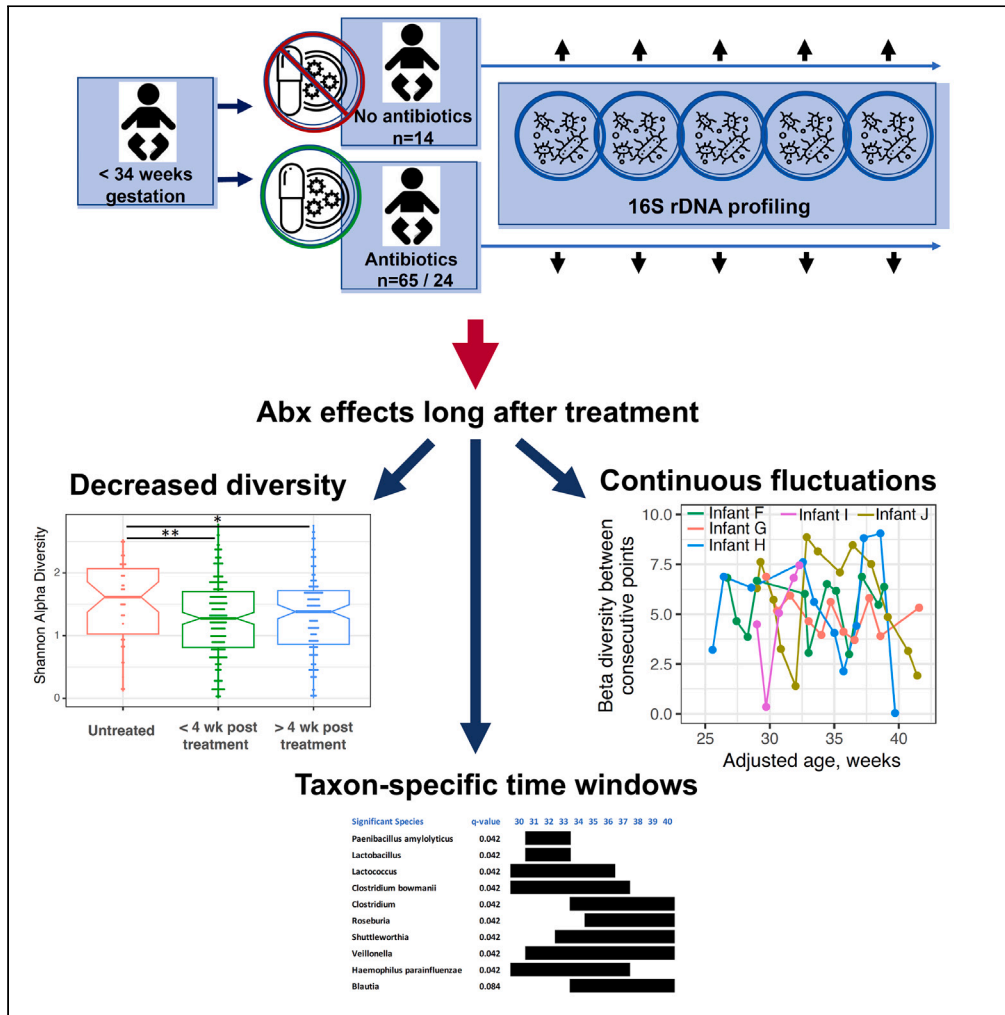**Article**

Long-term dysbiosis and fluctuations of gut microbiome in antibiotic treated preterm infants

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Highlights

We longitudinally profiled gut microbiome in 79 preterm infants throughout NICU stay

Antibiotic treatment led to very long-term dysbiosis and microbiome fluctuations

These effects were mainly due to depleting a few common taxa shared across infants

Bacterial taxa were depleted by antibiotics in taxon-specific developmental windows

Article

Long-term dysbiosis and fluctuations of gut microbiome in antibiotic treated preterm infants

Murat Cetinbas,[1,2] Julie Thai,[3] Evgenia Filatava,[4] Katherine E. Gregory,[4,5,*] and Ruslan I. Sadreyev[1,6,7,*]

SUMMARY

Postnatal acquisition of the microbiome is critical to infant health. In preterm infants, empiric use of antibiotics is common, with significant health consequences. To understand the influence of antibiotics on acquisition of the microbiome over time, we longitudinally profiled microbial 16S rRNA in the stool of 79 preterm infants during their hospitalization in the intensive care unit and compared antibiotic treated and untreated infants. Despite similar clinical presentation, antibiotic treated infants had strong deviations in the content, diversity, and most dramatically, temporal stability of their microbiome. Dysbiosis and fluctuations of microbiome content persisted long after antibiotic exposure, up to hospital discharge. Microbiome diversity was dominated by a few common bacteria consistent among all infants. Our findings may inform clinical practice related to antibiotic use and targeted microbial interventions aimed at overcoming the adverse influence of antibiotics on the microbiome of preterm infants at specific developmental time points.

INTRODUCTION

Antibiotics are the second most common class of drug prescribed in the US, with an estimated 154 million prescriptions written each year.[1,2] As many as one-third of these prescriptions are written inappropriately, overly exposing patients to antibiotics and significantly increasing the risk of antibiotic resistance and serious health conditions including *Clostridium difficile* diarrheal diseases. Antimicrobial stewardship initiatives have influenced the use of antibiotics, but some of the decreases in inappropriate use achieved prior to 2020 have been countered by the COVID-19 pandemic. Given the ongoing use of antibiotics, better understanding the impact of antibiotics on human health, specifically gut health, gut microbes, and the microbiome, is a public health priority.

During infancy and childhood, antibiotics are the most common medication prescribed. Nearly all preterm children are exposed to antibiotics via their mothers during labor and birth, immediately following birth, and then again in the first year of life[3–5] While antibiotics are known to prevent and treat life-threating infections in this vulnerable patient population, they are also associated with significant morbidity including necrotizing enterocolitis (NEC), late-onset sepsis, and death.[6] Empiric use of antibiotics in preterm children has been decreased by a better understanding of the risk factors for infection and the development of neonatal sepsis calculators,[7] but inappropriate use persists.[8] In short, antibiotics remain an important part of the treatment plan for many preterm infants in the neonatal intensive care units (NICUs) but there is only a limited knowledge of how they influence other aspects of preterm infant health, notably gut health, specific gut microbes, and the microbiome as a whole at specific developmental time points.

Numerous epidemiologic analyses have shown associations between antibiotic exposures early in life and immune-mediated health outcomes such as allergy, inflammatory bowel disease (IBD), and obesity.[9–12] These associations may be partially explained by the evidence suggesting that antibiotics influence the composition of the microbiome by decreasing overall species-richness and diversity,[13–15] as well as antibiotic resistance.[16,17] More specific analyses focused on the unique longitudinal differences in the content of the microbiome and presence of specific gut microbes in infants treated with antibiotics are needed to answer clinical questions about how antibiotics influence the microbiome and when these changes occur in the context of gestational age and early growth and development of the gut. In this study, we used longitudinal profiling of gut microbiome in a cohort of preterm infants throughout their NICU hospitalization to understand the association of antibiotics treatment with (1) specific bacterial composition, (2) diversity, and (3) temporal dynamics and stability of microbiome over the course of early infant development. Investigating these specific factors related to the influence of antibiotics on the preterm infant gut microbiome is critical to advancing the long-awaited development of next-generation antibiotics, as well as targeted microbial interventions aimed at restoring the composition of the preterm infant gut microbiome following antibiotic treatment.

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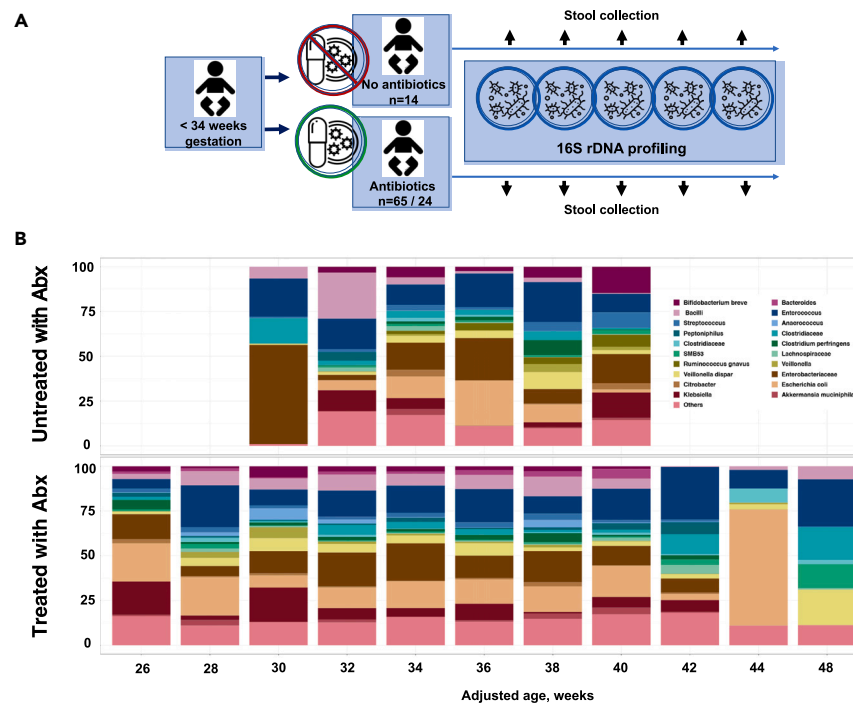https://doi.org/10.1016/j.isci.2023.107995

Figure 1. Longitudinal survey of microbial abundancies in preterm infants treated and not treated with antibiotics

(A) Schematic of experimental design. Among 65 infants treated with antibiotics, 34 infants received antibiotic treatment in the first week of hospitalization. As a parallel analysis, we focused on the subgroup of 24 antibiotic treated infants that did not have significant differences in clinical values from control group of 14 infants not treated with antibiotics.

(B) Bar plot of group-wise mean fractional abundances at specific adjusted ages (in weeks) in infants untreated (top, n = 14) and treated (bottom, n = 65) with antibiotics.

RESULTS

Longitudinal survey of gut microbiome in preterm infants treated with or without antibiotics

To understand the influence of antibiotic treatment on the developmental dynamics of gut microbiome in preterm infants (<34 weeks gestation), we analyzed the time dependence of microbial content, microbiome diversity, and temporal stability of the microbiome in infants treated with antibiotics and compared to those not treated with antibiotics during the NICU hospitalization from birth to discharge (mean duration of hospitalization = 81.6 ± 40 days).

In total, we profiled 363 stool samples from 65 preterm infants treated with antibiotics and 52 samples from 14 preterm infants not treated with antibiotics (Figure 1A; Table S1). A detailed summary of the whole patient cohort including clinical characteristics of both the infant and the mother is shown in Table 1 and further categorized by infant's antibiotic treatment (treated vs. untreated groups) in Table 2. All infants were born prior to 34 weeks of gestation, and as expected, the infants treated with antibiotics were born at earlier gestational ages and lower birth weights (mean = 28.45 ± 2.62 weeks, 1115.9 ± 480.1 g) when compared to their untreated counterparts (mean = 31.55 ± 1.68 weeks, 1441.1 ± 460.8 g). Other than gestational age, birth weight, and actual breast milk intake in the first 28 days, the groups did not significantly differ in clinical presentation (i.e., maternal antibiotic administration, mode of birth, human milk feeding, Table 2). In order to account for the differences, we additionally analyzed a subgroup of antibiotic treated infants (n = 24, marked in Table S1) that did not have significant differences from control group with respect to gestational age, birth weight, or breast milk intake (Table 3). These subgroup analyses are reported in the supplemental figures. As the temporal parameter of infant gut development, we used infant's adjusted age, as it has previously been shown to be a strong correlate of the development of gut microbiome across time.[18,19]

In infants treated with antibiotics, stool samples were obtained at various time points over the course of their hospitalization, beginning as early as 26 weeks and spanning to as late as 48 weeks of adjusted age (Figure 1B). Stool sampling in infants not treated with antibiotics spanned 30–40 weeks of adjusted age (Figure 1B), which was expected based on their gestational age at birth and overall shorter hospitalization. The resulting 16S rDNA data were analyzed using QIIME2[20] with operational taxonomic units (OTUs) defined by QIIME2 at the detailed resolution of species level when possible. However, as typical for QIIME2 analysis, many OTUs could not be resolved down to individual species and were reported at broader taxonomic levels of genus, family, etc. Figure 1B shows the temporal progression of mean fractional OTU abundances in the samples from infants untreated (top) and treated with antibiotics (bottom), binned by adjusted age at biweekly resolution. A similar progression for the subgroup of n = 24 antibiotic treated infants with no significant differences in clinical characteristics from control group is shown in Figure S1.

Table 1. Clinical characteristics of all mother-infant dyads (n = 79)

Infant characteristics	Mean $\pm$ SD or n (%)
Gestational Age at Birth (weeks)	29.00 $\pm$ 2.74
Birth Weight (grams)	1173.5 $\pm$ 490.0
Male Sex	37 (46.8%)
Length of Hospitalization (days)	81.6 $\pm$ 40.0
Antibiotic Exposure	65 (82.3%)
Sepsis	11 (13.9%)
Necrotizing Enterocolitis	7 (8.9%)
Bell Stage I	2 (2.5%)
Bell Stage II	4 (5.1%)
Bell Stage III	1 (1.3%)
Maternal characteristics	
Cesarean Delivery	59 (74.7%)
Singleton Gestation	50 (63.3%)
Mother's Antibiotic Exposure	76 (96.2%)

Continuous variables are indicated as mean $\pm$ standard deviation and categorical variables are indicated as absolute number (percentage).

Antibiotic associated differences in microbiome diversity and temporal stability

Consistent with previous reports,[18,21] we observed that the diversity of intestinal microbiome in preterm infants was much lower than typically observed in term infants. In most of these samples, the microbiome included a relatively small number of up to 10–20 OTUs and was often dominated by a few major OTUs in a given sample. Although this relatively low microbiome diversity was observed across the whole cohort regardless of antibiotics exposure, the microbiome diversity was especially low in infants treated with antibiotics (Figure 1B).

To understand the differences between these infant groups in quantitative detail, we analyzed the dynamics of (1) microbiome diversity and (2) stability of microbiome over the course of time in each individual infant. As two typical examples, Figure 2 shows the detailed dynamics of microbiome composition for an infant that was not treated with antibiotics (Figure 2A, infant 1084 in Tables S1 and S2) compared to an infant who received antibiotic treatment on the first week after birth (Figure 2B, infant 1120 in Tables S1 and S2). Temporal progression of microbiome content for each infant is shown as a heatmap of fractional abundance of OTUs (rows) as a function of adjusted age (columns), with colors indicating the abundance of a given OTU at a given time.

Both infants had a very sparse gut microbiome that was dominated by only a few highly abundant OTUs at each time point. However, these infants had strikingly different patterns of temporal microbiome development in terms of diversity and OTU content (Figure 2). In infants not treated with antibiotics, the number of major dominant OTUs was small but still larger than in antibiotic-treated infants. These infants showed a more expected gradual growth of microbiome diversity over time preserving the balance between fractional abundances. Acquisition of new OTUs typically did not affect previously acquired dominant OTUs but increased the diversity of the microbiome, similar to microbiome development in healthy term infants (Figure 2A). By contrast, in antibiotic treated infants, the number of highly abundant OTUs was smaller and the balance between the fractional abundance in the same infant was not conserved and abundances showed abrupt changes over time. Such irregular pattern of time evolution of OTU abundances with very little continuity between adjacent time points was only inherent to infants treated with antibiotics and the strong abrupt changes persisted over a long period of time, often several weeks and even months after the antibiotic treatment was discontinued (Figure 2B).

To further characterize these striking differences in microbiome diversity and stability, we analyzed the time evolution of Shannon diversity[22] and species-richness in individual infants as measures of microbiome diversity. Species-richness (richness for short) is the number of observed OTUs, whereas Shannon diversity takes into account both the number of OTUs and the evenness of the distribution of OTU abundances. Figure 3A shows typical examples of trajectories of Shannon diversity as a function of adjusted age for representative infants not treated with antibiotics. Figure 3B shows typical examples of trajectories of Shannon diversity for infants treated with antibiotics.

Infants not treated with antibiotics displayed a gradual increase in diversity metrics over time, consistent with a continual colonization with infant's age. Diversity fluctuations along these trajectories were relatively rare and minor and expected (Figure 3A). By contrast, the trajectories of microbiome diversity for antibiotic treated infants did not show such consistent gradual growth pattern and exhibited high-magnitude random fluctuations. Contrary to our expectations, this pattern of low diversity and strong fluctuations persisted throughout the hospitalization, often many weeks after antibiotic treatment (Figure 3B).

To understand the overall effect of antibiotics on microbiome diversity, we compared the distributions of diversity metrics among all samples in the two groups of infants regardless of the time that samples were collected. Infants treated with antibiotics had significantly lower Shannon diversity of their microbiome (Figure 3C, Wilcoxon p = 0.0066).

Table 2. Clinical characteristics of mother-infant dyads categorized by infant antibiotic exposure during hospitalization

	Infant antibiotic exposure		
Infant characteristics	Treated (n = 65)	Untreated (n = 14)	p-value
Gestational Age at Birth (weeks)	28.45 ± 2.62	31.55 ± 1.68	<0.0001
Birth Weight (grams)	1115.9 ± 480.1	1441.1 ± 460.8	**0.0191**
Male Sex	28 (43.1%)	9 (64.3%)	0.149
Race			
White	38 (58.5%)	6 (42.9%)	0.038
Black or African American	15 (23.1%)	2 (14.3%)	
Asian	0 (0%)	2 (14.3%)	
Other or Unknown	12 (18.5%)	4 (28.6%)	
Ethnicity			
Not Hispanic/Latina	48 (73.8%)	8 (57.1%)	0.342
Hispanic/Latina	16 (24.6%)	6 (42.9%)	
Unknown	1 (1.5%)	0 (0%)	
Length of Hospitalization (days)	88.2 ± 40.0	51.2 ± 22.7	**0.0015**
Sepsis	11 (16.9%)	0 (0%)	0.197
Necrotizing Enterocolitis	7 (10.8%)	0 (0%)	0.342
Bell Stage I	2 (3.1%)	0 (0%)	1.000
Bell Stage II	4 (6.2%)	0 (0%)	
Bell Stage III	1 (1.5%)	0 (0%)	
Dietary Intake			
1–28 days			
Actual BM intake (%)	80 ± 34	57 ± 39	**0.0036**
Infants with >50% BM	51 (78.5%)	8 (57.1%)	0.096
29 days - discharge			
Estimated BM intake (%)[a]	51 ± 45	53 ± 46	0.986
Infants with >50% BM[a]	28 (49.1%)	5 (50.0%)	0.959
Overall hospitalization			
Estimated BM intake (%)	64 ± 40	54 ± 38	0.171
Infants with >50% BM	41 (63.1%)	8 (57.1%)	0.678
Maternal characteristics			
Cesarean Delivery	47 (72.3%)	12 (85.7%)	0.499
Singleton Gestation	41 (63.1%)	9 (64.3%)	0.932
Maternal Antibiotic Exposure	62 (95.4%)	14 (100%)	1.000

Continuous variables are indicated as mean ± standard deviation and categorical variables are indicated as absolute number (percentage). p-values for statistical significance of differences between antibiotic treated and untreated groups were calculated using chi-square analysis and Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables. Statistically significant differences (p < 0.05) are highlighted in bold.
[a]Based on "Treated" (n = 57) and "Untreated" (n = 10) since 8 infants from "Treated" group and 4 infants from "Untreated" group were discharged prior to 29 days.

To further quantify and understand the observed fluctuations in microbiome content (Figure 2) and diversity (Figures 3A and 3B) we used Shannon beta diversity to calculate the distance between microbiome content in the same infant at any two consecutive time points. For each sampling time point *T*, we calculated beta diversity between the microbiome content in the sample taken at point *T* and the next sampling point *T*+1 in the same infant. The beta diversity values reflect the magnitude of short-term changes in microbiome content as it progresses through time. Figures 3D and 3E show typical examples of trajectories of beta diversity between adjacent time points as a function of adjusted age for representative infants not treated (Figure 3D) and treated with antibiotics (Figure 3E). In infants not treated with antibiotics the magnitude of the beta diversity was relatively low throughout the time, we observed smaller differences between consecutive time points indicating relatively high stability of microbiome throughout the time (Figure 3D). By contrast, in infants treated with antibiotics, the differences in microbiome content between consecutive time points were much larger, suggestive of much more unstable microbiome content rapidly changing

Table 3. Clinical characteristics of mother-infant dyads for the subgroup of antibiotic treated infants that had no significant differences from control infants

Infant characteristics	Infant antibiotic exposure		p-value
	Treated (n = 24)	Untreated (n = 14)	
Gestational Age at Birth (weeks)	30.9 ± 1.60	31.6 ± 1.68	0.226
Birth Weight (grams)	1570.5 ± 390.8	1441.1 ± 460.8	0.356
Male Sex	11 (45.8%)	8 (57.2%)	0.501
Race			
White	17 (70.8%)	6 (42.9%)	0.174
Black or African American	3 (12.5%)	2 (14.3%)	
Asian	0 (0%)	2 (14.3%)	
Other or Unknown	4 (16.7%)	4 (28.6%)	
Ethnicity			
Not Hispanic/Latina	18 (75%)	8 (57.1%)	0.253
Hispanic/Latina	6 (25%)	6 (42.9%)	
Unknown			
Length of Hospitalization (days)	60.4 ± 29.9	51.2 ± 22.7	0.458
Sepsis	0 (0%)	0 (0%)	–
Necrotizing Enterocolitis	2 (8.3%)	0 (0%)	0.522
Bell Stage I	0 (0%)	0 (0%)	0.522
Bell Stage II	2 (8.3%)	0 (0%)	
Bell Stage III	0 (0%)	0 (0%)	
Dietary Intake			
1–28 days			
Actual BM intake (%)	72 ± 39	57 ± 39	0.092
Infants with >50% BM	17 (70.8%)	8 (57.1%)	0.391
29 days - discharge			
Estimated BM intake (%)[a]	46 ± 44	53 ± 46	0.648
Infants with >50% BM[a]	9 (50%)	5 (50%)	1.000
Overall hospitalization			
Estimated BM intake (%)	62 ± 39	54 ± 38	0.389
Infants with >50% BM	15 (62.5%)	8 (57.1%)	0.744
Maternal characteristics			
Cesarean Delivery	15 (62.5%)	12 (85.7%)	0.160
Singleton Gestation	14 (58.3%)	9 (64.3%)	0.717
Maternal Antibiotic Exposure	22 (91.7%)	14 (100%)	0.522

Continuous variables are indicated as mean ± standard deviation and categorical variables are indicated as absolute number (percentage). p-values for statistical significance of differences between antibiotic treated and untreated groups were calculated using chi-square analysis and Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables.
[a]Based on "Treated" (n = 18) and "Untreated" (n = 10) since 6 infants from "Treated" group and 4 infants from "Untreated" group were discharged prior to 29 days.

through the whole period of observations (Figure 3E). This high level of fluctuations resulted in a significant difference between overall distributions of beta diversity in two infant groups (Figure 3F, Wilcoxon p = 0.00018).

This decreased diversity and persistent long-term fluctuations of microbiome content were also evident even in a smaller subgroup of n = 24 antibiotic treated infants that had no significant differences of clinical variables compared to control (Table 3). Figure S2 shows representative trajectories and overall significant differences from control in terms of Shannon alpha diversity (Wilcoxon p = 0.018) and beta diversity between adjacent time points (Wilcoxon p = 0.00044) for infants for this subgroup.

Very similar results were obtained using species richness as an alternative metric of microbiome diversity (Figure S3). The representative trajectories of richness for the two groups of infants are shown in Figures S3A and S3B, respectively. Infants treated with antibiotics had
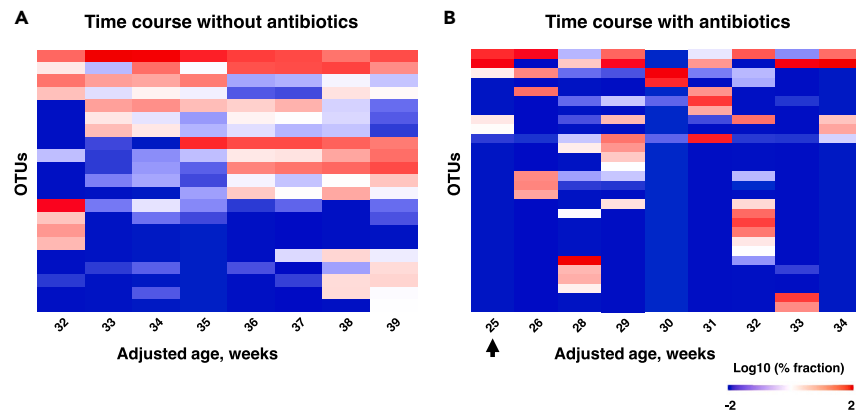
A **Time course without antibiotics**

B **Time course with antibiotics**

Figure 2. Antibiotic treatment is associated with lower diversity and strong temporal instability of gut microbiome

(A and B) Heatmaps of abundancies of individual OTUs (rows) in units of adjusted age (weeks, columns) for representative infants not treated (infant 1084, A) and treated with antibiotics (infant 1120, B). Time of antibiotic treatment is indicated by an arrow. Colors indicate the abundance (log2 of fractional abundance) of a given OTU (species or otherwise lowest resolved taxon) at the given age. In infants not treated with antibiotics, bacterial diversity tends to grow via the addition of new bacterial taxa while largely retaining previously acquired taxa. In infants treated with antibiotics, microbial diversity is lower and OTU abundances undergo strong short-term fluctuations. These fluctuations persist for a long time after antibiotic treatment.

significantly lower richness of their microbiome, either as a whole cohort (Figure S3C, Wilcoxon p = 0.0021) or as a balanced subgroup of 24 infants with no significant differences in clinical variables compared to control (Figure S3D, Wilcoxon p = 0.0066).

Common bacteria: a small subset of the most frequently observed and abundant microbes shared among all infants

Although a typical microbiome sample for a preterm infant contains up to 10–20 OTUs, only a few of the most abundant OTUs accounted for a major fraction of microbiome in a given sample. These dominant OTUs were common among all infants regardless of antibiotic exposure. The heatmap in Figure 4 shows OTUs as rows, ranked by the fraction of all infants where the given OTU was present at one or more timepoints. These fractions of infants are shown as a barplot on the left. The colors in the heatmap indicate the highest abundance of the given OTU (row) observed among all samples for a particular infant (column). The corresponding numerical values are listed in Table S2. Data presented in Figure 4 suggest two important observations. First, the OTUs that are most frequently present among all infants (top rows of the heatmap with highest bars in the barplot on the left) also have the highest fractional abundance in an infant. Second, these dominant OTUs are the same in both groups of infants regardless of antibiotic exposure. Taken together, the same group of OTUs emerges as both the most abundant and the most frequently present in our cohort of samples regardless of antibiotic treatment. We define this group as "common" OTUs. As a criterion of this definition, we chose the approximate position of the inflection point in the barplot of infant fractions where the given OTU was detected, which corresponded to approximately top 20 OTUs (Figure 4). These top 20 "common OTUs" were also substantially more abundant than the rest of OTUs, which we will refer to as "rare OTUs" (Figure 4). Common OTUs and the fractions of infants in both groups where these OTUs were detected are shown in Table 4. Six OTUs were identified in >90% of all infants: c. *Bacilli*, f. *Enterobacteriaceae*, *Enterococcus*, *Veillonella dispar*, *Escherichia coli*, and *Streptococcus*. Among these OTUs, *Enterobacter*, *Enterococcus*, *E. coli*, and *Streptococcus* are commonly found within the preterm infant gut microbiome. These bacteria are different from those typically found in the term infant gut microbiome, largely due to the hospital environment, feeding regimes, and common clinical practices such as antibiotic administration. While there were not striking differences in the top OTUs between the antibiotic treated and untreated groups, these common OTUs are relevant to preterm infant health outcomes in that they represent more pathogenic and less commensal bacteria, such as *Bifidobacterium* or *Lactobacilli*. The clinical relevance of specific identities of these common OTUs are two-fold. First, they highlight the more pathogenic nature of the gut microbiome in preterm infants. Second, they may provide a basis to guide microbial interventions during the newborn ICU hospitalization to optimize the gut microbiome in all infants.

A similar pattern of common OTUs was also evident when we restricted our analyses of antibiotic treated infants to the smaller subgroup of 24 infants with no significant differences of clinical values compared to control (Figure S4).

Lower microbiome diversity persists for weeks and is influenced by common bacteria

Focusing separately on the common and rare OTUs, we investigated the diversity and temporal dynamics of these two groups, as well as their differences associated with antibiotic treatment. Most preterm infants receive antibiotic treatment in the first week of hospitalization. The majority of preterm infants in our cohort (n = 34) received antibiotics during their first three days following birth and some of these infants (n = 6) were treated with antibiotics at later time points prior to hospital discharge. The remainder of infants in our antibiotic treated cohort (n = 31) were not administered antibiotics in the first days following birth but received antibiotics at a later point in their hospitalization. To understand the persistence of antibiotic effects after the treatment was discontinued, we compared diversity between three categories of
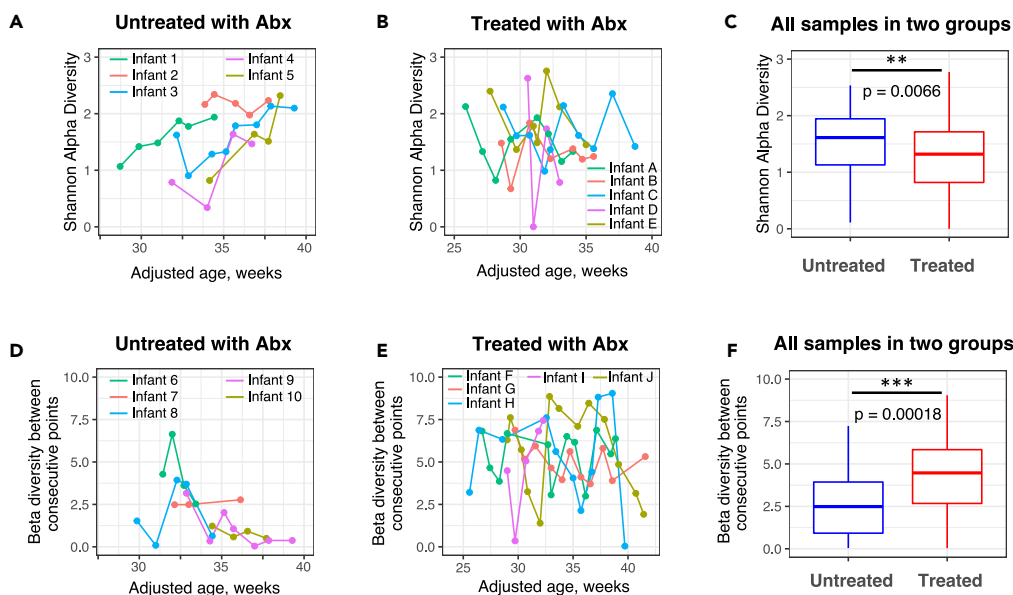
Figure 3. In infants treated with antibiotics, microbiome diversity is significantly lower and fluctuations in microbiome content are significantly stronger over the whole period of observation

(A and B) Representative trajectories of Shannon alpha diversity (y axis) as a function of adjusted age (weeks, x axis) in individual infants untreated (A) and treated with antibiotics (B).

(C) Boxplots comparing distributions of Shannon diversity among all samples from infants untreated (blue) and treated with antibiotics (red).

(D and E) Representative trajectories of distance (Shannon beta diversity) between the composition of microbiome in the same infant at consecutive time points, as a measure of instability of microbiome composition. Each line corresponds to an individual infant, with points indicating beta diversity distance between microbiome compositions at the given sampling time point and the next time point.

(F) Boxplots comparing distributions of distances (Shannon beta diversity) between all adjacent sampling time points for all individual infants untreated (blue) and treated with antibiotics (red). Boxplots represent median as a line, interquartile range (IQR) between Q1 and Q3 as a box, and the range between Q1 - 1.5 IQR and Q3 + 1.5 IQR as whiskers. Asterisks denote statistical significance: Wilcoxon test p-value <0.01 (**) and <0.001 (***).

infants: infants who did not receive antibiotic treatment, infants during or less than 28 days (4 weeks) after antibiotic treatment, and antibiotic-treated infants more than 28 days after the treatment (regardless of when the treatment started). As diversity metrics in these comparisons, we used Shannon diversity (Figure 5) and richness (Figure S5). As expected, the diversity of the whole microbiome (Figures 5A and S5A) in infants during antibiotic treatment was significantly lower than in infants who did not receive antibiotic treatment. Interestingly, this lower microbiome diversity persisted in the samples taken more than 4 weeks after antibiotic treatment (Figures 5A and S5A), suggesting that antibiotic exposure has an unexpected long-term effect on microbiome diversity.

Since both Shannon diversity and richness are calculated as sums of individual terms for each OTU, these sums over the whole microbiome can be divided and assessed separately among common and rare bacteria. The diversity of common bacteria showed a trend very similar to the whole microbiome (Figures 5B and S5B). Both infants during antibiotic treatment and infants >4 weeks post-treatment had significantly lower microbiome diversity than infants that did not receive antibiotic treatment. The diversity of rare bacteria was overall much lower but followed a similar trend (Figures 5C and S5C). Although this trend for a decrease was not statistically significant for Shannon alpha diversity (Figure 5C), the richness of rare bacteria was significantly depleted both <4 weeks and >4 weeks after antibiotic treatment (Figure S5C). Taken together, this suggests that although the set of the most common OTUs is shared between the infants regardless of antibiotic treatment (Figure 4), the diversity of these common OTUs is a dominant part of the antibiotic-associated differences observed in the overall microbiome diversity.

We also analyzed the long-term behavior of microbiome diversity in the subgroup of 24 infants that did not have significant differences of clinical variables compared to control (Figures S6 and S7). This smaller subgroup also showed a similar trend for suppression of microbiome diversity beyond 4 weeks after antibiotic exposure, measured by both Shannon alpha diversity (Figures S6A–S6C) and richness (Figures S7A–S7C).

Associations of antibiotic treatment with temporal progression of microbiome diversity and composition

To assess the association between antibiotic treatment and microbiome diversity at a longer timescale, we compared the diversity of the last microbiome sample before hospital discharge of infants who were and were not treated with antibiotics. Adjusted age at hospital discharge was 41.1 ± 4.3 weeks for untreated infants and 38.9 ± 2.5 weeks for infants treated with antibiotics (mean ± SD). Among antibiotic treated
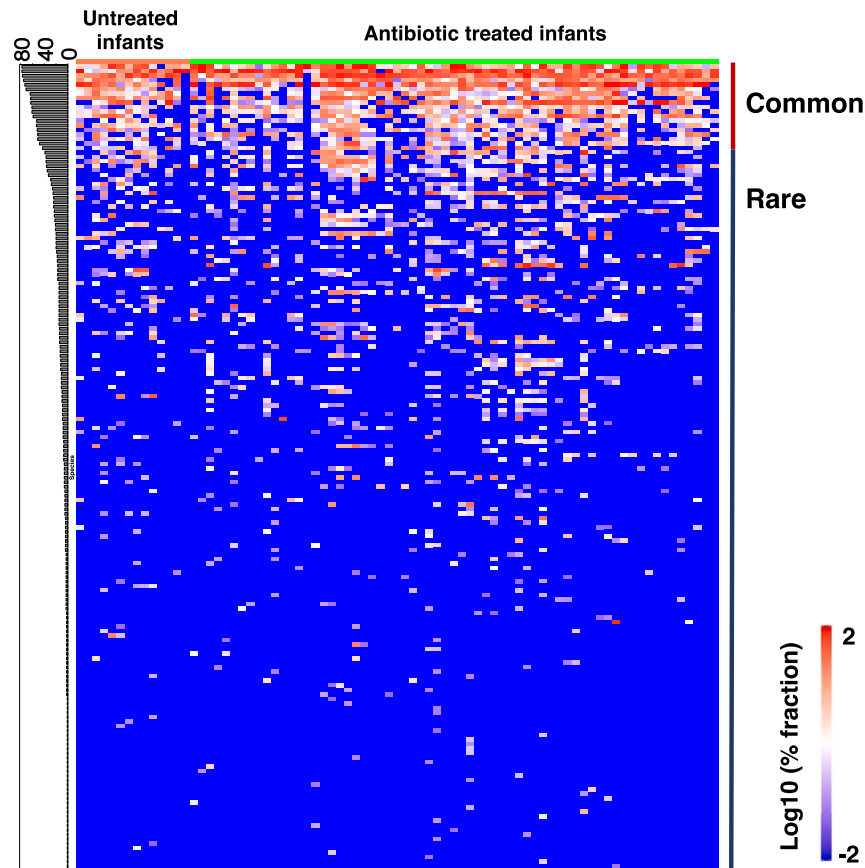
Figure 4. A small set of OTUs was common across the whole infant cohort

The heatmap of OTUs ranked by the fraction of infants where the OTU was detected (bar plot on the left). Rows: OTUs; columns: individual infants. For each infant and each OTU, the color indicates the highest fractional abundance of the OTU observed among all samples from the given infant. Approximately top 20 OTUs had the largest abundances across the vast majority of infants from both untreated and antibiotic treated groups ("common" OTUs marked by the red bar on the right). The remaining OTUs ("rare" OTUs, gray bar on the right) had lower abundances and were less frequently detected among infants (bar plot on the left).

infants, this last sample before discharge was typically taken a long time (41.6 ± 25.3 weeks) after antibiotic treatment. But even at this last time point, infants treated with antibiotics had a lower microbiome diversity quantified by Shannon diversity (Figure 5D, albeit this difference was above significance cutoff, Wilcoxon p = 0.13) and by richness (Figure S5D, significant difference with Wilcoxon p-value of 0.0011). Similar but less significant decreases of Shannon diversity (Figure S6D) and richness (Figure S7D) were also observed in a smaller subgroup of antibiotic treated infants with no significant differences of clinical variables compared to control. This surprisingly persistent difference may suggest unexpectedly long-term effect of antibiotic treatment on microbiome diversity, which continues beyond NICU care and hospital discharge.

To understand these long-term differences in microbiome diversity at a higher temporal resolution, we further analyzed detailed temporal progression of these differences as a function of adjusted age. In the infants not treated with antibiotics, the average Shannon diversity showed a distinct trend of increase, which was especially pronounced at older ages of 36–40 weeks (Figure 6A). By contrast, the infants treated with antibiotics did not show this trend at any time (Figure 6A). As a result, there was a significant difference between time evolution of microbiome diversity at the later stages, as detected by SS-ANOVA method[23] for the period of 34–38 weeks of adjusted age (p = 0.011, Figure 6B). Notably, when we analyzed the diversity within the group of common bacteria alone, it had remarkably similar trends (Figure 6C) and showed significant difference of temporal trajectories around 34–40 weeks of adjusted age (Figure 6D).

A trend toward the decrease of Shannon diversity at older ages (Figure S8A) and the corresponding difference compared to the control group (Figure S8B) was also observed among the smaller subgroup of 24 antibiotic treated infants whose clinical variables did not have significant differences from control. These trends among all bacteria were closely reproduced by the analysis focused on only common bacteria (Figures S8C and 8D).

These temporal differences did not occur among the subgroup of rare bacteria (Figure S9). Taken together, these results suggest that somewhat surprisingly, the short-term dynamics of microbiome at early stage of life and their antibiotic-associated differences are largely

Table 4. Common OTUs with the largest abundance and the highest fraction of infants where the OTU was detected

OTU	Number of_infants with OTU detected	% of all infants	% of untreated infants	% of treated infants
c. Bacilli	78	100.0	92.9	100.0
f. Enterobacteriaceae	78	100.0	100.0	98.5
Enterococcus	77	98.7	92.9	98.5
Veillonella dispar	75	96.2	78.6	98.5
Escherichia coli	73	93.6	85.7	93.8
Streptococcus	71	91.0	85.7	90.8
Clostridium perfringens	64	82.1	92.9	78.5
f._Clostridiaceae	63	80.8	92.9	76.9
Klebsiella	63	80.8	64.3	83.1
Finegoldia	61	78.2	64.3	80
Veillonella	61	78.2	64.3	80
Bifidobacterium breve	59	75.6	64.3	76.9
Propionibacterium	53	67.9	50.0	70.8
SMB53	53	67.9	85.7	63.1
Peptoniphilus	51	65.4	42.9	69.2
Haemophilus parainfluenzae	51	65.4	71.4	63.1
Anaerococcus	48	61.5	42.9	64.6
o. Actinomycetales	43	55.1	71.4	50.8
Citrobacter	39	50.0	42.9	50.8
Actinomyces	37	47.4	50.0	46.2

For top 20 OTUs by the fraction of infants, the number and percentage of all infants where the OTU was detected in at least one sample are indicated along with the separate percentages among infants treated and untreated with antibiotics.

driven by a small group of most dominant common bacteria. This finding may help guide future development of microbial interventions focused on specific bacteria that are absent from the microbiome at specific points in time following antibiotic treatment.

Next, we analyzed the temporal differences in the abundance of specific OTUs. Using SS-ANOVA method, we detected 10 OTUs that show significant differences in their abundance in time between infants with and without antibiotic exposure (Figure 6E). All of these OTUs showed depletion of their abundances in antibiotic treated infants. This depletion occurred at different intervals of adjusted age for different OTUs (Figure 6E). In the smaller subgroup of 24 antibiotic treated infants whose clinical variables did not have significant differences from control, SS-ANOVA detected an expectedly smaller set of 7 differential OTUs, four of which were shared with the full cohort (Figure S8E).

This antibiotic-associated depletion in specific windows of adjusted age provides insights on when and which bacteria might need a corrective intervention to increase their abundance within the microbiome of these preterm infants. For example, the lack of *Lactobacillus*, a commensal microbe shown to protect against infection, may create an especially high vulnerability for the preterm infant who is prone to infection with hospital-based pathogens at this specific time when the TLR4 receptors have been shown to be upregulated in the preterm gut. Furthermore, *Lactobacillus* has been shown to protect against NEC related intestinal injury,[24] suggesting that the lack of *Lactobacillus* at a time when preterm infants are most prone to NEC (i.e., 32–34 weeks gestation[25]) creates additional vulnerability in the preterm infant. Another notable finding relates to *Firmicutes* (i.e., *Clostridium* and *Veillonella*). The microbiome of infants who develop NEC has been shown to have a lower abundance of *Firmicutes*[26] which, based on our findings, may be attributed to antibiotic treatment. This finding is not only relevant to the pathophysiology of NEC, but also diminished short-chain fatty acid (i.e., butyrate) production that relies on bioenergetic *Firmicutes*.

DISCUSSION

Acquisition of new bacteria is essential for healthy gut development during infancy and early childhood. Delays or abnormal perturbations in this process are associated with various short- and long-term outcomes including abdominal distension, gastric retention, gut inflammation, NEC, sepsis and immune-mediated diseases such as allergy.[27] Preterm infants are an especially vulnerable population of children because of their immature organ systems at the time of birth and specifically, their immature gut development and extremely low diversity of their microbiome.[16,27–31] Microbiome composition early in life is influenced by various factors including gestational age, mode of delivery, and diet (formula vs. mothers' milk), among others.[27] In addition, medications, most notably antibiotic exposures, have been shown to have adverse effects on human gut microbiome in general and on preterm infant microbiome in particular.[16,28–31] The adverse consequences of early antibiotic exposure in preterm infants can affect longer-term development of gut microbiome, influencing overall gut and immune health throughout childhood and beyond.[16,30]
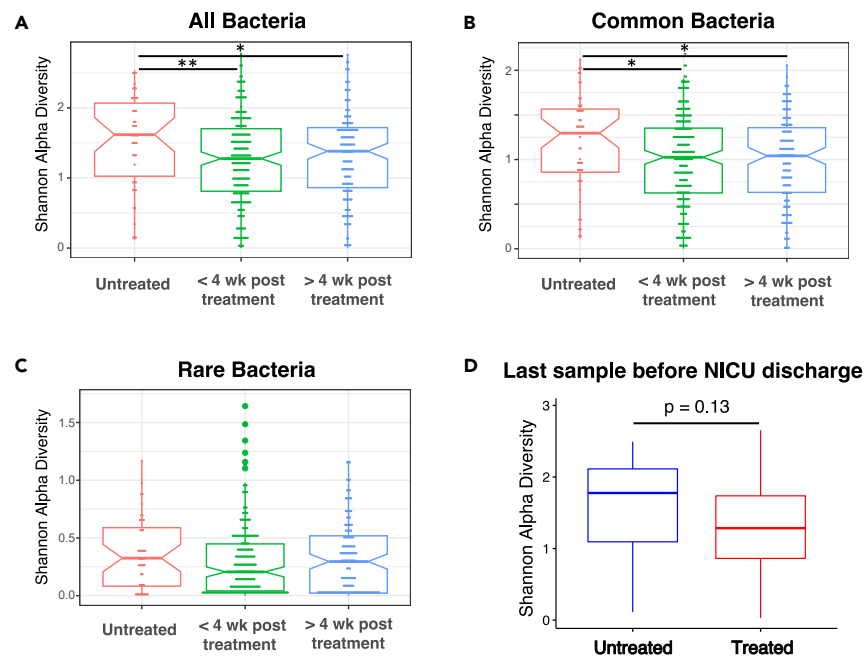
Figure 5. Low microbiome diversity persists for a long time after antibiotic treatment

(A) Boxplots comparing the distributions of Shannon alpha diversity among samples taken from infants untreated with antibiotics (red), samples from antibiotic-treated infants within 28 days after antibiotic treatment (<4 weeks, green, Wilcoxon p = 0.007), and samples from antibiotic-treated infants after 28 days post treatment (>4 weeks, blue, Wilcoxon p = 0.015).

(B) Distributions of Shannon alpha diversity calculated only for the subset of common OTUs. Significant differences in the diversity of common bacteria (Wilcoxon p = 0.016 and 0.03 for <4 weeks and >4 weeks post treatment, respectively) suggest that common OTUs are a major contributor to the differences of the whole microbiome diversity observed in A.

(C) Distributions of Shannon alpha diversity calculated only for the subset of rare OTUs. The differences are not significant, although a similar trend is observed.

(D) Boxplots comparing Shannon diversity of the last microbiome sample before hospital discharge in infants untreated (blue) and treated with antibiotics (red). Although the difference is not significant (p = 0.13), microbiome diversity in infants treated with antibiotics tends to be lower up to the time of discharge. Boxplots represent median as a line, interquartile range (IQR) between Q1 and Q3 as a box, and the range between Q1 - 1.5 IQR and Q3 + 1.5 IQR as whiskers. Asterisks denote statistical significance: Wilcoxon test p-value <0.05 (*) and <0.01 (**). See also significant difference of species richness in Figure S5.

In this study, we collected and analyzed longitudinal gut microbiome samples in a cohort of 79 preterm infants throughout their NICU hospitalization. Sampling across multiple consecutive time points allowed us to monitor intrinsic microbiome dynamics of each individual infant. We analyzed these longitudinal data to understand the associations of antibiotic treatment with the changes in composition, diversity, and temporal stability of the gut microbiome.

A few bacterial OTUs have the strongest presence regardless of antibiotic exposure

We compared the composition and diversity of gut microbiome in the groups of antibiotic treated and untreated infants. With the exception of gestational age, birth weight, duration of hospitalization, and incidence of NEC, all of which were expected differences consistent with the current practice of antibiotic use in this patient population, both groups had a similar clinical presentation (Table 2). Importantly, there were no significant differences in maternal antibiotic treatment, mode of birth, or proportion human milk vs. infant formula feeding, all factors that have been shown to significantly influence the preterm infant gut microbiome.[21] To account for potential effects of differences in gestational age, weight, and breast milk intake, we performed parallel microbiome analyses in a smaller subgroup of antibiotic treated infants that did not have significant differences from the control group in these values.

Regardless of antibiotic exposure, intestinal microbiome composition in both infant groups was generally sparse and dominated by a few taxa (Figures 2, 3A–3C, and S2A–S2C), in contrast to a higher microbiome diversity in healthy term infants.[32,33] This extremely low microbiome diversity is consistent with previous reports in preterm infants suggesting that gut microbiome of preterm infants is composed of only a few bacterial species that belong to a handful of taxonomic phyla.[34–36]

Another common pattern between these two infant groups was the presence of the same set of bacterial OTUs that stood out by both the highest abundance across the time course and the frequency of occurrence among all infants (Figures 4 and S4). Most of these OTUs are well-characterized bacterial species that are generally present in infants and in hospitalized preterm infants in particular.[29] We defined the set of top 20 most abundant and frequently observed taxa as "common" as opposed to what we defined as "rare", i.e., the remaining taxa.
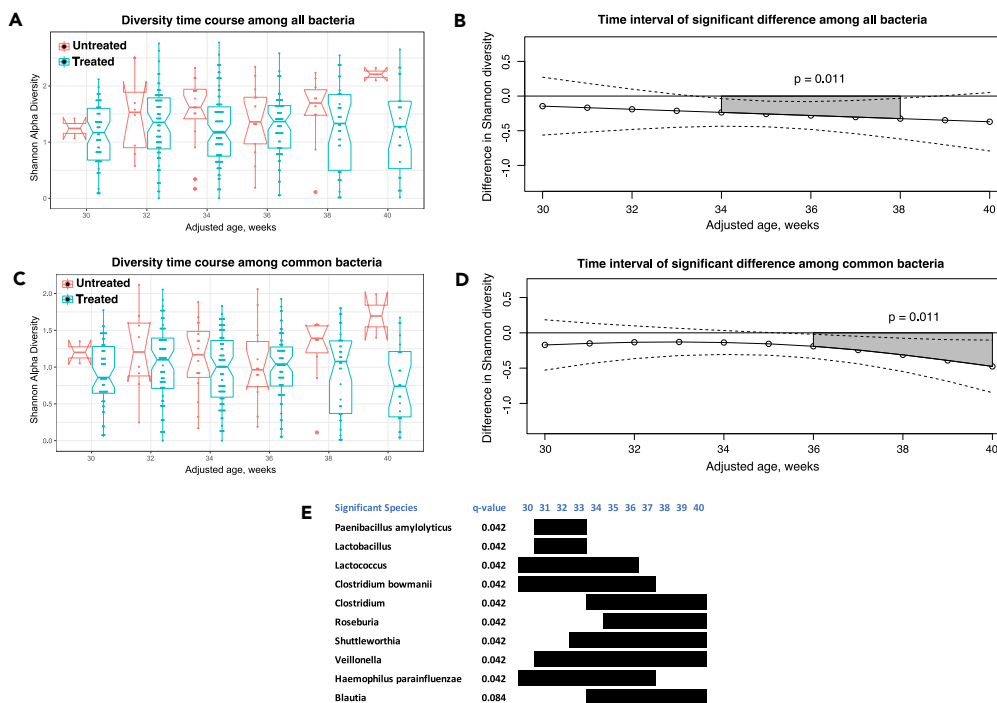
Figure 6. Longitudinal analysis of microbiome diversity and composition during infant development

(A) Boxplots at biweekly intervals show gradual increase in Shannon diversity with adjusted age for untreated infants (red) in contrast to infants treated with antibiotics (green).

(B) Longitudinal analysis with SS-ANOVA detected significant difference between Shannon diversity trajectories of treated and untreated groups at the adjusted age of 34–38 weeks (p = 0.011). X axis: adjusted age; y axis: difference between average Shannon diversities in two groups.

(C and D) The subset of common OTUs is the major contributor to the differences observed in A, B.

(C) Boxplots at biweekly intervals for Shannon diversity calculated only for common OTUs in untreated infants (red) compared to infants treated with antibiotics (green).

(D) SS-ANOVA analysis applied only to the common OTUs detected a significant difference between Shannon diversity trajectories of two groups at the adjusted age of 36–40 weeks (p = 0.011). Boxplots represent median as a line, interquartile range (IQR) between Q1 and Q3 as a box, and the range between Q1 - 1.5 IQR and Q3 + 1.5 IQR as whiskers. SS-ANOVA line plots represent average difference as a solid line and 95% confidence interval as dotted lines.

(E) Individual OTUs show significant antibiotics-associated depletion during specific age intervals. Temporal range of significant depletion in antibiotic treated group is indicated by a bar, with adjusted p-value (q-value) indicated on the left.

Antibiotics influence microbiome diversity, temporal stability, and abundance of specific bacteria

We then characterized the antibiotic associated microbiome differences between the two groups of infants and the contributions of common and rare bacteria to these differences. We observed three major aspects of antibiotic associated microbiome differences in: microbial diversity, temporal stability of microbiome dynamics, and abundance of specific bacteria.

We found that microbial diversity in infants treated with antibiotics was significantly lower (Figures 2, 3A–3C, S2A–S2C, and S3). Remarkably, this difference maintained after antibiotic treatment (Figures 5A, S5A, S6A, and S7A) and was still pronounced weeks later, even at the time of hospital discharge (Figures 5D, S5D, S6D, and S7D).

The temporal resolution of our study allowed us to discover previously unreported and surprising antibiotic associated differences in the stability of microbiome dynamics. Infants not treated with antibiotics had a relatively stable gut microbiome over time (Figure 2A) where previously acquired OTUs were retained and new OTUs were acquired in a relatively gradual fashion. This gradual colonization was consistent with a relatively steady increase in microbial diversity (Figures 3A, S2A, and S3A), with relatively modest fluctuations. By contrast, infants treated with antibiotics had a very unstable microbiome composition that was continually undergoing strong irregular changes between consecutive time points (Figure 2B). These irregular fluctuations of composition resulted in widely fluctuating microbial diversity without consistent increase in diversity (Figures 3B, S2B, and S3B). Quantifying this instability by the differences between OTU compositions at consecutive time intervals, as measured by beta diversity index (Figures 3D–3F and S2D–F), confirmed that the irregular short-term fluctuations were highly pronounced in individual infants (Figures 3E and S2E) and the antibiotic treated group as a whole (Figures 3F and S2F). In sum, the newly acquired bacteria in the antibiotic treated group rarely gained the ability for long-term colonization. Rather, they were quickly replaced by other types of bacteria within the gut (Figures 2B, 3E, 3F, S2E, and S2F). This led to temporal instability (Figures 3B, S2B, and S3B) and significantly lower diversity of the microbiome. Our results suggest that the unexpectedly irregular colonization pattern in infants treated

with antibiotics may shed light on the intestinal biology underpinning the long-term consequences of perturbed gut colonization early in life and the role that antibiotics play in later immune-mediated disease.

Our time-dependent analysis of microbial abundances revealed a group of OTUs whose abundance was significantly depleted in antibiotic treated infants during specific windows of gestational age (Figures 6E and S8E). To our knowledge, this is the first time that longitudinal preterm infant microbiome data have been reported in this context, pointing out specific time windows of adjusted ages when a microbial intervention would have therapeutic potential following antibiotic treatment.

We observed significant differences in the temporal progression of abundance of ten bacterial types within three-to nine-week adjusted age windows. The differences that persisted for the shortest period of time, between 31 and 33 weeks, were found in *Paenibacillus amylolyticus* and *Lactobacillus*. The differences that persisted for the longest period of time, between 31 and 40 weeks, were found in *Veillonella* and *Shuttleworthia*. Other notable differences identified between 30 and 40 weeks were found in two types of *Clostridium*.

Identifying these specific time-dependent differences of bacterial abundances in preterm infants is especially informative when superimposed on the time-dependent development of the preterm gut. For example, the preterm gut is characterized by immature immune regulation, notably in TLR4 signaling, predisposing it to NEC.[37] The depletion of specific bacteria at specific windows of gestational age in antibiotic treated infants may contribute to the immature immune regulation within the preterm gut. Breast milk and probiotics have been shown to upregulate the expression of genes that inhibit the TLR4 pathways, protecting the infant from NEC.[37] Antibiotic treatment depleting the bacteria present in breast milk (i.e., *Firmicutes*[38]) is a likely contributor to NEC, in addition to the array of internal biological factors in the preterm infant gut[39,40]

In addition to these individual types of bacteria discussed above, it is important to understand the role of broader communities of co-existing microbes, their impact on early microbiome development, and their clinical relevance for improved antibiotic use and possible therapeutic applications. Increasing microbiome diversity via acquisition of new bacterial types is essential for healthy gut development. However, if this acquisition is not balanced with the maintenance of the previously acquired microbial community, it may result in the loss of microbiome equilibrium and dysbiosis, delaying the normal progression of microbiome composition. Therefore, both acquiring new species and maintaining the general stability of existing microbial community against external perturbations (acquisition of new species, diet, etc.) are crucial for microbiome development early in life. Our results suggest that in preterm infants, this balance is strongly perturbed even by a single antibiotic treatment.

Most preterm infants are treated with antibiotics in the first week of hospitalization, and it would be reasonable to assume that the immediate effects of antibiotics would be limited to 1–2 weeks after the treatment. Surprisingly, we found that the effect of antibiotics on microbiome composition and in turn diversity and stability persisted throughout the hospitalization, which for many infants can last for months after birth. The microbiome of antibiotic treated infants did not regain balance even at the time of hospital discharge and was still less developed and much less stable than in infants not treated with antibiotics.

The dynamics of microbiome diversity in an individual infant was dominated by a few common OTUs. We found that the differences between cohorts in terms of both overall microbial diversity (Figures 5A, 5B, S5A, S5B, S6A, S6B, S7A, and S7B) and temporal progression of diversity metrics (Figures 6A–6D and S8A–D) could be largely reproduced by considering only these few common bacteria, which suggests that despite their universal presence in both groups of infants, the relative abundance of these common bacteria was a major factor contributing to the microbiome differences in antibiotic treated infants. This may suggest the possibility that some of these common bacteria are a part of a small core microbial community at the early stages of microbiome development. Such a core community would be beneficial by contributing to the temporal stability of microbiome while allowing for the gradual acquisition of new microbes and serving as a seed for further microbiome growth. The disruption of this core community by early antibiotic treatment might help explain the unexpectedly long-lasting effects of antibiotics in preterm infants. After disruption by antibiotics, regaining these core microbes would be challenging, especially for a preterm infant in the NICU environment. This long-term absence of the core seed community might contribute to prolonged dysbiosis and microbiome instability.

Another highly relevant question is the association of these core microbes with the production of specific metabolites[28] that could be beneficial for normal microbiome development and regulation of the cascade of immune cells within the gut, similar to the pathophysiology recently reported in the adult IBD gut.[41–43] The scope of our current data, however, does not allow for a quantitative investigation of these questions, which will be important to address in future broader studies.

In sum, our results suggest strong and surprisingly long-lasting association of antibiotics treatment with the content, diversity, and temporal stability of gut microbiome in preterm infants. In addition to the insights into temporal progression of microbiome at the earliest postnatal stages, this and further studies should inform improved clinical guidance on antibiotic use that would take into account the morbidity risks of long-term dysbiosis and delays in stable gut colonization. Our results also suggest candidate microbes and the corresponding developmental windows for the design of possible pre- and probiotic interventions to facilitate healthy microbiome development in preterm infants after antibiotic exposure.

Limitations of the study

Similar to many studies involving preterm infants and the microbiome, the number of profiled infants was modest, given access to the patient population and the challenges of sample collection in NICU environment. The limited sample size and number of samples collected possibly limited statistical power of the study. In addition, the study design did not provide the opportunity for follow-up data collection and microbiome analyses post-hospitalization. Additional follow-up and longer-term analyses would enhance the study since antibiotic effects

continued as late as the time of discharge. Another temporal limitation in the study was a weekly resolution of the longitudinal analyses. Although these analyses throughout the hospital stay provided unprecedented insight into microbiome dynamics, a higher temporal resolution (i.e., daily resolution) may provide more granular details of these dynamics, especially given the magnitude of fast microbiome fluctuations after antibiotic treatment. Profiling bacterial 16S rRNA as opposed to whole-genome sequencing introduced well-known limitations in the taxonomic resolution and also did not allow for comprehensive analyses of genes and functional pathways across developing microbiome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107995.

AUTHOR CONTRIBUTIONS

K.E.G. conceived and supervised the clinical study. R.I.S., K.E.G., M.C. conceived the main idea and designed the analysis. J.T., E.F., K.E.G. collected the data and performed statistical analyses of clinical values. M.C. performed Bioinformatics analyses. M.C., R.I.S., K.E.G. wrote the paper.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
QIIME2 v. 2018.2.0	(Caporaso et al.[20])	https://qiime2.org
DEBLUR plugin in QIIME2	(Amir et al.[44])	https://qiime2.org
MAFFT plugin in QIIME2	(Katoh et al.[45])	https://qiime2.org
FastTree2 plugin in QIIME2	(Price et al.[46])	https://qiime2.org
Pre-trained Greengenes 13_8 99% Database	(McDonald et al.[47])	https://docs.qiime2.org/2022.2/data-resources/
R	R Core Team, 2020	https://www.r-project.org
Shannon Alpha and Beta Diversity Calculations with R	(Marcon et al.[22]) (R Core Team, 2020)	https://www.r-project.org
S-ANOVA implementation in metagenomeSeq package	(Paulson et al.[23])	http://www.cbcb.umd.edu/software/metagenomeSeq
Critical commercial assays		
QIAamp DNA Stool Mini Kit	Qiagen, Germany	Cat. No./ID: 51604 www.qiagen.com
Agilent Genomic DNA ScreenTape Assays	Agilent, Santa Clara, CA	www.agilent.com
Nextera XT Index Kit	Illumina, San Diego, CA	FC-131-1096 www.illumina.com
Aline PCRClean DX beads	Aline Biosciences, Waltham, MA	SKU: C-1003 www.alinebiosciences.com
Deposited data		
16S rDNA sequencing data	This paper, NCBI BioProjects, SRA	BioProject accession number PRJNA899559

RESOURCE AVAILABILITY

Lead contact and materials availability

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Ruslan I. Sadreyev (sadreyev@molbio.mgh.harvard.edu). Requests for experimental information should be directed to Katherine E. Gregory (katherine.gregory.2@bc.edu). This study did not generate new unique reagents.

Data and code availability

- The sequencing data have been deposited with links to BioProject accession number in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/); BioProject: PRJNA899559.
- This study did not generate new unique computational tools. The used codes were previously published as described in method details.
- Any additional information required to reanalyze the data reported in this paper is available upon request from the lead contact, Ruslan I. Sadreyev (sadreyev@molbio.mgh.harvard.edu).

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All study procedures followed Protocol #2016-P-001020 as approved by the Mass General Brigham's Human Research Committee (IRB). Written informed consent was obtained from the parent or guardian of the participating infants using an IRB approved consent form and following the IRB approved protocol Participant inclusion criteria were: birth prior to 34 weeks' gestation at a single level III newborn intensive care unit (NICU) and anticipated survival based on clinical assessment within the first 48 h following birth. Maternal and infant demographic and clinical data was collected from the electronic health record following standardized definitions and procedures. Information on race and ethnicity in all cohorts is included in Tables 2 and 3. Fecal samples were collected from infant diapers on a weekly basis from birth until discharge. Briefly, samples were collected following an aseptic technique and stored at −80°C until DNA extraction, as reported in other studies[48,49]

METHOD DETAILS

Sample preparation and sequencing

DNA isolation and PCR amplification were performed by first thawing and centrifuging samples at 10,000 g × 20 min at 0°C. The pellets were used for total DNA isolation using the QIAamp DNA Stool Mini Kit (Qiagen, Germany). Isolated DNA was then arrayed into 96-well plates,

quality determined (agarose gel), quantity normalized (by PicoGreen) and stored at −20°C until rRNA sequencing and analysis. The 16S V3-4 regions were amplified from the metagenomic DNA [primers 515F (5'- CCTACGGGAGGCAGCAG -3') and 806R (5'- CCGTCAAT TCMTTTRAGT -3' with unique barcodes) primers with sequencing adapters; targeted read depth of 10,000 reads/sample. To construct sequencing libraries, amplicons were purified and pooled to equimolar concentrations (PicoGreen), then sequenced using an Illumina MiSeq sequencer (paired-end 250-bp reads with v2 500 cycles kit).

Bioinformatics analyses

Sequencing data (fastq files) were processed with QIIME software package v. 2018.2.0.[20] Reads with low quality score (on average less than 25) were truncated to 240bp. Reads were then filtered using *deblur* method with default settings.[44] The remaining high-quality reads were aligned with *mafft* plugin.[45] The aligned reads were masked to remove highly variable positions and a phylogenetic tree was generated from the masked alignments by FastTree.[46]

Taxonomy assignment was performed using QIIME2 *feature-classifier* plugin and pre-trained Naive Bayes classifier, which was trained on the Greengenes 13_8[47] 99% operational taxonomic units (OTUs). OTUs were defined at the species level where possible, otherwise OTUs were reported at higher taxonomic levels of genus, family etc. Shannon alpha diversity[22] in a given sample was calculated as

$$S_\alpha = \sum_{n=1}^{N} p_n \, log_2 p_n$$

where p_n is the fractional abundance of OTU n, and N is the total number of OTUs observed in the sample. As a measure of variation between samples, we calculated Shannon beta diversity[22] as

$$S_\beta = \frac{1}{2} \sum_{n=1}^{N} \left(p_n \, log_2 \frac{p_n}{q_n} + q_n \, log_2 \frac{q_n}{p_n} \right)$$

where p_n and q_n ($n = 1, \ldots, N$) are the fractional abundances of OTU n in the two compared samples. Longitudinal differential analysis of OTU abundances and Shannon alpha diversity was performed using SS-ANOVA implementation in metagenomeSeq package.[23] Wilcoxon test was used to assess statistical significance of differences between groups. Multiple testing correction was performed using Benjamini-Hochberg false discovery rate (FDR).[50] The FDR threshold was set at 0.05.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used Chi-square test and Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables. Distributions of continuous variables over a group of samples were represented as boxplots indicating median as a line, interquartile range (IQR) between Q1 and Q3 as a box, and the range between Q1 - 1.5 IQR and Q3 + 1.5 IQR as whiskers.