Antibiotics may influence gut microbiome signaling to the brain in preterm neonates

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Abstract

Antibiotic use in neonates can have detrimental effects on the developing gut microbiome, increasing the risk of morbidity. A majority of preterm neonates receive antibiotics after birth without clear evidence to guide this practice. Here we present microbiome and metabolomic results from the Routine Early Antibiotic use in SymptOmatic preterm Neonates (REASON) study, which is the first trial to randomize symptomatic preterm neonates to receive or not receive antibiotics in the first 48 hours after birth. Using 16S rRNA sequencing of stool samples collected longitudinally for 91 neonates, we show that antibiotics alter trends in microbiome diversity and development between randomized neonates and illustrate that type of nutrition helps shape the early infant gut microbiome. Integrating multi-omic data for the gut microbiome, stool immune markers, stool metabolites, and inferred metabolic pathways, we identified an association between *Veillonella* and the neurotransmitter gamma-aminobutyric acid (GABA). Our results suggest early antibiotic use may impact the gut-brain axis with the potential for consequences in early life development.

Main

Premature infants are particularly susceptible to infections secondary to increased need for invasive procedures and immaturity of the immune system, skin, and gastrointestinal tract[1–3]. Increasingly, there is growing concern that risk factors for mortality may originate from underlying pathologies that could also be responsible for premature birth[4]. Symptoms of prematurity are difficult to discern from symptoms of infection which, compounded by the increased risk of infection, have led to most premature infants being exposed to antibiotics early

in life[5–7]. Despite high mortality rates, the incidence of culture positive early onset sepsis (EOS) is relatively low, between 0.2-0.6%[8]. In the absence of a positive culture, a majority of preterm infants receive antibiotics immediately after birth based on maternal risk factors (e.g. intra-amniotic infection) or laboratory abnormalities (e.g. elevated serum C-reactive protein (CRP) because of the risk of mortality[8]. Given the low incidence of culture positive EOS in this population, it is possible that such high rates of antibiotic use are unnecessary and may increase morbidity in these infants[9]. Other morbidities in the neonatal intensive care unit (NICU) such as necrotizing enterocolitis (NEC) also have high mortality rates and have been associated with prolonged antibiotic exposure.[10,11]. Nevertheless, antibiotics remain the most commonly prescribed medication in the NICU[12,13].

The gut microbiome comprises a highly volatile community structure early in life[14]. Microbial colonization is influenced as early as birth by mode of delivery, and perhaps even in the uterine environment by maternal factors[15,16]. Not surprisingly, antibiotic use has been shown to also change the composition of the preterm gut community[17–20]. Furthermore, antibiotic use early in life has increasingly been associated with adverse outcomes both short- and long-term[21,22]. One possible consequence is the disruption of the gut-brain axis (GBA), which involves bi-directional transmission of bio-molecular signals between the gut microbiota and the nervous system[23]. Aberrations in the GBA have been associated with altered immune homeostasis, as well as psychiatric, behavioral and metabolic conditions in adulthood[24]. It is therefore imperative to determine if such high rates of antibiotic use in preterm infants is necessary, as it could have lifelong consequences on future health.

Randomized clinical trials have the advantage of controlling for many of the numerous covariates that could interfere with answering whether preemptive antibiotic use in preterm

infants affects outcomes. The Routine Early Antibiotic use in SymptOmatic preterm Neonates (REASON) study is the first to randomize symptomatic premature infants to either receive or not receive antibiotics soon after birth. Previously reported results from this study demonstrate the feasibility of such a trial and that withholding antibiotics did not lead to a significant increase in neonatal mortality or morbidity[25]. By employing a multi-omic approach, this randomized trial also provides the unique opportunity to understand how antibiotic intervention perturbs the early life gut microbiome, metabolome, and inflammatory environment in ways that may be consequential to health and development.

Results

Cohort and study description

Ninety-eight infants were enrolled in the study over the two-year period: group A – antibiotics indicated (n=32), group B – antibiotics not indicated (n=11), and group C – eligible for randomization (n=55). Twenty-eight infants were randomized to C1 (antibiotics x 48 hours) and 27 infants were randomized to group C2 (no antibiotics). For 13 infants (48%) in group C2, antibiotics were prescribed in the first 48 hours after birth. Neither sex nor mode of delivery were significantly different between all groups and were not significantly different between the two randomized subgroups using the chi-square test. Both weight (p-value=0.0001) and gestational age (p-value=0.0002) were significantly different between groups overall, with group A infants on average with lower gestational age (GA) and at lower birth weights. Neither birth weight nor GA were significantly different between the randomized subgroups by ANOVA. A summary of the infants in this analysis is provided in Table 1. A full description of enrollment has been described previously[25].

Six hundred ninety-three stool samples were collected longitudinally for 91 of the total 98 infants enrolled. Stool data was not available for 7 infants due to early mortality. Sequencing data for 16S rRNA was able to be obtained for 656 of those samples. After rarefying to an even sequencing depth of 10,000 reads per sample, 642 samples remained for further analysis. The breakdown of samples by group is provided in Table 1. The aim for this analysis is to test the effects of randomization to antibiotics vs. no antibiotics has on the developing gut microbiome, metabolome and inflammatory environment using high-throughput 16S rRNA sequencing, quantitative PCR (qPCR), metabolomics, pathway inference, immune marker analysis and open-source statistical tools.

Antibiotic use affects trends in early gut microbiome development over time

The within-sample diversity differed by enrollment groups A, B, C1, and C2. Using amplicon sequencing variants (ASVs), the richness, and the alpha diversity were not significantly different between enrollment groups overall (Kruskal-Wallis, Richness: $p = 0.31$, Shannon: $p = 0.90$) and did not differ across time using a linear mixed effects model (LME) through Qiime2[26–28] (Shannon p-values: group A = 0.98, group B = 0.70, group C1 = 0.83, group C2 = 0.926). Though not statistically significant, scatter plots of each sample's alpha diversity, including regression lines for each group, indicate trends in diversity development over time between the groups (Fig. 1a, Shannon diversity). These trends are expected given the variation in antibiotic levels used by each group. For example, group B (antibiotics not indicated) tended to have the highest trend in diversity over time and is the group with the lowest antibiotic use. In contrast, group A (antibiotics indicated) infants saw very little diversity development over time while receiving the most antibiotics. Certain considerations need to be made when comparing infants

by enrollment group. For example, group A infants had significantly lower GA and birth weights, particularly compared to group B infants who on average had the highest GAs and birth weights, and the shortest stays in the NICU. Furthermore, infants in randomized group C2 who were changed to receive antibiotics in the first 48 hours after birth can no longer be considered as receiving no antibiotics and would be more similar to group C1. To account for this in testing the effect of antibiotic use, we focus only on the randomization group, taking into consideration those infants who were changed to receive antibiotics by instead separating infants based on any antibiotic use within 48 hours after birth (any_abx).

Samples from group C infants that were collected during antibiotic use (abx_during_sample = TRUE) were compared with those during periods of no treatment (abx_during_sample = FALSE). As anticipated, samples taken during periods of antibiotic use had lower richness and lower bacterial load, as determined by quantitative PCR (qPCR) of 16S rRNA copies per gram of stool (Figs. 1b,c). As this result may be driven by samples collected early in life, just those samples collected in the first week of life were also compared and were not significantly different in richness (Wilcoxon, $p = 0.70$), alpha diversity (Wilcoxon, $p = 0.43$) and bacterial load (Wilcoxon, $p = 0.50$).

Taking into consideration those infants in group C2 who were changed to antibiotic use, bacterial richness was plotted as a function of time (Collection_week) considering whether the infant received antibiotics (any_abx) (Fig. 1d). Pairwise comparisons of the antibiotic and no antibiotic infants at each collection week were not statistically significant (Wilcoxon test, $p > 0.05$). Initially within the first few weeks of life, bacterial richness is similar regardless of antibiotic

use. However, beginning at the fifth collection week (30 days after birth on average) infants who never received antibiotics trended towards higher bacterial richness compared to infants that were treated. Average bacterial richness remained higher among the no antibiotic infants through collection week 8 (53 days after birth on average). Again using linear mixed effects modelling, we found that both bacterial richness and Shannon diversity trended higher in infants who never received antibiotics compared with those that did, though neither diversity metric was significant as a function of time (Richness: $p = 0.35$, Shannon: $p = 0.26$) (Figs. 1e,f). As with the infants in group C, infants who were randomized to not receive antibiotics and were not changed to receive antibiotics (any_abx = FALSE) had shorter stays in the NICU. Furthermore, although the randomized groups are almost evenly powered without considering change in antibiotic use status, removing C2 infants who received antibiotics underpowers the group of infants who never received antibiotics, limiting the strength of comparisons.

Changes in overall bacterial community structure were apparent between groups when assessing beta-diversity. Both the Bray-Curtis and Jaccard distance indices were used to assess community structure taking into account quantitative ASV abundance and qualitative presence/absence information, respectively. Beta dispersion was not significantly different between groups using the Bray-Curtis metric (ANOVA; Df = 3, Sum of Squares = 0.01589, Mean Squares = 0.0052974, $F = 2.547$, $p = 0.055$) but was significant using Jaccard (ANOVA: Df = 3, Sum of Squares = 0.0315, Mean Squares = 0.01050, $F = 8.4013$, $p = 1.75E-05$), specifically group A versus all other groups (TukeyHSD, B-A $p = 0.0153$, C1-A p-value = 0.0001, C2-A $p = 0.0028$). With the test assumption met for the Bray-Curtis index, PERMANOVA also showed distance

dissimilarity to be significant between groups (PERMANOVA; Df = 3, Sum of Squares = 4.642, R^2 = 0.01632, F = 3.544, p = 0.001). Principle coordinates analysis (PCoA) suggests an effect of antibiotic use by enrollment group. The infants exposed to the least amount of antibiotics through the NICU course (group B and group C2 that were not changed to receive antibiotics) had tighter clustering using both Bray-Curtis and Jaccard, whereas groups A and C1 had the highest variability, suggesting less antibiotic use led to greater microbiome stability (Figs. 2a,b). Similarly, group C1 had greater spread by PCoA using Bray-Curtis but not Jaccard (Figs. 2c,d). Infants in group C2 who were changed to receive antibiotics in the first 48 hours but did not receive antibiotics again had much tighter clustering, indicating the outcome of early antibiotics in the randomized group and the effect antibiotics use has on microbiome stability (Figs. 2e,f).

Feeding patterns drive changes in gut diversity and bacterial load

For preterm infants, diet generally consists of mother's breast milk, pasteurized donor breast milk, and/or formula. To control for any effect of antibiotics, only samples from group C2 infants that were not changed to receive antibiotics in the first 48 hours were used. Using the calculated alpha diversity metrics used previously, feeding type was found to significantly associated with bacterial community richness (Kruskal-Wallis, p = 0.0016) and Shannon diversity (Kruskal-Wallis, p = 6.2E-05) (Figs. 3a,b). In addition, feeding type had an effect on total bacterial load measured by the number of 16S rRNA copies per gram of stool for each sample (Kruskal-Wallis, p = 0.003) (Fig. 3c). As expected, stool collected during times of no enteral feeding (NPO) had the lowest richness and total bacterial load. Samples collected during formula feeding had just as high of richness and diversity as samples during feeding with

mother's milk. However, samples collected during feeding with mother's milk tended to have the higher bacterial load (Wilcoxon, $p = 0.0057$). Donor breast milk (DBM), which is pasteurized prior to use, tended to have lower richness, diversity, and bacterial load compared to other feeding types except NPO. Interestingly however, combining formula with DBM trended towards increased diversity and bacterial load, though not significantly, most likely due to the limited number of samples collected during feeding with DBM + formula from group C (n= 2).

Clustering analysis by PCoA also showed trends in gut composition based on feeding type (Figs. 3d,e). Stool collected while feeding with mother's milk had the widest variability in overall composition by both Bray-Curtis and Jaccard, whereas donor milk had the tightest clustering. Notably, samples taken during formula or formula mixed with mother's milk had similar clustering by Bray-Curtis, but formula feeding alone led to more variability with Jaccard, suggesting addition of mother's milk had little effect on the abundance of bacteria but formula feeding did contribute more variability in the presence or absence of certain bacteria. Not considering samples from infants in group C2 who were changed to antibiotics, feeding type had a significant effect on community structure by both Bray-Curtis (PERMANOVA, Df = 7, Sum of Squares = 5.527, $R_2 = 0.133$, $F = 1.98$, $p = 0.001$) and Jaccard (PERMANOVA, Df = 7, Sum of Squares = 4.692, $R_2 = 0.118$, $F = 1.720$, $p = 0.001$). Along the same lines, pairwise comparisons identified significant differences in beta diversity between mother's milk vs. formula, donor milk vs. formula, mother's milk vs. donor milk, and mother's milk vs. donor milk mixed with formula (PERMANOVA, $p < 0.05$). These results indicate feeding type as a significant source of variation in the composition of the preterm infant gut in addition to antibiotic use.

Gut microbial community development is highly variable and unique to each infant

Although the infants in this study were enrolled into 4 groups, each infant's stay in the NICU is highly personalized, by type, frequency, number or length of antibiotic use, type and length of feeding patterns, and adverse clinical events over time. To aid in visual identification of patterns throughout the NICU course, detailed charts were created for each infant that depict both clinical and laboratory data over time (Days Post Birth) integrated into a single graphic per infant (Fig. 4 and Supplementary Figure 1). This includes results from the 16S rRNA analysis as pie charts for each stool sample, color coded by bacterial taxonomy and sized based on the $\log_{10}$-transformed number of 16S rRNA copies per gram of stool, as well as adverse clinical events coded by a single letter code (Fig. 5). With these visualizations, patterns are more easily observed between antibiotic treatments, feeding types, and the gut microbiome. For example, anti-fungal fluconazole administration resulted in lower bacterial loads and diversity. Also, the effects of antibiotic use and feeding patterns become visually apparent. To illustrate, infant 5 was exclusively fed mother's milk from day 14 through day 71 post birth. At day 31, antibiotics vancomycin and piperacillin were administered (Supplementary Figure 1). During treatment, *Veillonella* was entirely removed from the stool microbiome, falling from 3.23E+06 cells per gram (73%) from the pre-treatment time point to undetected while antibiotics were used. At day 47, 9 days after antibiotic treatment ceased, *Veillonella* again dominated the stool at 3.83E+06 cells per gram (80%), almost a complete replacement of levels before treatment. Also, the proportions of the other 2 genera found in the stool, *Escherichia* and an unclassified Enterobacteriaceae spp., were nearly identical after treatment and continuation of mother's milk

as before treatment (pre-treatment: *Escherichia* – 21%, Enterobacteriaceae spp. – 4.7%; post-treatment: *Escherichia* – 15%, Enterobacteriaceae spp. – 3.3%), demonstrating that mother's milk effectively restored the stool microbiome to its pre-treatment state.

Bacterial genera correlate with stool metabolites and inferred metabolic pathways

In addition to 16S rRNA profiling, 90 stool samples from 10 infants were sent for metabolomic profiling. Each of the four enrollment groups were included in these samples for comparison. Peak height responses were recorded for 454 identifiable metabolites. To determine if gut bacteria were associated with relative concentrations of metabolites in stool, the top 10 most abundant bacterial genera associated with identified metabolites were determined. Spearman correlation values were plotted using a heatmap, which indicated numerous significant, positive and negative, associations between bacteria and metabolites (Fig. 6a). Next, 6 of the top 10 bacterial genera were correlated between infant groups (Fig. 6b). Interestingly, *Veillonella* was positively associated with the neurotransmitter 4-aminobutanoate (GABA) (Spearman R = 0.48, p = 0.003) and *Veillonella* counts were significantly different between groups A and C2 (Wilcoxon, p = 0.0475). *Veillonella* counts were plotted using a boxplot, along with the normalized concentrations of GABA between groups (Figs. 6c,d). Furthermore, using PICRUSt2, functional pathway abundances were inferred based on the rarefied 16S rRNA data[29]. The *Veillonella* counts of predicted pathways were strongly correlated with biosynthesis of the GABA precursor L-glutamate and L-glutamine (Spearman R = 0.88, p = 3.02E-27) (Fig. 7). Thus, *Veillonella* may be at least partially responsible for GABA neurotransmitter production and that this function is negatively impacted by antibiotic use early in life. Alternatively, a

negative correlation between *Bifidobacterium* counts and glycocholic acid was observed (Spearman R = -0.39, p = 0.0098), which was impacted by antibiotic use between groups (Supplementary Figure 2). Furthermore, bifidobacteria were negatively associated with other conjugated bile acids including taurocholic (Spearman R = -0.45, p = 0.0021) and glycocholic acids (Spearman R = -0.39, p = 0.0098), but positively associated with deconjugated cholic (Spearman R = 0.36, p = 0.0203). Thus, gut microbiota affected by antibiotic use may be responsible for modification of neuroactive metabolites (i.e. deconjugated bile salts) in addition to production of neurotransmitters.

Immune markers in stool correlate with bacterial abundance

Antibiotic use was examined for its correlation with inflammatory marker level. These levels were also correlated with gut bacterial abundances. Twelve immune markers were measured in 110 samples across 18 of the first enrolled infants. Twelve bacterial genera had at least one significant correlation with an immune marker ($p < 0.05$) (Fig. 8a). Significant correlations between the bacterial genera and stool immune markers were classified as either inflammatory or anti-inflammatory based on the known function of the marker (Fig. 8b). Interestingly, *Enterococcus* was consistently negatively correlated with most of the immune markers measured. *Enterococcus* counts were lower in group A compared to the randomized groups (Wilcoxon, A vs. C1 p = 1.6E-06, A vs. C2 p = 9.4E-07) (Fig. 8c). *Enterococcus* counts did not differ significantly between infants who received or did not receive antibiotics (Wilcoxon, p = 0.2) (Fig. 8d). *Enterococcus* had a significant negative correlation with IL8:IL10 ratios, whereas this genus was positively correlated with IL1a:IL1ra ratios, suggesting that mechanisms of

controlling inflammation can be unique depending on the microbe. Notably, MIP1a was the only marker that differed significantly depending on antibiotic use (Wilcoxon, p = 0.039). *Citrobacter* was positively correlated with MIP1a (Spearman R = 0.47, p = 3.74E-05), and was significantly higher in group C1 compared to group C2 (Wilcoxon, p = 7.2E-08). *Citrobacter* was higher in those infants that received antibiotics compared to those that did not (Wilcoxon, p = 0.037).

Discussion

There is an urgent need for evidence supporting or refuting the widespread practice of routine antibiotic use after birth in symptomatic preterm neonates. The REASON study represents a significant step as it is the first randomized controlled trial to test the feasibility of randomizing symptomatic preterm infants to antibiotics versus no antibiotics, evaluating the effect of antibiotic treatment on the developing gut microbiome, metabolome, and inflammatory environment. Our results expand upon previous reports with new evidence that early routine antibiotic use leads to greater beta-diversity dissimilarity and lowered trends in richness and Shannon diversity development over time, even after discontinuation of antibiotics[17,30,31]. The power to detect significant associations was decreased mainly because many of the infants randomized to not receive antibiotics were changed to antibiotic administration. A larger multi-center randomized study is needed to validate and expand upon the extended effect of antibiotics on the developing gut microbiome.

Our results confirm that feeding type also have a significant influence on gut microbiome richness, diversity and bacterial load[32–34]. Exclusive or partial feeding with mother's own milk tended to associate with high bacterial load, which is supported by evidence that breast milk harbors maternal-originating bacteria, as well as nutritional components (prebiotics) that support

bacterial proliferation in the intestinal tract[35,36]. Interestingly, formula-fed infants had comparable levels of richness, diversity and bacterial load as mother's milk. This supports the notion that mother's milk drives early colonization of a limited set of dominant microbes through nutrient and antimicrobial-mediated selection[37–39].

Integrating detailed and personalized records of clinical and laboratory data led us to identify overlooked patterns in the data. One such peculiar pattern was that stool samples taken during administration of the anti-fungal fluconazole had lowered copies of 16S rRNA, suggesting lower bacterial load. A previous study reported that fluconazole, though not inherently bactericidal, increased the bactericidal activity of neutrophils[40]. Immune marker data were collected for 18 of the first enrolled infants, and one or more of those markers, such as calprotectin which is secreted by neutrophils, may help explain this pattern[41]. However, unfortunately only 3 of the 18 infants that had immune marker data received fluconazole, and therefore more data is needed to test this hypothesis. Interestingly, counts of *Enterococcus* were often negatively correlated with levels of many of the immune markers tested, both pro- and anti-inflammatory, an odd finding considering Enterococci have been associated with risk for infection in preterm neonates[42,43]. On the other hand, *Citrobacter* counts were associated with increased levels of the macrophage chemokine MIP1α and counts were significantly higher in infants who received antibiotics. Increased levels of MIP1α are likely related to recruitment of intestinal macrophages leading to a heightened inflammatory environment, suggesting that antibiotic use may select for bacteria which lead to intestinal inflammation[44]. Further work including a larger sample size will be needed to understand how changes in the preterm infant gut caused by routine antibiotics impacts the gut inflammatory environment.

Neurological development can be impaired in infants born very prematurely compared to their full-term counterparts; a trend that extends into delayed cognitive and behavioral development through childhood[45–47]. Could routine antibiotic use in preterm neonates play a role in this association? Intestinal microbes produce a plethora of metabolites and bio-active compounds that can be absorbed by the host[48]. Some of these compounds have direct neurologic implications including neurotransmitters such as GABA, which is reduced in preterm infants, is critical for early brain development, and possesses immunomodulatory properties[49,50]. Our results indicate that antibiotic use negatively affected the abundance of *Veillonella* and that *Veillonella* were positively correlated with GABA concentrations in the gut. Furthermore, *Veillonella* correlated strongly with the L-glutamine biosynthesis pathway, the precursor to GABA production. Aside from production of neurotransmitters, we identified negative correlations between *Bifidobacterium* abundance and concentrations of conjugated bile acids, particularly glyco- and taurocholic acid, which were significantly different based on antibiotic use. Bifidobacteria, which were more abundant in infants that did not receive antibiotics, are known to deconjugate bile acids to primary forms including cholic acid, which was positively correlated with bifidobacteria abundance[51,52]. Cholic acid can passively diffuse into the brain where it blocks signaling in the $GABA_A$ receptor[53]. Bifidobacteria may therefore be essential in regulating GABA signaling in the developing brain. These are significant findings, for they suggest routine antibiotic use could be disrupting processes involved in the gut-brain axis and immunomodulatory pathways critical for neonatal and future childhood development.

Evidence-based antibiotic use to prevent infection in preterm neonates is critical in preventing unnecessary treatment that may be doing more harm than good. Routine overuse of antibiotics can change the developmental trajectory of the infant gut microbiome during a time of critical

establishment and interaction. Given the potential for extensive crosstalk between gut microbiota and the host, changes in microbiome composition could have both short- and long-term effects on outcomes and overall health and development. Future randomized studies with greater infant enrollment will be crucial in our understanding of the effects current neonatal practice has on health which will allow for the reevaluation of practices. Such trials will need to expand on the findings from this pilot study from a multi-omic standpoint to identify direct links between antibiotic-induced dysbiosis and health outcomes.

Materials and Methods

Experimental design, enrollment, and clinical sample and data collection

The REASON study was conducted from January 2017 - January 2019 at the University of Florida and was approved by the institutional review board (IRB201501045). This study is funded by the NIH (R21HD088005). A detailed description of the study design including enrollment, group selection, randomization, and collection of clinical samples and data including outcomes has been previously described[25]. Briefly, 98 premature infants were enrolled in the study and placed into one of three groups according to previously described criteria: group A with indication for antibiotic use, group B without indication for antibiotic use, and group C eligible for randomization to antibiotics (C1) or no antibiotics (C2) in the first 48 hours after birth. Infants not receiving antibiotics in the first 48 hours after birth (group B, C2) could be changed to receive antibiotics at any time at the medical team's discretion. Clinical samples relevant to this analysis include weekly fecal collection starting with meconium when possible (all stored at -80°C) and results of bacterial and fungal cultures (blood, urine, sputum, and cerebrospinal fluid - when available) and laboratory measurements of CRP, white blood cell count and immature to neutrophil ratio. Clinical metadata from the mothers such as antepartum

antibiotic use, type, duration, and proximity to delivery were recorded. Pertinent clinical metadata from the infants include group placement, antibiotic use status, antibiotics and antifungal use including type and duration throughout NICU course, feeding type and duration, GA at birth, sex, mode of delivery and any serious adverse events (SAEs) including NEC, late onset sepsis, spontaneous intestinal perforations, bronchopulmonary dysplasia, and death.

Stool DNA extraction, 16S rRNA PCR and Sequencing Analysis

DNA extraction and 16S rRNA barcoded PCR was carried out exactly as described previously[54]. Approximately 60 gigabases of nucleotide sequencing data was generated across 5 Illumina Miseq flowcells for stool samples collected from 91 (of the 98 total) study participants where samples were collected (ICBR, Gainesville, FL, USA). The resulting sequencing reads were merged, demultiplexed, trimmed, filtered for quality and processed into amplicon sequencing variants (ASVs) as previously described with no alterations in method[54]. Briefly, sequences were processed to ASVs using the DADA2 package in R (https://www.R-project.org) and assigned taxonomy using the SILVA_v132 training dataset[55–59]. Samples were rarefied to 10,000 reads per sample, leaving 642 of the total 656 individual longitudinal stool samples for analysis.

Total bacterial quantification by universal 16S qPCR

Total bacterial load per gram of stool was determined by universal 16S rRNA qPCR using the same primer set used for amplicon sequencing (341F and 806R). QPCR assays were performed on a QuantStudio 3 system (Applied Biosystems, Life Technologies, USA). The reaction mixture contained 12.5 μl PowerUp SYBR Green 2X Master Mix (Applied Biosystems), 1 μl each of forward (341F) and reverse (806R) primer (10 μM), 1 μl of DNA template, 0.1 μg/μl BSA and brought to a final volume of 25 μl with nuclease free water. Cycling conditions were identical to

those of the endpoint PCR used for sequencing, however with a total of 40 cycles and replacing the final elongation step with a melt curve. Each sample reaction was performed in triplicate and these values were averaged for each sample copy calculation. A standard curve was generated for copy quantification using known concentrations of the expected PCR product amplified from a similar stool sample. Copies of 16S rRNA per gram of stool was calculated by multiplying the average copy number per replicate reaction (i.e. 1 μl DNA template) by the total DNA extraction volume (75 μl) and dividing this value by the mass of stool extracted in grams.

Absolute bacterial abundance by copy number correction

Absolute bacterial abundance was calculated on a per gram of stool basis by correcting the relative sequencing abundance by the variable number of copies of the 16S rRNA gene in each observed organism. This correction was done using the "Estimate" tool provided as part of the rrnDB copy number database[60]. Briefly, after rarefying each sample to an even sequencing depth, the ASV sequences were submitted through the rrnDB online portal where they were classified down to the genus level using the RDP classifier version 2.12 and copy number adjusted using rrnDB copy number data version 5.6[60,61]. The copy number adjusted relative abundance for each observed taxon was multiplied by the total number of 16S rRNA copies obtained by qPCR, resulting in the absolute abundance of each taxon per gram of stool.

Fecal inflammatory markers

Inflammatory markers were analyzed using a combination of multiplex technologies using the Bio-Rad Bio-Plex platform (Bio-Rad, California, USA). The markers evaluated include common markers of intestinal inflammation including calprotectin and S100A12, in addition to other markers such as IL-6, TNF, IL-10 and other cytokines and chemokines that may play a role in

inflammatory or anti-inflammatory processes. The data were analyzed using direct comparisons of all infant groups using analysis of variance (ANOVA) and subsequent individual comparisons. Fecal calprotectin and S100A12 levels were measured using the fCal ELISA kit from BUHLMANN Laboratories AG (Schonenbuch, Switzerland) and the Inflamark S100A12 kit from Cisbio Bioassays (Codolet, France), respectively, according to the manufacturer's instructions. Samples were then analyzed for the presence of both pro-inflammatory and anti-inflammatory cytokines/chemokines using Multiplex Human Cytokine Magnetic kit, Milliplex MAP Kit (Millipore, Billerica, MA, USA). Twelve cytokines/chemokines, including EGF, IL-10, IL-1RA, IL-B, IL-4, IL-6, IL-8, IP-10, MCP-1, MIP-1a, TNFα, and VEGF were analyzed according to the manufacturer's instructions. Plates were read using the MILLIPLEX Analyzer 3.1 xPONENT™ System (Luminex 200). Cytokine concentrations were determined using BeadView software (Millipore, Billerica, MA, USA).

Metabolomics

The infant stool samples were suspended in 400 µl 5 mM ammonium acetate. Homogenization was done 3 times for 30 seconds each time using a cell disruptor. Protein concentrations of the homogenates were measured using Qubit Protein Assay. Samples were normalized to 500 µg/ml protein at 25 µl for extraction. Each normalized sample was spiked with 5 µl of internal standards solution. Extraction of metabolites was performed by protein precipitation by adding 200 µl of extraction solution consisting of 8:1:1 acetonitrile: methanol: acetone to each sample. Samples were mixed thoroughly, incubated at 4°C to allow protein precipitation, and centrifuged at 20,000 x g to pellet the protein. 190 µl supernatant was transferred into clean tube and dried using nitrogen. Samples were reconstituted with 25 µl of reconstitution solution consisting of

injection standards, mixed, and incubated at 4° C for 10-15 min. Samples were centrifuged at 20000 x g. Supernatants were transferred into LC-vials.

Global metabolomics profiling was performed as previously described using a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler[62]. Briefly, samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 µm column with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The flow rate was 350 µl/min with a column temperature of 25°C. 4 µl was injected for negative ions and 2 µl for positive ions.

Data from positive and negative ions modes were processed separately. LC-MS files were first converted to open-format files (i.e. mzXML) using MSConvert from Proteowizard[63]. Mzmine was used to identify features, deisotope, align features and perform gap filling to fill in any features that may have been missed in the first alignment algorithm[64]. Features were matched with SECIM internal compound database to identify metabolites. All adducts and complexes were identified and removed from the data set prior to statistical analysis.

Statistical Analysis

The ASV and taxonomy tables resulting from DADA2 were manipulated using the phyloseq R package v1.30.0[65]. Inferred metabolic pathway abundances were determined from the rarefied 16S rRNA data using PICRUSt2[29]. Alpha diversity measures, including the observed number of ASVs and the Shannon diversity index, as well as Spearman correlations and associated heatmaps, were calculated and drawn using the microbiome R package v1.8.0 (https://bioconductor.org/packages/devel/bioc/html/microbiome.html). Violin and box plots (including statistical testing where applicable) were generated using the ggpubr R package v0.2.4

(https://github.com/kassambara/ggpubr), which serves as a wrapper for ggplot2[66]. The linear mixed effects modeling, volatility analysis and associated plots were done using the longitudinal plugin "q2-longitudinal" offered in Qiime2 v2019.4[26–28]. The biomformat R package (https://biom-format.org) was used to convert data in phyloseq format to BIOM format for import into Qiime2[67]. Bray-Curtis and Jaccard distance dissimilarities were calculated using the vegan R package v2.5.6 (https://github.com/vegandevs/vegan) and PCoA plots were made using ggplot2 v3.3.0[66]. Individual infant charts were also generated using ggplot2. Non-parametric statistical tests including the Wilcoxon and Kruskal-Wallis tests were used for pairwise and overall comparisons of 3 or more factors, respectively[68,69]. The permutational analysis of variance (PERMANOVA) test was used in the vegan package to compare overall microbiome dissimilarities between antibiotic use, feeding type, and enrollment groups. P-values were adjusted for false discovery rate (FDR) via the Benjamin-Hochberg method[70].

Data availability

The demultiplexed 16S rRNA sequencing data generated in this study is deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA515272.

Trial Registration

This project is registered at clinicaltrials.gov under the name "Antibiotic 'Dysbiosis' in Preterm Infants" with trial number NCT02784821.

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J.N. designed and oversaw the study. J.L.R. aided in implementation in the NICU, review of clinical events for the cohort, preparation for the DSMB and IRB reviews, and analyzed clinical data. J.N, J.L.R., D.C., and C.B. carried out patient enrollment/consent/group allocation, provided care for the infants in the NICU during the study. J.L.R, C.B. L.P. recorded clinical data. N.L. maintained/distributed the samples and performed the stool immune marker assays. T.J.G performed the stool metabolomics assays and analysis. K.L.M. assisted with stool DNA extraction, 16S PCR/qPCR, and figure generation. J.T.R. performed the stool microbiome

analysis, data integration, figure generation and wrote the manuscript. R.A.P. and E.W.T. assisted with data interpretation. All authors reviewed the manuscript before submission.

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Ethics Declarations

Competing Interests

Dr. Josef Neu is the principal investigator of a study with Infant Bacterial Therapeutics and on the Scientific Advisory Boards of Medela and Astarte. No other authors have conflicts of interest to disclose.

Tables and Figures

	Group A	Group B	Group C1	Group C2	Changed from C2
Total	32	11	28	27	13
Changed to antibiotics (bailed)	NA	1	NA	13	NA
Sex (M::F)	14::14	4::7	13::15	17::10	10::3
Delivery mode (V::C)	13::19	8::3	11::17	8::19	11::2
Gestational age range (median)	23 – 32 (28)	29 – 32 (32)	23 – 32 (29)	23 – 32 (29)	24 – 32 (28)
Maternal antibiotic exposure (Y::N)	23::9	6::5	21::7	19::8	12::1
Number of samples post-rarefying	232	42	171	197	97

Table 1 – Summary of infant enrollment and covariates

Summary of the number of enrolled infants per group used in this analysis and the number of infants changed from groups C2 and B (no antibiotics). Enrollment groups are also summarized by infant sex (male::female), mode of delivery (vaginal::caesarean), gestational ages and maternal antibiotic exposure (yes::no). The number of individual stool samples used in the analysis are also summarized by group.

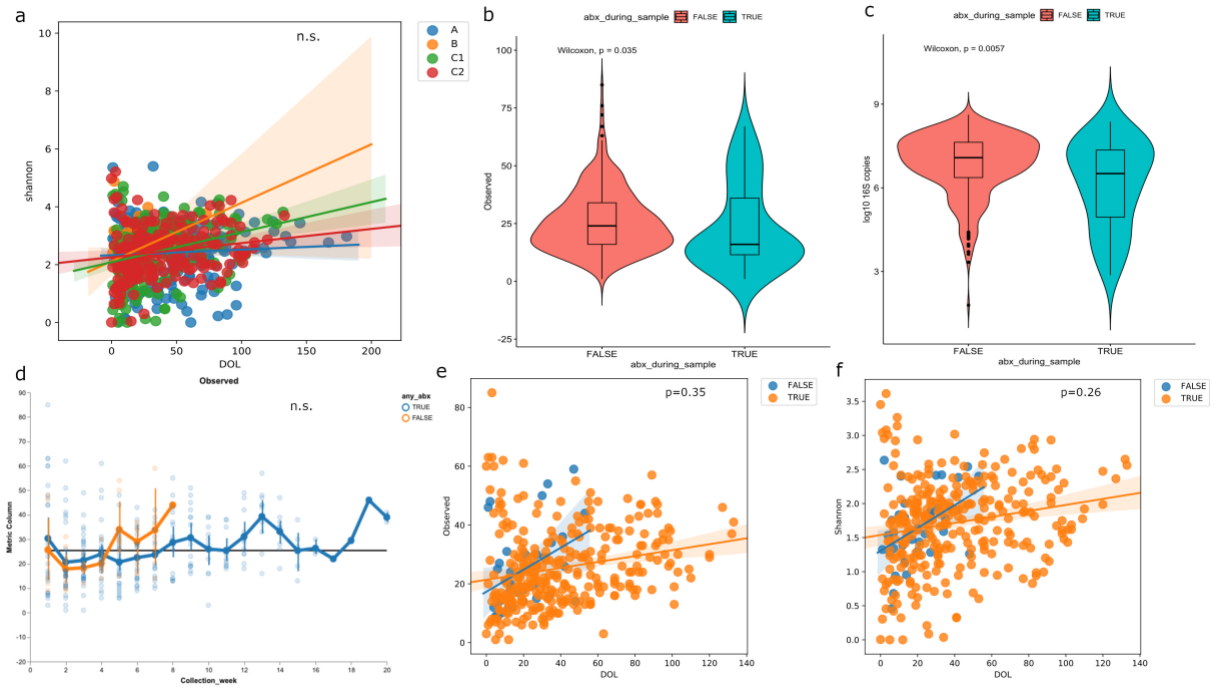


Figure 1 – Antibiotic use affects early gut richness, bacterial load and trends in diversity

a, Regression scatter plot applying linear mixed effects (LME) modeling to test the relationship between enrollment group and Shannon diversity longitudinally. (group A n=232, group B n=42, group C1 n=171, group C2 n=197). Time scale on the x-axis is day after birth (DOL). Greyed area represents a 95% confidence interval. **b**, **c**, Violin boxplots showing the **(b)** observed number of ASVs (richness) and **(c)** the bacterial load ($\log_{10}$ 16S rRNA copies) of samples which were taken when antibiotics either were (TRUE, n=83) or were not (FALSE, n=562) administered (abx_during_sample). Comparisons performed using the Wilcoxon test. **d**, Volatility plot of the observed number of ASVs by whether the infants in the randomized group received antibiotics (any_abx, TRUE, n=323 vs. FALSE, n=45). Pairwise comparisons at each timepoint done using the Wilcoxon test. **e**, **f**, Regression scatter plots applying LME to the **(e)** observed number of ASVs and **(f)** Shannon diversity longitudinally for infants in group C that

either received (TRUE, n=323) or did not receive (FALSE, n=45) antibiotics (any_abx). Greyed area represents a 95% confidence interval.

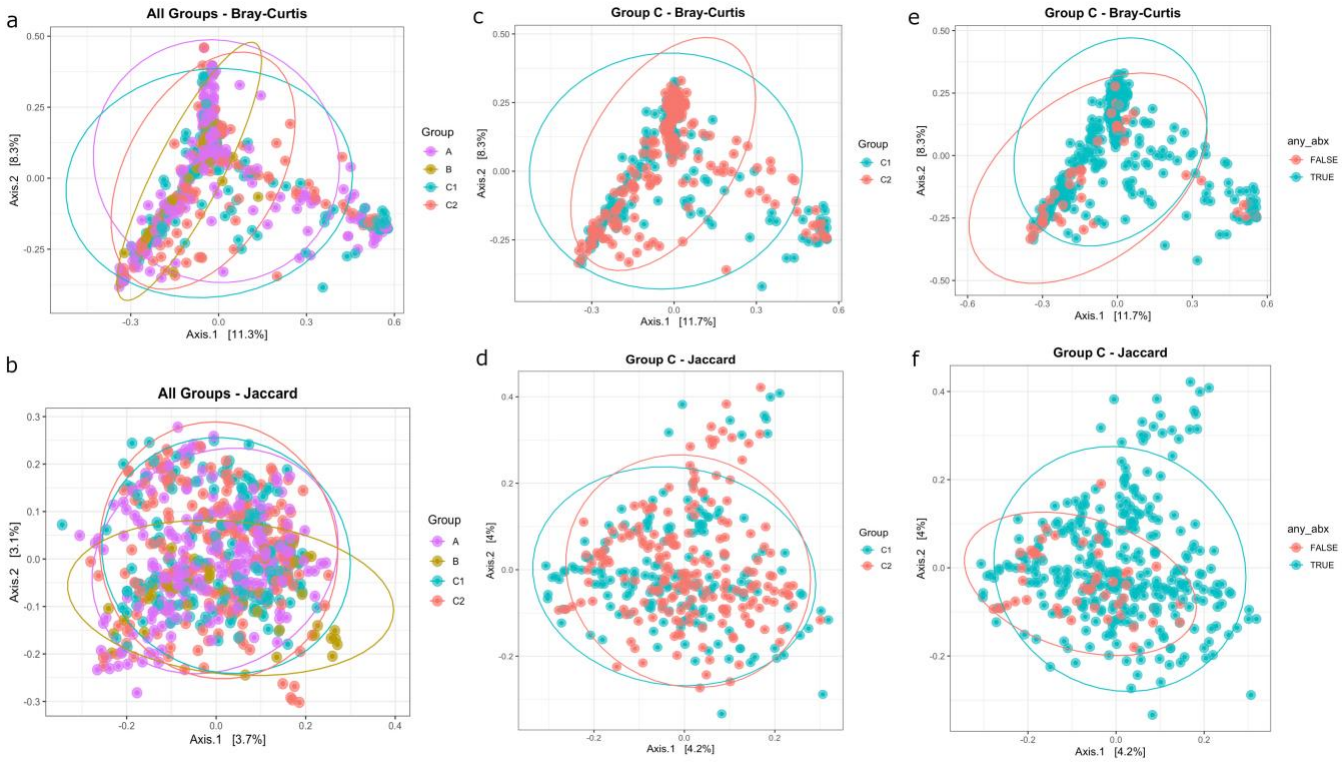


Figure 2 – Beta-diversity shows effects of antibiotics on the infant gut communities

a, **b**, **c**, **d**, **e**, **f**, PCoA ordination of stool samples by **(a, b)** enrollment group, **(c, d)** group C1 or C2, and **(e, f)** whether they received antibiotics (C1 plus C2 changed: any_abx = TRUE, n=323) or not (C2 not changed: any_abx = FALSE, n=45) using the quantitative Bray-Curtis distance index **(a, c, e)** and the qualitative Jaccard distance index **(b, d, f)**. (group A n=232, group B n=42, group C1 n=171, group C2 n=197). Ellipses are calculated based on a 95% confidence interval.

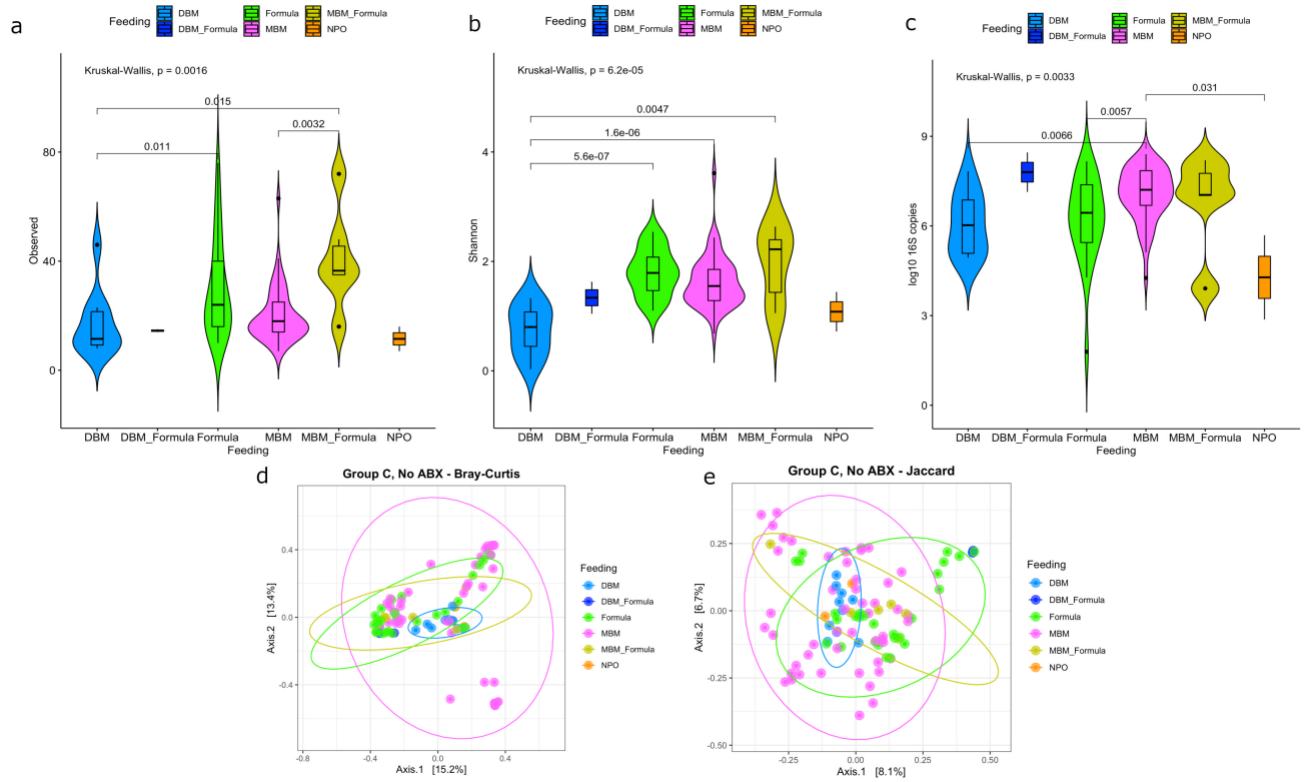


Figure 3 – Feeding patterns influence bacterial community richness, diversity, size and structure

a, **b**, **c**, Violin boxplots comparing the **(a)** observed number of ASVs (richness), **(b)** Shannon diversity, and **(c)** $\log_{10}$-transformed number of 16S copies (bacterial load) of samples across different feeding types. Overall comparisons between feeding types was performed using the Kruskal-Wallis test and pairwise comparisons by the Wilcoxon test. Only significant pairwise comparisons are shown for clarity ($p < 0.05$). **d, e**, PCoA ordination of stool samples collected during different feeding types using the **(d)** Bray-Curtis and **(e)** Jaccard distance indices. Ellipses are calculated based on a 95% confidence interval. DBM: donor breast milk, MBM: mother's breast milk, NPO: no enteral nutrition, ABX: antibiotics. (MBM n=49, DBM n=10, Formula n=27, MBM_Formula n=6, DBM_Formula n=2, NPO n=2).

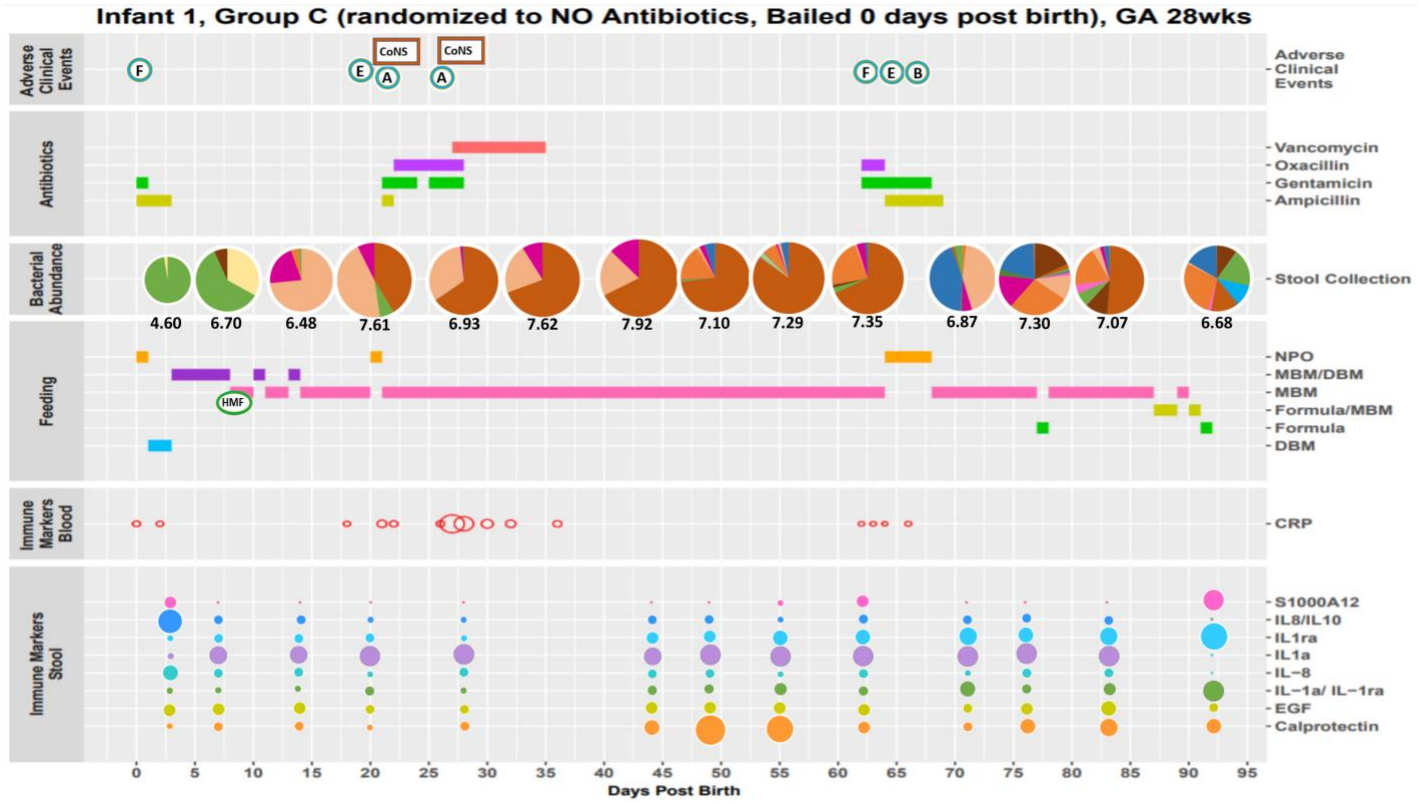


Figure 4 – Integration of clinical and laboratory data gives detailed view of infant stay in NICU

Extensive clinical and laboratory data, when combined, provide a detailed summary of each infant's stay in the NICU. Data included in each chart from top to bottom include: the infant ID, group assignment, antibiotic change status (bail), gestational age, any adverse clinical events (which are further described in Supplementary Figure 1), the type and duration of antibiotic use (if any), the copy-number corrected absolute composition of each weekly stool sample and it's $\log_{10}$-scale number of bacterial 16S rRNA copies, the type and duration of each feeding including administration of human milk fortifier, the relative levels of C-reactive protein measured from blood, and relative concentrations of measured stool immune markers (for infants where these measurements were performed). DBM: donor breast milk, MBM: mother's breast milk, NPO: no enteral nutrition, CRP: C-reactive protein, EGF: epidermal growth factor.

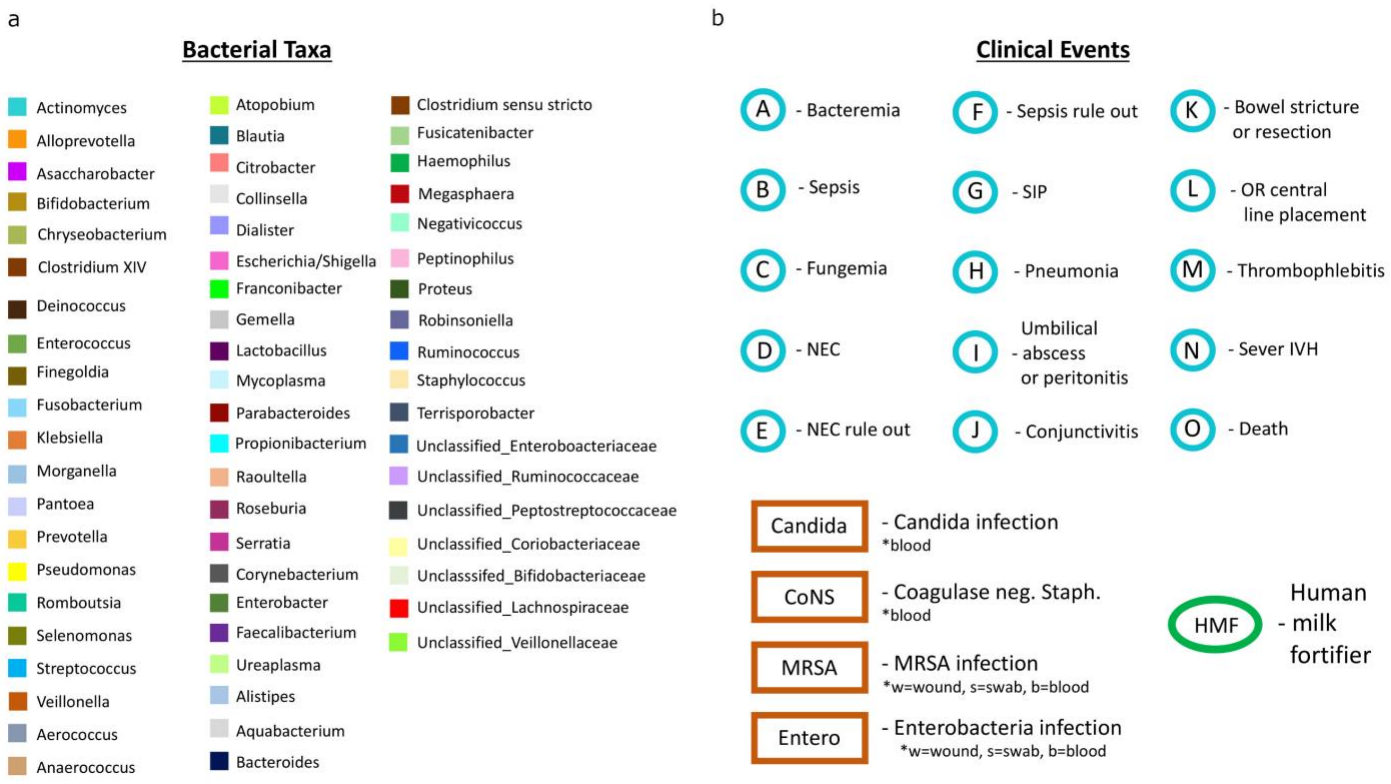
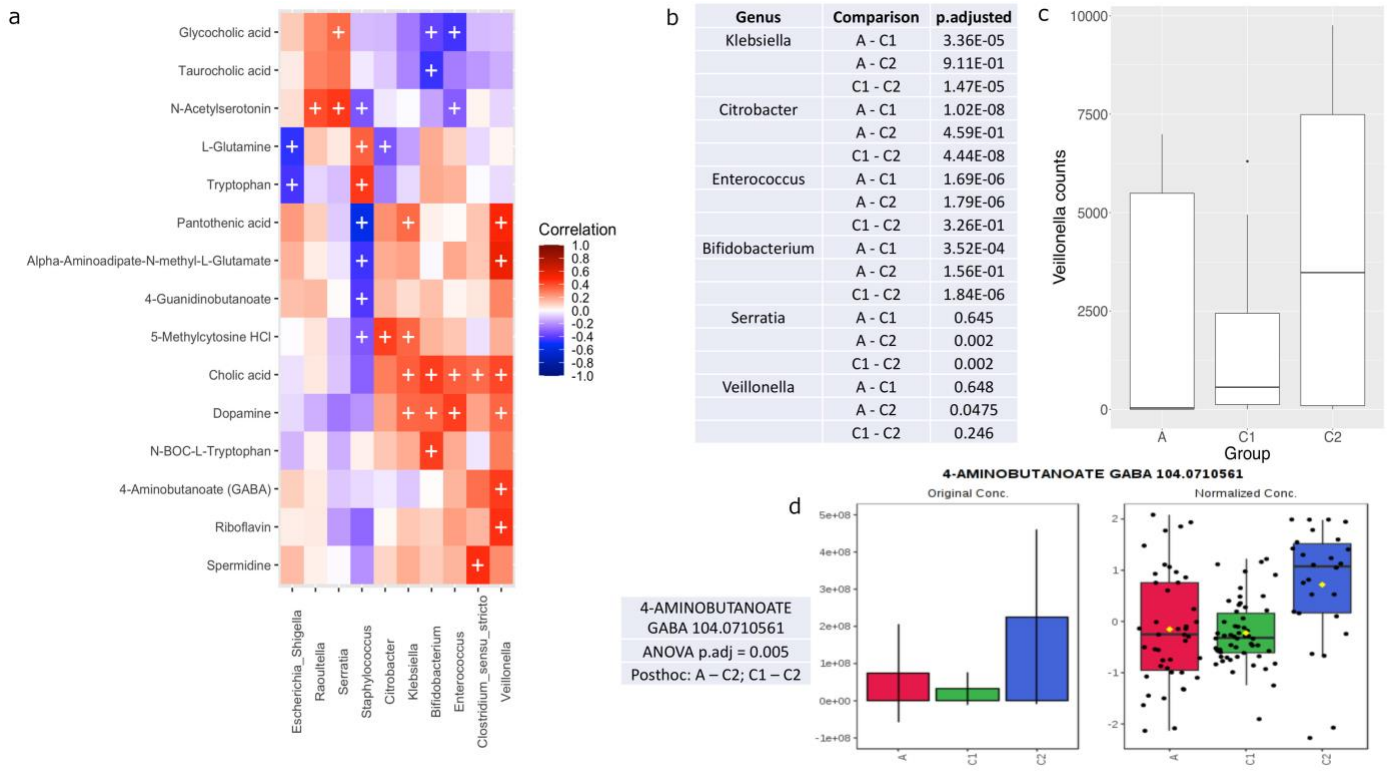


Figure 5 – Bacterial taxa and adverse clinical event key for infant charts

(a) Key for the color-coded bacterial taxa used in the stool 16S rRNA copy-number corrected composition pie chart for each infant chart. **(b)** Key for the adverse clinical events (including infections by body site) and the administration of human milk fortifier for each infant chart.



Genus	Comparison	p.adjusted
Klebsiella	A - C1	3.36E-05
	A - C2	9.11E-01
	C1 - C2	1.47E-05
Citrobacter	A - C1	1.02E-08
	A - C2	4.59E-01
	C1 - C2	4.44E-08
Enterococcus	A - C1	1.69E-06
	A - C2	1.79E-06
	C1 - C2	3.26E-01
Bifidobacterium	A - C1	3.52E-04
	A - C2	1.56E-01
	C1 - C2	1.84E-06
Serratia	A - C1	0.645
	A - C2	0.002
	C1 - C2	0.002
Veillonella	A - C1	0.648
	A - C2	0.0475
	C1 - C2	0.246

Figure 6 – Metabolites in stool correlate with abundance of bacterial genera

a, Heatmap of Spearman correlation coefficients between peak response heights of identified metabolites in stool and the top 10 bacterial genera from the same samples (n=90 stool samples). Only genera with at least one significant correlation with an identified metabolite are displayed (9 of 10). Significant correlations are indicated by a '+' with FDR-corrected p-values < 0.05. **b**, Bacterial genera which had significantly different counts when comparing at least 2 enrollment groups (at least 1 pairwise comparison). P-values are FDR-adjusted. **c**, Boxplot comparing the number of rarefied *Veillonella* counts between the enrollment groups. **d**, Original and normalized concentrations of the neurotransmitter 4-aminobutanoate (GABA) by enrollment group and significant comparisons of normalized concentrations between groups by ANOVA.

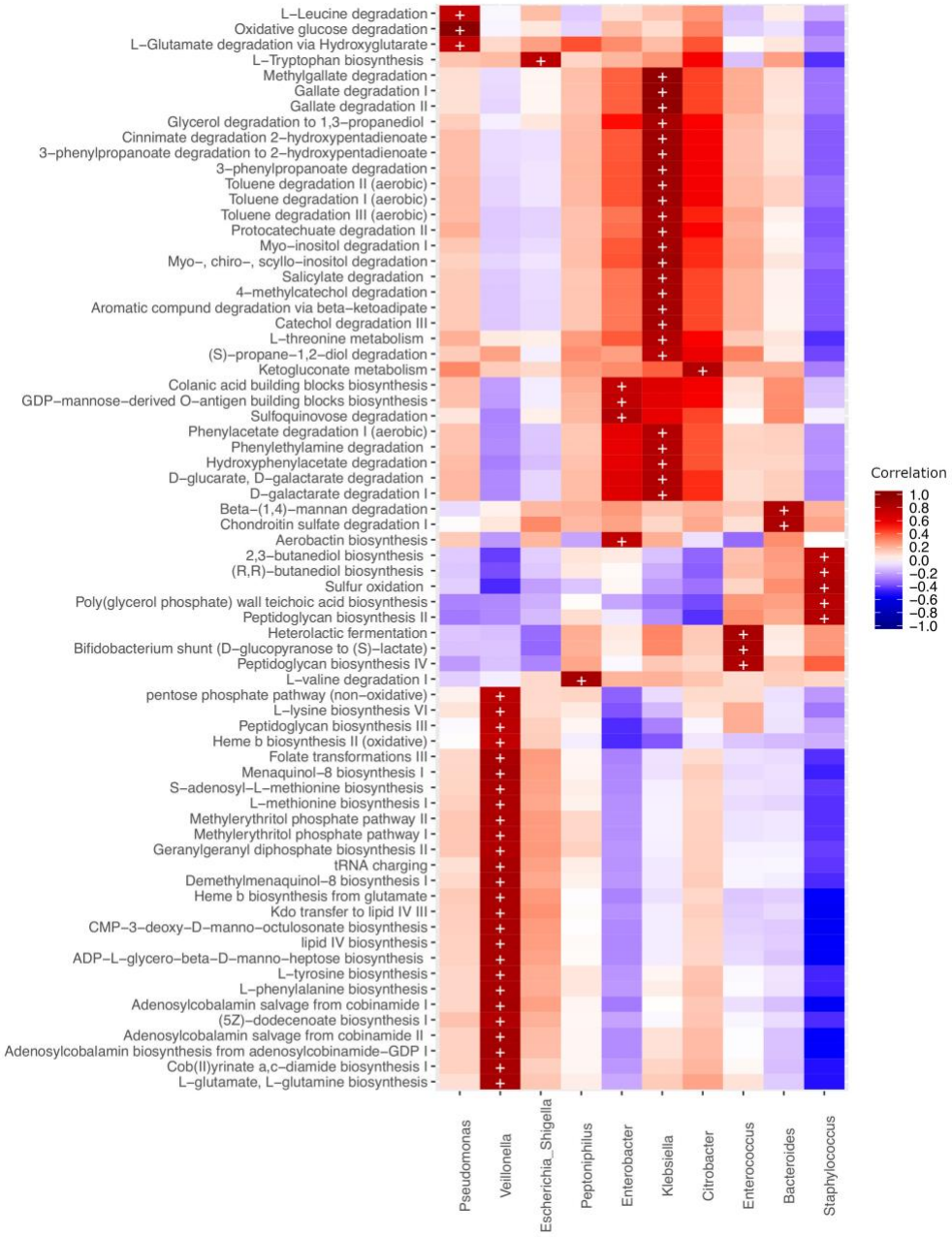
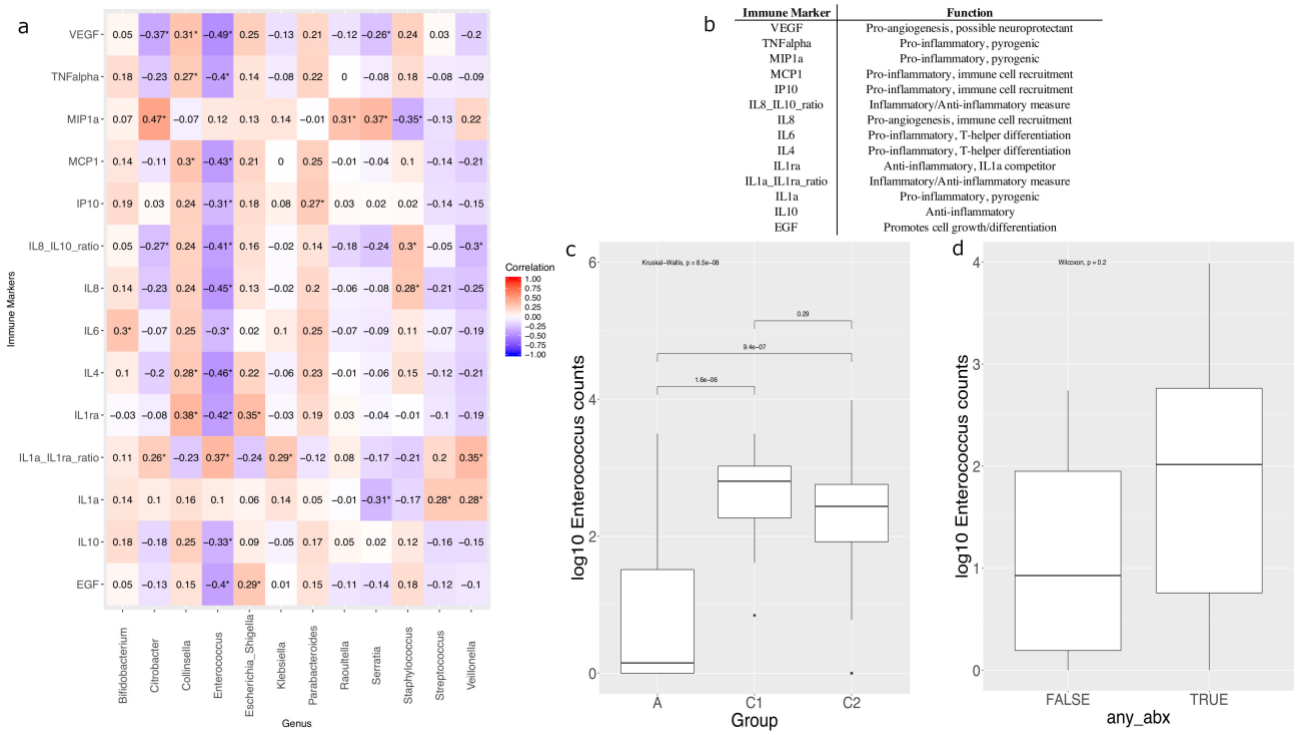


Figure 7 – Abundance of top bacterial genera correlate with abundances of inferred metabolic pathways

Heatmap of Spearman correlation values between counts of the most highly abundant bacterial genera and abundance of inferred metabolic pathways using PICRUSt2. Only bacterial genera with at least one significant correlation are depicted (p-values < 0.05). n=90 stool samples.



Immune Marker	Function
VEGF	Pro-angiogenesis, possible neuroprotectant
TNFalpha	Pro-inflammatory, pyrogenic
MIP1a	Pro-inflammatory, pyrogenic
MCP1	Pro-inflammatory, immune cell recruitment
IP10	Pro-inflammatory, immune cell recruitment
IL8_IL10_ratio	Inflammatory/Anti-inflammatory measure
IL8	Pro-angiogenesis, immune cell recruitment
IL6	Pro-inflammatory, T-helper differentiation
IL4	Pro-inflammatory, T-helper differentiation
IL1ra	Anti-inflammatory, IL1a competitor
IL1a_IL1ra_ratio	Inflammatory/Anti-inflammatory measure
IL1a	Pro-inflammatory, pyrogenic
IL10	Anti-inflammatory
EGF	Promotes cell growth/differentiation

Figure 8 – Stool immune marker levels show modest correlation with gut microbiota

a, Heatmap of Spearman correlation coefficients between immune markers measured from stool and the most abundant bacterial genera from the same samples (n=110 stool samples). Only the bacterial genera with at least one significant correlation with an immune marker are displayed (n=12). Significant correlations are marked with an '*' by the coefficient, with FDR-adjusted p-values < 0.05. **b**, Table listing the immune markers used for correlation analysis and their commonly known general functions. **c**, Comparison of $\log_{10}$-transformed number of *Enterococcus* counts by enrollment group and their significance by Kruskal-Wallis. **d**, Comparison of $\log_{10}$-transformed number of *Enterococcus* counts by whether the infant in received any antibiotics (any_abx) and their significance by the Wilcoxon test. VEGF: vascular endothelial growth factor, TNFalpha: tumor necrosis factor alpha, MIP1a: macrophage inflammatory protein 1a, MCP1: monocyte chemoattractant protein 1, IP10: interferon inducible protein 10, EGF: epidermal growth factor.