant the sample came from). In the case of health status, permutations were restricted so samples from a single infant were always assigned the same group to disentangle the effects from the origin of sample. Only samples with three or more longitudinal samples were included in analysis.

	Df	Pseudo-F	Variance explained (R^2)	P-value
Health	1	2.6	0.05	0.24
Individual	9	2.57	0.43	< 0.001
Residuals	39		0.52	
Total	40		1.000	

Table 2. PERMANOVA based on Bray-Curtis dissimilarity of metabolite profiles among samples.

PERMANOVA indicates that metabolomic profiles of fecal samples are personalized to the individual. Fecal samples from infants with NEC were not different from other infants based on global metabolomics profile. Samples were permuted across health status (NEC versus control or LOS) or by individual (which infant the sample came from). In the case of health status, permutations were restricted so samples from a single infant were always assigned the same group to disentangle the effects from the origin of sample. Only samples with three or more longitudinal samples were included in analysis.