The microbiome and metabolome of pre-term infant stool is personalized, and not driven by health outcomes including necrotizing enterocolitis and late-onset sepsis

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Abstract

The assembly and development of the gut microbiome in infants has important consequences for immediate and long-term health. Preterm infants represent an abnormal case for bacterial colonization because of early exposure to bacteria and frequent use of antibiotics. To better understand the assembly of the gut microbiota in preterm infants, fecal samples were collected from 32 very low birthweight preterm infants over the first six weeks of life. Infant health outcomes included healthy, late-onset sepsis, and necrotizing enterocolitis (NEC). We characterized the bacterial composition by 16S rRNA gene sequencing and metabolome by untargeted gas chromatography mass spectrometry. Preterm infant fecal samples lacked beneficial *Bifidobacterium* and were dominated by *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* due to the near uniform antibiotic administration. Most of the variance between the microbial community compositions could be attributed to which baby the sample came from (Permanova R^2=0.48, p<0.001), while clinical status (healthy, NEC, or late-onset sepsis), and overlapping time in the NICU did not explain a significant amount of variation in bacterial composition. Fecal metabolomes were also found to be unique to the individual (Permanova R^2=0.43, p<0.001) and weakly associated with bacterial composition (Mantel statistic r = 0.23 ± 0.05 (p = 0.03 ± 0.03). No measured metabolites were found to be associated with necrotizing enterocolitis, late-onset sepsis or a healthy outcome. Overall, preterm infants gut microbial communities were personalized and reflected antibiotic usage.

Importance

Preterm infants face health problems likely related to microbial exposures including sepsis and necrotizing enterocolitis. However, the role of the gut microbiome in preterm infant health is poorly understood. Microbial colonization differs from healthy term babies because it occurs in

the NICU and is often perturbed by antibiotics. We measured bacterial compositions and metabolomic profiles of 77 fecal samples from thirty-two preterm infants to investigate the differences between microbiomes in health and disease. Rather than finding microbial signatures of disease, we found the preterm infant microbiome and metabolome were both personalized, and that the preterm infant gut microbiome is enriched in microbes that commonly dominate in the presence of antibiotics. These results contribute to the growing knowledge of the preterm infant microbiome and emphasize that a personalized view will be important to disentangling the health consequences of the preterm infant microbiome.

Introduction

Early life exposure to microbes and their metabolic products is a normal part of development, with enormous and under-explored impact on the immune system. The intestinal microbiota of infants initially assembles by exposure to the mother's microbiota and microbes in the environment (1–4). In healthy breast-fed infants *Bifidobacteria longum* spp. infantis capable of digesting human-milk oligosaccharides dominate the infant gut (5). When infants are born preterm, they are exposed to environmental and human associated microbes earlier in their development than normal, and rarely harbor *Bifidobacteria* spp. in their gut communities. We do not yet understand the effects of altering the timing of initial bacterial exposure. With numerous emerging health consequences related to the microbiome, understanding factors that influence this initial assembly of the microbiome will be important.

Preterm infants are routinely treated with antibiotics, enriching for microbes that can colonize in the presence of antibiotics (4, 6, 7). While antibiotics have tremendously reduced infant mortality, their effect on microbiota assembly and resulting health consequences is not fully understood. Prenatal and postnatal antibiotics have been shown to reduce the diversity of the

60 infant intestinal microbiota (8, 9). Children under two years old are prescribed antibiotics at a
61 higher rate than any other age group, and 85% of extremely low birthweight infants (< 1000 g)
62 are given at least one course of antibiotics (10). Even if an infant is not exposed to antibiotics
63 after birth, approximately 37% of pregnant women use antibiotics over the course of the
64 pregnancy (11).

65 Perturbing the microbiota of infants can have immediate and long-lasting health consequences.
66 In the short term, infants can be infected by pathogenic bacteria that results in sepsis, which is
67 categorized as early-onset or late-onset depending on the timing after birth. Preterm infants are
68 also at high risk to develop necrotizing enterocolitis (NEC), which is a devastating disease that
69 causes portions of the bowel to undergo necrosis. NEC is one of the leading causes of mortality
70 in preterm infants, who make up 90% of NEC cases (12). The incidence of NEC among low
71 birthweight preterm infants is approximately 7% and causes death in about one third of cases.
72 The exact causes of NEC are not known, but an excessive inflammatory response to intestinal
73 bacteria may be involved (13).

74 Many of the long-term consequences of microbial colonization are believed to be mediated by
75 interactions between the intestinal microbiota and the immune system. In addition to direct
76 interactions, the microbiota interacts with the immune system through the production of
77 metabolites that can be taken up directly by immune and epithelial cells (14, 15). For example,
78 bacterial production of short chain fatty acids can affect health and integrity of the intestinal
79 epithelia and immune cells (16–18). However, few studies use metabolites alongside bacterial
80 community profiling. In fact, the healthy composition of an infant fecal metabolome is
81 understudied.

In this retrospective study, we follow the changes in the gut microbiota over time in 32 very low birth weight (< 1500 grams) preterm infants born at Children's Hospital Orange County. We simultaneously track the bacterial composition and metabolite profile over time. Infants were classified into three groups based on health outcomes: healthy infants, late-onset sepsis, and NEC. The composition of the intestinal microbiota was measured by 16S rRNA gene sequencing of fecal samples taken over time. Preterm infant guts were dominated by *Enterobacteriaceae* and *Enterococcus*, and *Staphylococcus*. Untargeted metabolomics analysis of the fecal samples by gas chromatography mass spectrometry (GC-MS) revealed a personalized metabolome that was weakly associated with the bacterial composition.

Results

Patient cohort

A total of 77 fecal samples were collected from 32 very low birth weight infants in the NICU at Children's Hospital Orange County from 2011 to 2014 (**Table 1, Figure 1**). Birthweights ranged from 620 – 1570 grams. Fecal samples were collected between day 7 and 75 of life. Sampling time and number of fecal samples varied. Three or more longitudinal samples were available from ten of the infants, while one or two samples were available from the remaining 22 infants. Three infants developed NEC, eight developed late-onset sepsis, and 21 remained healthy. 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. Twenty-four infants received antibiotics at some point during the sampling period, the most common being ampicillin and gentamycin.

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 and were not associated with health outcome (**Supplemental figure 1**). The high variation in bacterial load is likely due to the near uniform use of antibiotics. Bacterial communities were composed of mostly *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, and *Bacteroides* (**Figure 2a**). Most samples were dominated by one to three genera of bacteria. Only three infants (two fed breastmilk, one fed breastmilk and formula) were colonized at greater than 1 % relative abundance by *Bifidobacteria*, which emerging evidence suggests is a key member of the infant microbiome. We computationally confirmed that the primers used are able to detect 90% of known *Bifidobacteria* species (19). No single bacterial OTU or community composition was consistently found for infants that became sick (NEC or late-onset sepsis) compared to the infants that remained healthy.

Longitudinal sampling revealed that over the course of days, the bacterial composition could change dramatically (**Figure 2a, 2b**). Permutational Multivariate Analysis of Variance (PERMANOVA) was applied to determine which of the known clinical factors explained the most variance in the bacterial community composition. The individual explained 48% ($p < 0.001$) of the variance in the samples, meaning that about half of the total variance among all tested fecal samples could be attributed to the infant the fecal sample came from (**Supplemental table 1**). None of the other factors explained a significant amount of variation in the bacterial composition, including infant health, overlapping dates in the NICU, delivery mode, or feeding

126 mode. Four of the infants in the study are twins. Twin set 1 (infants 12 and 13) had a similar
127 microbial composition while the other three sets did not (**Supplemental Figure 2**).

128 Diversity of the bacterial communities was low as expected for preterm infants. Alpha diversity
129 as measured by Shannon index increased overall with age, but the trend was not significant
130 (linear model $R^2 = 0.005$, $p = 0.52$) (**Figure 3a**). Other clinical factors including health outcome,
131 feeding (breastmilk versus breastmilk and formula), antibiotic use, and delivery mode were
132 tested for an effect on the alpha diversity (**Figure 3b-e**). None of the factors were associated with
133 a difference in alpha diversity except recorded antibiotic use, in which Shannon diversity was
134 unexpectedly lower on average in infants that did not have a record of receiving antibiotics
135 (Wilcoxon rank sum test p=0.06). It should be noted that although six infants did not have a
136 record of antibiotic use, records may be incomplete due to hospital transfers. All four infants that
137 were colonized with B*acteroides* were born vaginally, although five other vaginally born infants
138 were not colonized. Only vaginally born infants were colonized by *Bacteroides* (four out of nine
139 infants) while none of the twenty-two infants born by C-section were colonized.

140 Metabolomics

141 Metabolite profiles of infant fecal samples were analyzed by gas chromatography mass
142 spectrometry, which measures small primary metabolites. Over 400 small molecules were
143 detected from each fecal sample and 224 metabolites were known compounds. Metabolites were
144 grouped into the following categories: amino acid metabolism, bile acids, central metabolism,
145 fatty acids, fermentation products, lipid metabolism, nucleotide metabolism, organic acids,
146 sterols, sugars, sugar acids, sugar alcohols, and vitamin metabolism (**Figure 4, Supplemental**
147 **table 2**). No metabolites or categories of metabolites were found to be associated with
148 necrotizing enterocolitis or late-onset sepsis. The metabolite profile of each infant was seen to

vary over time, similar to the amount of variation seen in the bacterial composition (**Figure 5**). PERMANOVA analysis to determine which factors explain the most variance in the metabolite profile indicate that the individual explains 43% ($p < 0.001$) of the variation (**Supplemental table 1**).

To determine which metabolites might be useful for tracking bacterial metabolism in the infant gut, we examined metabolites with consistent abundance among infants versus those that varied (**Supplemental figure 3**). In general, sugars, central metabolites, and amino acids were variable while fatty acids, sterols, organic acids, and bile acids were more consistent. Infant 23, which developed necrotizing enterocolitis at day 16 of life, had low abundances of amino acid metabolites the two days prior to disease onset (**Figure 4**). However, several of the healthy control infants also had similarly low abundances of amino acid metabolites. The individual signal of each infant's metabolome is far more evident than any trends due to clinical factors (**Supplemental table 1**).

Bacterial composition associated with metabolite profile

Bacterial metabolism in the gut is expected to contribute to the abundances of metabolites detected in fecal samples. We wanted to know if fecal samples with a similar bacterial composition were also similar in their metabolite profile. We employed a Mantel test using Pearson correlations between distances among bacterial compositions of samples and distances among metabolite profiles of samples. Because bacterial compositions and metabolite profiles are personalized, using multiple samples from a single infant would skew the result. Therefore, one sample from each infant was randomly selected 100 times to remove the effect of the individual and the Mantel test was applied to each subset. The average Mantel statistic of $r = 0.23 \pm 0.05$ ($p = 0.03 \pm 0.03$) indicates a weak but significant association between the bacterial

172 composition and metabolite profile. Also, within individual infants, shifts in the bacterial
173 composition are accompanied by shifts in the metabolome. Infants 17, 23, and 31 have dramatic
174 shifts in both bacterial composition and metabolome profile over time, while infants 10 and 37
175 remain stable in both bacterial composition and metabolome.

176 To investigate the correlations driving this overall association, we calculated correlations
177 between bacterial abundances and metabolite intensities (**Figure 6**). *Staphylococcus* had the most
178 positive correlations including several classes of sugar metabolites, organic acids, and central
179 metabolites. Fatty acids, lipid metabolism, and amino acids were positively correlated with the
180 commonly abundant gut colonizers *Enterobacteriaceae* and *Bacteroides*, and negatively
181 correlated with the commonly low abundance colonizers *Staphylococcus* and *Enterococcus*. We
182 also looked more specifically at individual metabolites correlated with bacterial abundances
183 (**Figure 6**). Bacteroidetes were found to be positively correlated with succinate ($r = 0.85$). Many
184 other weak correlations ($r < 0.5$) exist between bacterial abundances and metabolite intensities,
185 but the sample size is not large enough to distinguish signal from noise.

186 **Discussion**

187 Bacterial compositions in this cohort were consistent with the emerging picture from other
188 studies that show the preterm infant gut harbors communities dominated by facultative anaerobes
189 including *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* (1, 2, 20). These communities
190 appear to be enriched in commonly antibiotic resistant organisms (21). While we expected to
191 find associations between bacterial community composition and health outcome in this cohort,
192 we were surprised to find that there were not clear signatures of microbiome composition linked
193 to NEC or sepsis. In larger cohorts, associations between particular bacteria or metabolites with
194 NEC have been reported, however, they are not universal signatures across patients, and may

reflect patient variation more than disease etiology (22–25). In fact, the strongest signal in both the microbiome and metabolome data from this cohort was the infant from whom the sample was taken. Overall, preterm infant microbiomes in this study were shaped by antibiotics, which have a strong impact on all patients, regardless of health outcome.

Although the bacterial composition of infant guts varied over time, we saw longitudinal samples from individual infants remained highly personalized over several weeks; nearly half of the variation in the microbial community compositions can be explained by which individual the sample came from. The stability of animal-associated microbiomes is an active area of research, with studies finding that the individual microbiome of an adult remains stable through time (26), but can be perturbed by extreme changes in diet or antibiotics (27–29). The bacterial composition in the adult gut largely returns to its previous state one month after antibiotic treatment, but altering the initial assembly of the microbiota in infants can have long lasting health consequences (7, 27, 30, 31). Previous work has found ampicillin and gentamycin (the most common antibiotics taken by infants in this study) to have an inconsistent effect on bacterial diversity, sometimes increasing and sometimes decreasing diversity (1). Similarly, in these infants, ampicillin and gentamycin resulted in more variation in bacterial diversity, but there was no clear trend of increasing or decreasing diversity. However, antibiotics change the dominant members of the microbiota which could have profound effects on immune development and growth (7, 31–33).

Evidence is emerging that a healthy infant gut microbiota is dominated by *Bifidobacteria* with the capacity to digest human milk oligosaccharides in breastmilk (5, 34, 35). The lack of a core *Bifidobacteria* community in infants could leave the microbiota open to colonization by facultative anaerobes like we observed in these infants (36). Proteobacteria such as

Enterobacteriaceae are commonly seen to increase in abundance after antibiotic administration (21). Indeed, infants in this study had microbiomes that were shaped by antibiotic use. Although six of the thirty-two infants in this study did not have recorded antibiotic use around sampling time, the microbiota can still be affected by prenatal antibiotics taken by the mother (7, 31, 37).

Microbiome studies have become widespread, so that a typical bacterial composition is well characterized in a range of sample cohorts. However, the same cannot be said for the metabolome. There is a dearth of knowledge about what a consensus healthy infant fecal metabolome should be, making comparisons for the cohort in this study difficult. To add to the complexity, each metabolomic approach detects subsets of metabolites, and depends on sample extraction and other method choices. Increasing the frequency of metabolomics data collection in microbiome studies would be a huge step forward for the field. Baseline knowledge about the typical connections between metabolite abundances and bacterial metabolism should be collected, so that molecules that have consistent abundances in a healthy state could give context to data generated from clinical samples in different disease states.

Untargeted metabolomics can survey many metabolites in a biological sample to provide a snapshot of the active metabolism in a system such as the human gut. Metabolite profiles of preterm infants in this study were found to be personalized to a similar degree as the bacterial composition. This is in contrast to a previous study on full term infants that showed the metabolomic profile to be stable, and weakly associated with bacterial composition, over the first few years of life (38). Personalized metabolic signatures of disease hold great promise to complement microbiota profiling in human systems (18, 36). However, analyzing metabolomic data is challenging because an array of inputs contribute to the abundances of metabolites in fecal samples including bacterial metabolism, host biology, and food intake.

We report a number of correlations between bacteria and metabolites in preterm infant feces, and bacterial metabolism has been previously shown to contribute to metabolite abundances in humans and mice (15, 39, 40). Short chain fatty acids are now commonly measured and associated with bacterial fermentation in the gut (41). In this study, the only short-chain fatty acid detected was succinate, which we found to be correlated with the presence of *Bacteroides*, which produces acetate and succinate from carbohydrate fermentation (42). We also detected several medium-chain fatty acids, which were generally correlated with the abundance of *Bacteroides* and *Enterobacteriaceae*. None of the twenty-two C-section born infants in this study were colonized by *Bacteroides*, possibly due to a lack of vertical transmission from the mother during birth (3).

Overall, we find preterm infant microbiomes are shaped by shared exposures especially to antibiotics, leading to the dominance of antibiotic resistant facultative anaerobes such as *Enterococcus* spp.. The anaerobic, milk degrading *Bifidobacteria* were largely absent, even in preterm infants with access to breastmilk, possibly due to a lack of exposure to microbes from family members in the sterile hospital environment along with antibiotics. Our understanding of the health consequences of microbial colonization under these antibiotic-enriched circumstances is still in its infancy.

Materials and methods

Sample Collection

Stool samples from diapers of preterm infants in the neonatal intensive care unit at Children's Hospital Orange County were collected by nurses over three years from 2011 to 2014. 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.

DNA extraction and 16S rRNA gene sequencing

Stool samples were thawed once and DNA was extracted from 10 mg using a fecal Zymo Fecal DNA MiniPrep Kit (#D6010). The V3 and V4 region of the 16SrRNA gene was amplified with two-stage PCR. The first PCR amplified the V3 to V4 region of the 16S rRNA gene using 341F and 805R primers: forward primer (5'- CCTACGGGNGGCWGCAG-3') and reverse primer (5'-GACTACHVGGGTATCTAATCC -3') (43). These primers also added an overhang so that barcodes and Illumina adaptors could be added in the second PCR. 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, primers with sample specific barcodes and Illumina adapter sequences 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. Amplicon pool was run on an agarose gel and the 500bp fragment was cut out and gel extracted using Millipore Gel Extraction Kit (#LSKGEL050). The sequencing 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 producing a total of 2.4 million paired-end reads.

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 to quantify DNA from 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).

Sequence processing

Sequences were quality filtered using PrinSeq to remove adapters as well as any sequences less than 120 base-pairs, containing any ambiguous bases, or with a mean PHRED quality score of less than 30 (44). Reads were found to drop steeply in quality after 140 base pairs, so all reads were trimmed to 140 base pairs. The forward read contained the V3 region in the high quality first 140 base pairs, while the V4 region was captured in the low-quality region of the reverse reads. Therefore, we used only the forward reads for subsequent analyses.

Bacterial community analysis

Quantitative Insights Into microbial Ecology (QIIME) was used for de novo OTUs picking using the Swarm algorithm with a clustering threshold of 8 (45, 46). This resulted in 2,810 OTUs among all samples. OTUs containing only one sequence were filtered out, leaving 212 OTUs. Taxonomy was assigned to each OTU using QIIME and the Greengenes 13_8 database. An OTU

table was constructed and used for downstream analysis. Ten rarefactions were performed on the OTU table down to 2000 reads per sample, which was the largest number of reads that allowed retention of most samples. QIIME was used to calculate alpha diversity by Shannon index and beta diversity by the average weighted UniFrac distance of the ten rarefactions. Community composition barplots, Principal Coordinate Analysis (PCoA) plots, and alpha diversity plots were created using R and the ggplot2 package (47, 48). All R scripts are included in the supplemental information.

Untargeted metabolomics by GC-MS

When fecal samples were thawed for DNA extraction, approximately 50 mg was collected and refrozen at -80 ° for metabolomics. Samples were sent on dry ice to the West Coast Metabolomics Center (WCMC) at UC Davis for untargeted metabolomics by gas chromatography time-of-flight mass spectrometry. Metabolites were extracted from fecal samples with a 3:3:2 mixture of isopropanol, acetonitrile, and water respectively before derivatization and GC-MS analysis by Fiehn Lab standard operating procedures (49–51). Metabolite profiles were compared by calculating Manhattan distances between samples based on all metabolite intensities and visualized by PCoA using the vegan and ape packages in R (52, 53).

Permutational multivariate analysis of variance (PERMANOVA)

PERMANOVA was used to determine factors that explained variance in bacterial community composition and metabolite profile. PERMANOVA was performed using the adonis function in the vegan package in R. The input for PERMANOVA was UniFrac distances of the 16S data and Manhattan distances of the metabolite profiles. Briefly, PERMANOVA quantifies the variation

among samples explained by the given groupings compared to randomized groupings. To measure the variance explained by individual infant, we excluded samples that had fewer than three longitudinal samples, leaving ten infants. When performing PERMANOVA for factors other than individual, we accounted for the longitudinal sampling by repeatedly subsampling one sample from each infant and averaging the results.

Correlations between bacteria and metabolites

Pearson correlations between bacterial abundances and normalized metabolite intensities were calculated using the cor function in R. Correlations were calculated between the relative abundances of all bacterial classes and all metabolite intensities among all samples in all infants. Only the four most highly abundant general of bacteria were used to ensure no results were skewed by taxa present in only one or a few samples. For each class of metabolite, the average of all correlations between metabolites in that class and each taxon was calculated so that trends between bacterial taxa and classes of metabolites could be visualized by heatmap.

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 randomly subsampled down to one sample per infant. A Bray-Curtis dissimilarity matrix was computed for the bacterial composition and Manhattan distances calculated for metabolite intensities. The Mantel function in the vegan package of R was used to calculate the Mantel statistic for a Pearson correlation between the two dissimilarity matrices. The average and standard deviation of the Mantel statistic r and p-value for the 100 Mantel tests was reported.

Data availability

346 Raw sequence data will be uploaded to the SRA. OTU tables, raw metabolomics data, a
347 markdown file of sequence processing workflow, and R scripts used for analyses are available at
348 https://github.com/swandro/preterm_infant_analysis.

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532

533 **Table 1.** Clinical and sampling information for all infants.

Infant	# samples	Age sample(s) collected (days)	Age at disease onset (days)	Group	Fetal age at birth	Birth weight (g)	Antibiotics	Delivery mode	Feeding type	Twin set
1	2	7,7		control	27w4d	875		c-section	breastmilk	
2	3	15,15,36		control	31w	1570	ampicillin,gentamycin	c-section	breastmilk and formula	
3	1	19		control	26w	980	ampicillin,gentamycin	c-section	breastmilk	
4	2	11,11		control	30w3d	1335		vaginal	breastmilk	
5	2	18,18		control	24w5d	630		c-section	breastmilk	
6	4	25,26,28,43		control	28w5d	860	ampicillin,gentamycin	c-section	breastmilk and formula	
7	3	10,21,24		control	25w2d	885		c-section	breastmilk and formula	
8	1	10		control	25w4d	940	ampicillin,gentamycin	vaginal	breastmilk	
9	1	8		control	27w2d	1205		vaginal	breastmilk	
11	2	29,29		control	27w4d	850	ampicillin,gentamycin	vaginal	breastmilk	
12	1	22		control	26w2d	880	ampicillin,gentamycin	c-section	breastmilk and formula	1
13	1	23		control	26w2d	925	ampicillin,gentamycin	c-section	breastmilk and formula	1
14	1	8		control	31w4d	1190	ampicillin,gentamycin	c-section	breastmilk	
15	3	18,40,40		control	28w1d	1270	ampicillin,gentamycin	c-section	breastmilk and formula	2
16	1	19		control	28w1d	1355	ampicillin,gentamycin	c-section	breastmilk and formula	2
17	3	18,32,54		control	26w2d	660	ampicillin,cefotaxime	c-section	breastmilk	
21	1	10		control	28w6d	1180	ampicillin,gentamycin	c-section	breastmilk	
22	1	25		control	28w6d	1360	ampicillin,cefotaxime	vaginal	breastmilk and formula	
24	2	27,73		control	26w	740	ampicillin,gentamycin	c-section	breastmilk	3
25	1	28		control	26w	780	ampicillin,gentamycin	c-section	breastmilk	3
35	2	18,18		control	25w5d	920		c-section	breastmilk and formula	
23	7	14,15,27,28,30,30,56	27	NEC	26w6d	1080	ampicillin,gentamycin, cefotaxime,vancomycin	vaginal	breastmilk	
28	4	31,32,33,48	31	NEC	26w	1060	vancomycin,piperacillin	c-section	breastmilk and formula	
30	4	21,41,42,56	41	NEC	23w6d	620	cefazolin,azithromycin,ampicillin	vaginal	breastmilk and formula	
20	1	21	26	septic	24w5d	815	ampicillin,gentamycin	c-section	breastmilk	
10	6	15,35,36,37,39,40	27	septic	26w5d	940	ampicillin,gentamycin,vancomycin	vaginal	breastmilk and formula	
26	1	22	22	septic	24w4d	660	ampicillin,gentamycin,cefotaxime,vancomycin	c-section	breastmilk	4
27	2	22,31	29	septic	24w5d	650	ampicillin,gentamycin	c-section	breastmilk	4
29	2	20,26	26	septic	26w1d	980	cefotaxime,vancomycin	c-section	breastmilk	
31	5	10,34,35,38,45	34	septic	27w	710	ampicillin,gentamycin	c-section	breastmilk and formula	
32	4	32,32,53,75	32	septic	27w5d				breastmilk and formula	
37	3	8,17,18	13	septic	24w1d	680	ampicillin,gentamycin,cefazolin,oxacillin	vaginal	breastmilk	

534

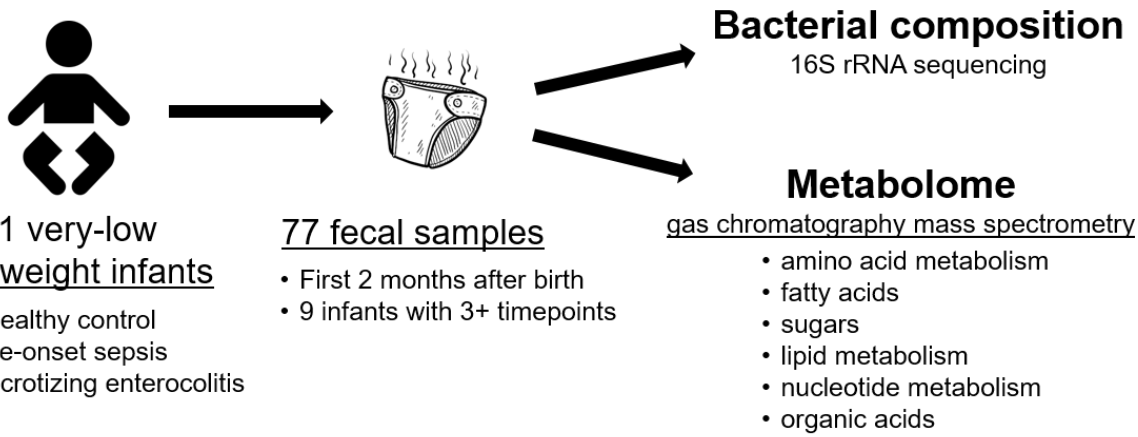


Figure 1. Study design schematic. Longitudinal fecal samples were collected over the first 75 days of life from very low birthweight infants in the NICU. Bacterial compositions and metabolomes were characterized.

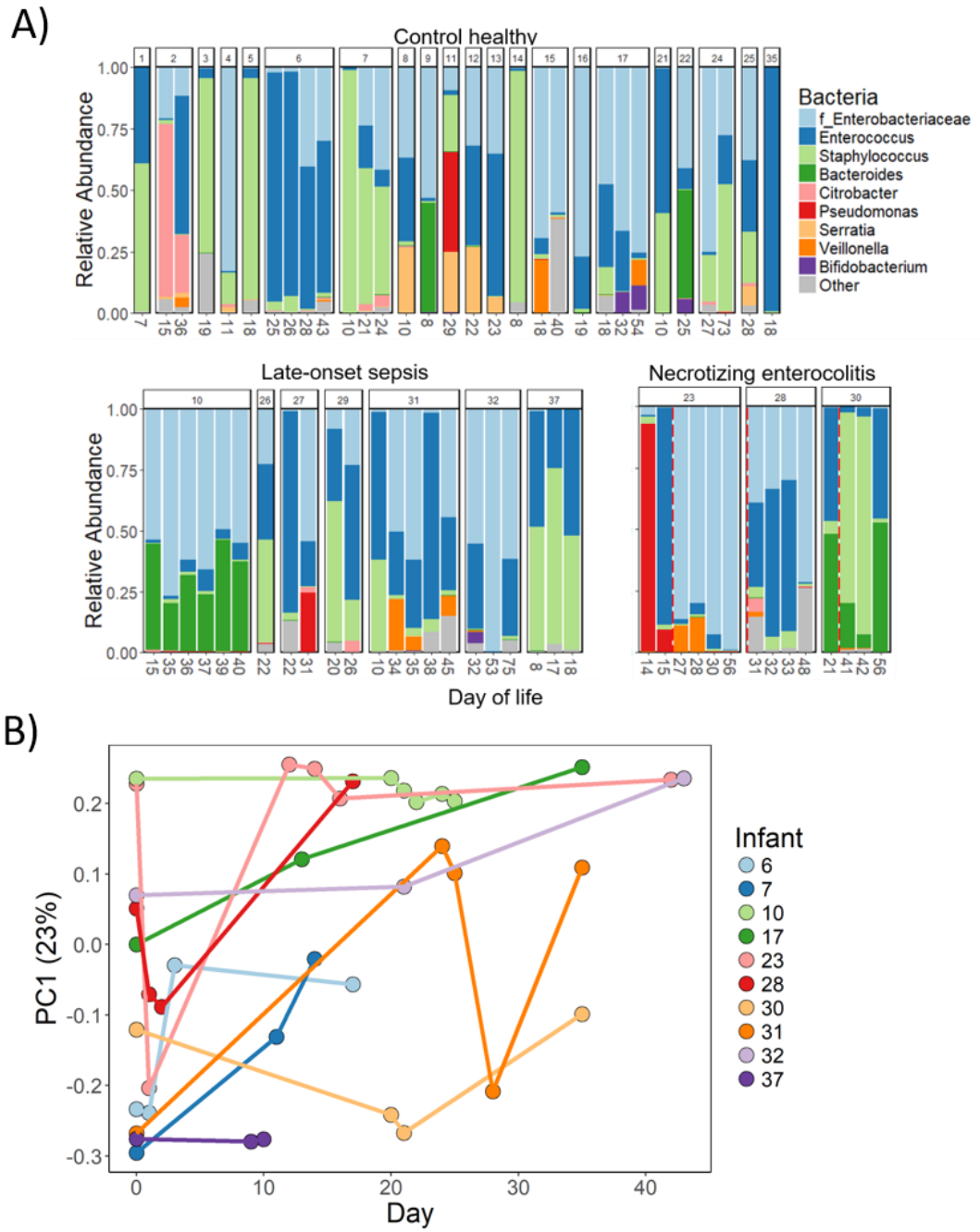
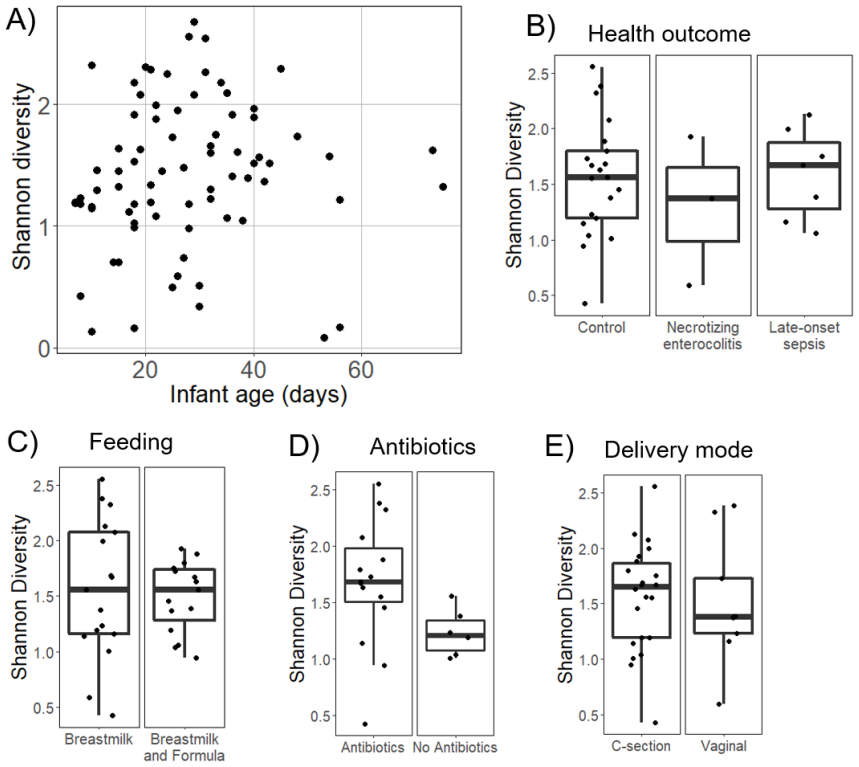
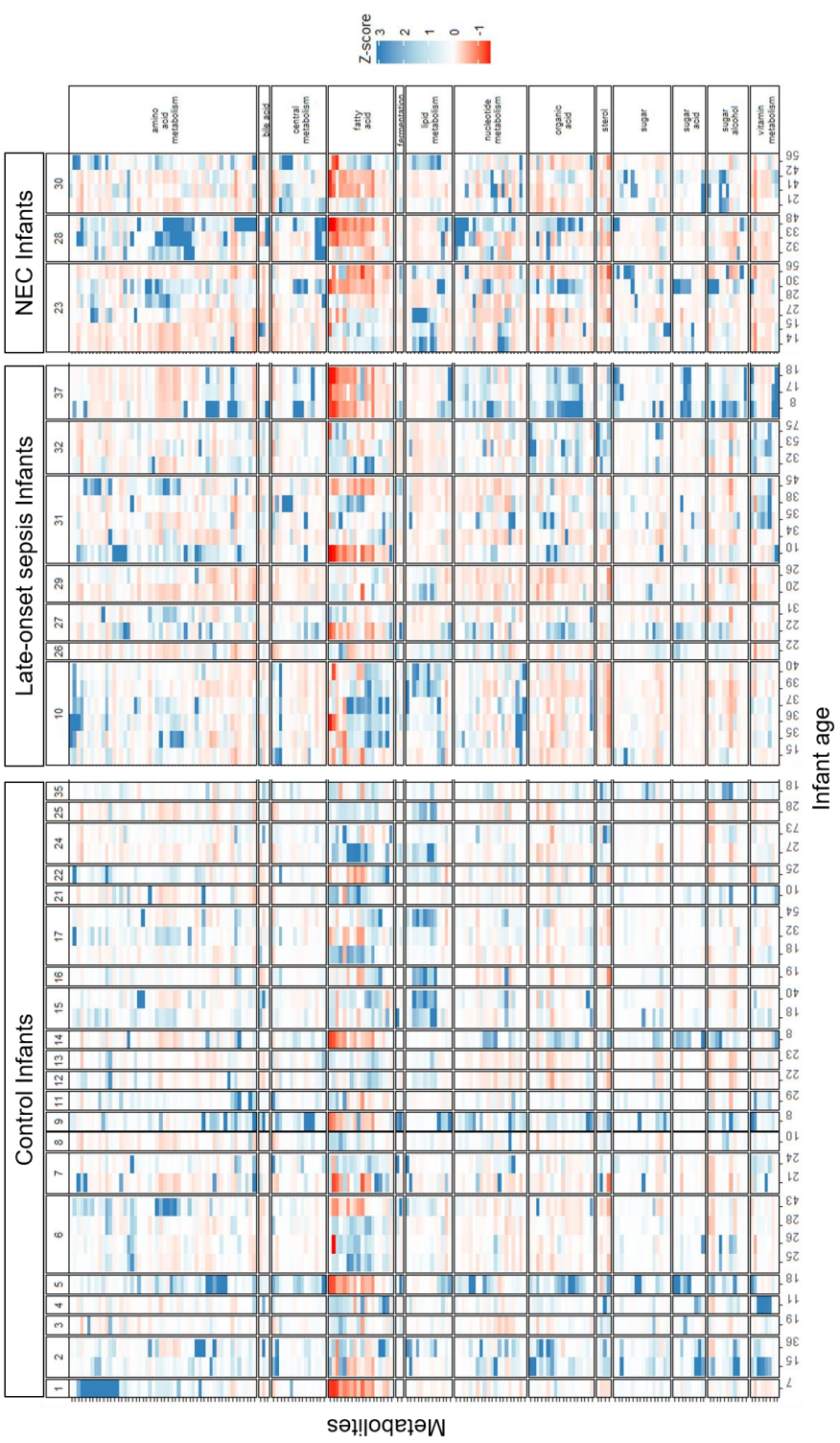


Figure 2. Bacterial composition of preterm infant guts. A) Stacked barplot of relative abundance of bacterial genera in all infant samples. The family *Enterobacteriaceae* is included because genus level resolution was not available. Infants are grouped together by health outcome. The timing of necrotizing enterocolitis diagnosis is indicated by a vertical dotted line. B) First axis of PCoA based on weighted uniFrac distances between bacterial communities plotted over time. Only infants with three or more longitudinal samples shown in B.



548

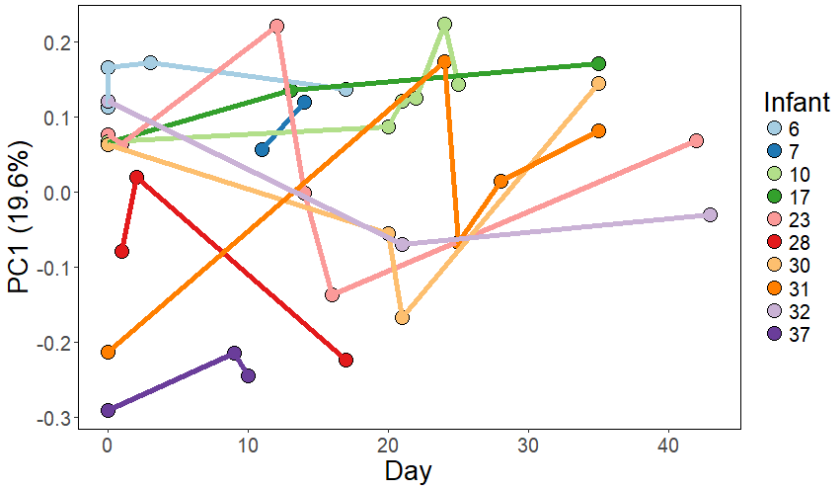
549 **Figure 3.** Alpha diversity measured by Shannon index of bacterial composition. A) Alpha diversity of all
550 samples over the age of the infant. Boxplots of the average alpha diversity of each infant separated by B)
551 health outcome, C) infants that were fed only breastmilk or a combination of formula and breastmilk, D)
552 record of antibiotic usage, or E) delivery mode.



553

554 **Figure 4.** Metabolite profile of preterm infant fecal samples. Color is the modified z-score which is based
555 on the median intensity for each metabolite in all infant samples. Red cells indicate standard deviations
556 below the median and blue indicate standard deviations above the median value for each metabolite.
557 Measured metabolites that could be assigned to a category are shown. Samples on the x-axis and grouped
558 by infant and ordered longitudinally. Metabolites within each category are listed in the supplemental data.

559



560

561 **Figure 5.** First component of PCoA of metabolite profile over time. Manhattan distances between
562 samples were calculated and visualized by PCoA. The first principal component which explains the most
563 variation among the samples is shown over time. Each dot represents a single fecal sample and is colored
564 by infant. Lines connect samples for each infant to show change over time. Only infants with three or
565 more longitudinal samples shown.

566

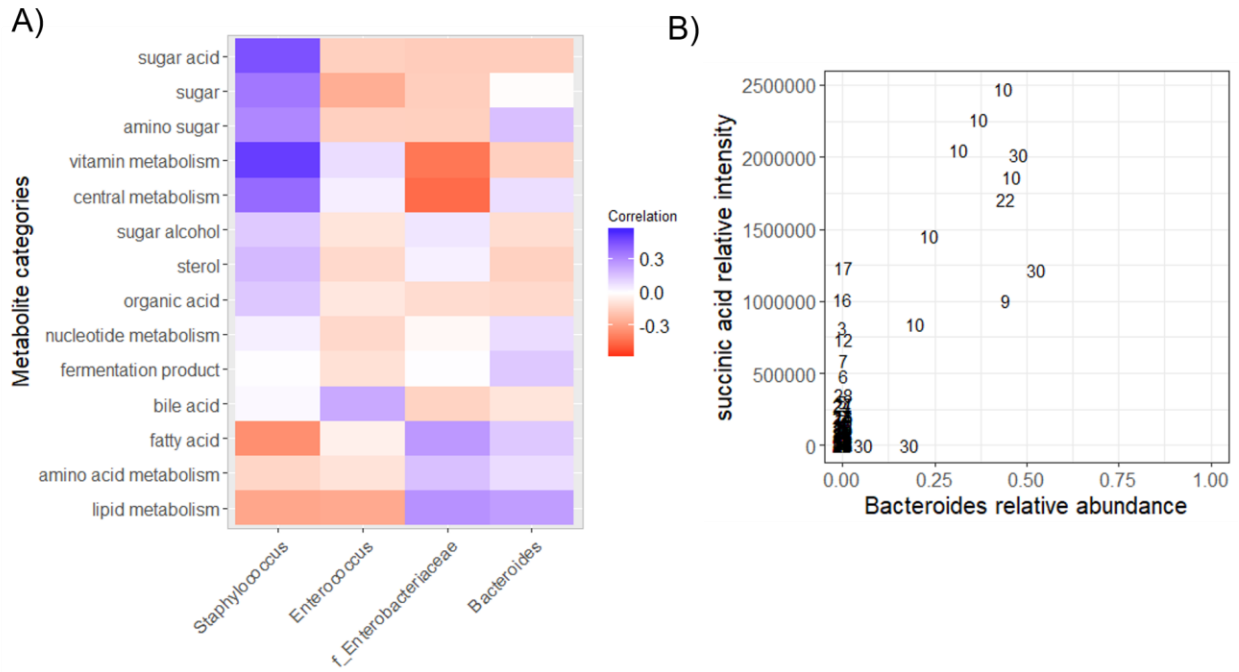


Figure 6. Correlations between bacteria abundances and metabolite intensities. A) Average of correlations between bacterial abundances and all metabolites in each metabolite category. B) Correlation between *Bacteroides* abundance and succinic acid intensity in all samples. Numbers indicate infant number.