Microbiome

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Antibiotic exposure is associated with minimal gut microbiome perturbations in healthy term infants

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Abstract

Background The evolving infant gut microbiome influences host immune development and later health outcomes. Early antibiotic exposure could impact microbiome development and contribute to poor outcomes. Here, we use a prospective longitudinal birth cohort of $n = 323$ healthy term African American children to determine the association between antibiotic exposure and the gut microbiome through shotgun metagenomics sequencing as well as bile acid profiles through liquid chromatography-mass spectrometry.

Results Stool samples were collected at ages 4, 12, and 24 months for antibiotic-exposed ($n = 170$) and unexposed ($n = 153$) participants. A short-term substudy ($n = 39$) collected stool samples at first exposure, and over 3 weeks following antibiotics initiation. Antibiotic exposure (predominantly amoxicillin) was associated with minimal microbiome differences, whereas all tested taxa were modified by breastfeeding. In the short-term substudy, we observed microbiome differences only in the first 2 weeks following antibiotics initiation, mainly a decrease in *Bifidobacterium bifidum*. The differences did not persist a month after antibiotic exposure. Four species were associated with infant age. Antibiotic exposure was not associated with an increase in antibiotic resistance gene abundance or with differences in microbiome-derived fecal bile acid composition.

Conclusions Short-term and long-term gut microbiome perturbations by antibiotic exposure were detectable but substantially smaller than those associated with breastfeeding and infant age.

Keywords Infant gut microbiota, Antibiotics, Amoxicillin, *Bifidobacterium*, Metagenomics, Bile acid

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Background

The establishment of the gut microbiome in early life has important long-term implications on overall health [1], including the development of the host immune system [2]. The infant gut microbiome is shaped by host and environmental factors [3], in particular breastfeeding [4, 5]. Antibiotics might also alter the human gut microbiome during infancy, with substantial microbiome associations observed in humans [6–8] and model organisms [9]. Several epidemiological studies report associations between early life antibiotic exposure and development of allergic, inflammatory, and metabolic disorders later in life [10], as well as risk for childhood obesity [11].

The impact of postnatal antibiotics on the infant microbiome has been investigated in previous observational studies [7, 8, 12–16] and randomized trials [17–19]. Studies to date have focused on a variety of antibiotics and span a range of infant health backgrounds. Evidence points to a reduction in gut microbiome diversity following antibiotic use [7, 8]. However, whether antibiotic-induced microbiome differences persist longer than weeks after exposure is unclear. Moreover, infant antibiotic studies indicate a reduced abundance of *Bifidobacterium* [13–16, 19, 20]. Some species members of *Bifidobacterium* are breastfeeding-associated gut commensals, and it is uncertain whether antibiotic exposure alters gut microbiome response to breastfeeding.

Widespread antibiotic use in early life raises concerns about the development of antibiotic resistance [21]. Evaluating the impact of antibiotic exposure on antibiotic resistance genes (ARGs) in the developing gut microbiome may be important to inform antibiotic stewardship efforts. In addition to ARGs, antibiotics may dislodge low-abundance species in the infant gut microbiome and potentially disrupt the unique metabolic functions of the gut microbiota, such as the capacity to modify bile acids, with a detrimental effect on gut homeostasis [22, 23].

To address these knowledge gaps, we examined antibiotic exposure and gut microbiome in a prospective, longitudinal cohort of 323 healthy African American term infants enrolled in the Infant Growth and Microbiome (IGram) Study, designed to examine infant gut microbiome development in a demographic group at high-risk for childhood obesity. We also present a nested short-term substudy of $N=39$ infants. We hypothesized that antibiotic exposure would perturb the gut microbiome over several weeks but that differences would not persist long-term until 24 months of age. Further, we hypothesized that the minimal short-term perturbations would not affect the functional capacity of the microbiome to modify bile acids.

Methods

Study design

Subjects were enrolled in IGram, a prospective, longitudinal, observational birth cohort study of African American women and their infants. Pregnant women in the 3rd trimester were enrolled between July 2014 and July 2017. Those with early pregnancy BMI in the healthy weight or obese range, with uncomplicated term pregnancies were eligible. Healthy term infants were enrolled at birth and evaluated until 24 months of age. See Supplement eMethods for eligibility criteria. The study was approved by the Institutional Review Board (IRB) at Children's Hospital of Philadelphia (CHOP).

Medical history was obtained at birth. Feeding mode (exclusively breastmilk, breastmilk and formula, and exclusively formula) and medication use was obtained by interview every month until the infant reached a year of age and then every 3 months until the age of two. Breastfeeding was defined as any amount of breastmilk, regardless of formula supplementation. Stool samples were collected at 4, 12, and 24 months, stored on ice, then aliquoted and stored at − 80 °C.

In the short-term substudy, we obtained stool samples from children prescribed antibiotics by their physician. Parents collected stool samples at the time of first antibiotic exposure and then at 1, 2, and 3 weeks post antibiotic initiation.

Microbiome sequencing and analysis

DNA was extracted from fecal and negative control samples using the PowerSoil-htp kit (MO BIO Laboratories, Carlsbad, CA), libraries were generated using the NexteraXT kit (Illumina, San Diego, CA, USA) and sequenced on the Illumina HiSeq to produce 125 bp paired-end reads as previously described [24] across 11 runs. Three types of negative controls were added to each plate and processed along with experimental samples. Clean swab and empty well samples were used to assess environmental and reagent contamination. DNA-free water was used to assess potential PCR contamination. A mock community consisting of *Vibrio campbellii* and Lambda phage were included as positive controls.

Shotgun metagenomic data were analyzed using the Sunbeam metagenomics pipeline [25]. Sequence reads were processed with default parameters to remove regions with low-quality scores, sequences of low complexity, and reads aligning to the human or phage ϕX174 genomes. The abundance of bacteria was estimated using Kraken [26]. Reads were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) [27] database and Comprehensive Antibiotic Resistance Database (CARD) [28] to estimate the abundance of bacterial gene orthologs and antibiotic resistance genes, respectively.

Taxonomic and KEGG ortholog tables were rarefied to 50,000 counts per sample to calculate beta diversity using Bray–Curtis dissimilarity.

Bile acid quantification and analysis

Bile acids were quantified using a Waters Acquity uPLC System with a Cortecs UPLC C-18 + 1.6 μm 2.1 × 50 mm column and a QDa single quadrupole mass detector as previously described [29]. Bile acids were categorized into conjugated, unconjugated, primary, and secondary bile acids. The levels over the four timepoints were tested using linear mixed-effects models with baseline age and breastfeeding status as covariates.

Statistical analysis

Data visualization and statistical analysis were implemented in the R environment for statistical computing. Community-level differences between groups were assessed using a PERMANOVA test [30] of Bray–Curtis distance between samples, using 999 restricted permutations in the short-term substudy. Batch effects were not detected based on the PERMANOVA test on Bray–Curtis distances using visit, breastfeeding status, and run number as model parameters. Gene and taxon abundance was assessed using linear models (main cohort) and linear mixed-effects models (short-term substudy). One-tenth of the smallest non-zero proportion was added to each relative abundance and log transformed prior to comparison. Linear models on log-transformed bacterial abundances for the main cohort were confirmed with additional tests: linear models on centered log ratio (clr) transformed data, beta regression on proportions, and generalized linear models on the read count data with a quasibinomial link function. Taxa and genes with 1% mean abundance across samples were tested. Antibiotic resistance genes annotated as conferring resistance to the penam class of antibiotics in the CARD database were analyzed. When multiple testing was conducted, *p*-values were adjusted using the Benjamini–Hochberg method [31] to control for a 5% false discovery rate.

Subjects in the main cohort were grouped by age of first antibiotic exposure: before 4 months (early exposure), 4–12 months (mid exposure), 12–24 months (late exposure), or unexposed at 24 months (unexposed group). Early, mid, and late exposure groups were compared to the unexposed group separately at each timepoint. Breastfeeding was included in all statistical models. In the short-term substudy, baseline age was also included as a variable.

We carried out sensitivity analyses to ensure that our results were not influenced by other clinical factors. To evaluate the effect of intrapartum antibiotics and delivery mode, we ran the statistical models separately for subjects with or without intrapartum antibiotics and subjects born by vaginal delivery or C-section. We added gestational age as a covariate in the models. To isolate the effect of amoxicillin from other antibiotics, we repeated the analyses excluding all subjects who were exposed to any antibiotics other than amoxicillin within the first 2 years of life. From the main study, an antibiotics naïve cohort of 39 subjects was matched for age and breastfeeding status to the short-term substudy cohort and used as baseline in the linear models. The subjects younger than 8 months ($n=8$) at T1 were matched to 4 M, younger than 16 months ($n=28$) were matched to 12 M, and older subjects ($n=3$) were matched to 24 M timepoints.

Results

In the longitudinal prospective cohort, we collected stool samples from $n=323$ healthy African American term infants at 4, 12, and 24 months after birth (Supplement Table 1). Of these, 153 (47%) were in the unexposed group. Of the infants prescribed antibiotics, 32 (10%) were in the early exposure group, 70 (22%) in the mid-exposure group, and 68 (21%) in the late exposure group (Fig. 1A). Infants exposed to antibiotics received 2.1 ± 1.4 courses, with 55% having more than one exposure. The most common antibiotics were amoxicillin or amoxicillin and clavulanate (68% of prescribed antibiotics, Fig. 1B).

The short-term substudy enrolled 39 infants and collected four samples within 20–45 days after antibiotics initiation (Fig. 1C). The median infant age was 11 months, and 12 infants (32%) were breastfeeding. Antibiotic exposure was predominantly amoxicillin (31 infants, 79%), mirroring the longitudinal prospective cohort.

Antibiotic exposure was associated with minimal long-term differences in microbiome composition

To examine the effect of antibiotic exposure on gut microbial composition, we compared the unexposed group to each antibiotic exposure group (early, mid, late) at each timepoint (Fig. 2A). Given known breastfeeding effects on microbial composition, we included breastfeeding in our comparisons. The percentage of breastfed infants declined with age (32% at 4 months; 19% at 12 months, and 5% at 24 months). There were no differences in breastfeeding status between antibiotic exposure groups at any timepoint ($P=0.4$–1.0, Fisher's exact). We found no effect of antibiotic exposure on the overall taxonomic composition of the microbiome between antibiotic-exposed and unexposed groups (Fig. 2B). As expected from prior studies, breastfeeding was associated with overall taxonomic composition at 4 and 12 months for all exposure groups (Supplement Fig. 1A,

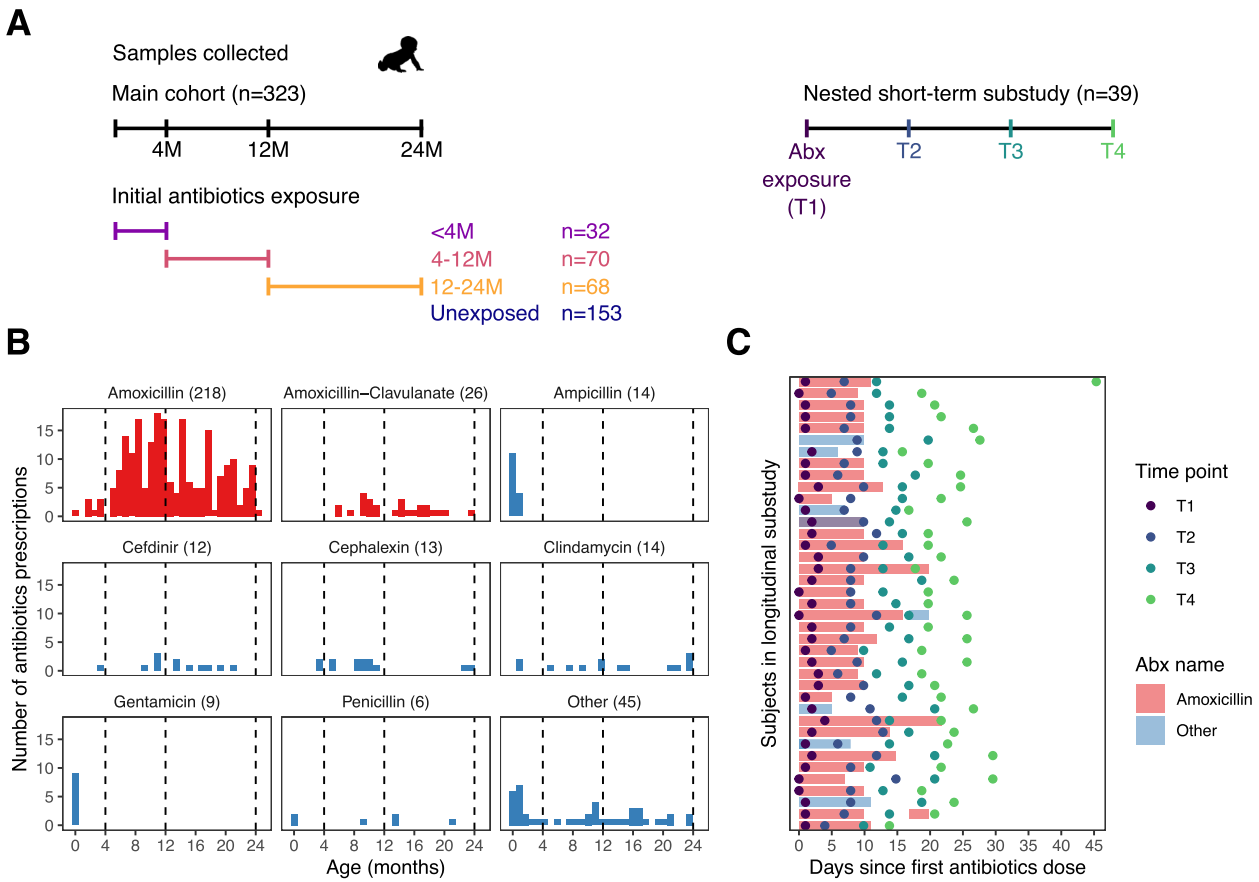

Fig. 1 Longitudinal prospective cohort of healthy term infants and nested short-term substudy. **A** Overview of the main study and the short-term substudy. **B** The age at which various antibiotics were initiated in the main study. Amoxicillin and amoxicillin-clavulanate are shown in red. **C** Sample collection overview for the short-term substudy. Each row represents an infant enrolled in the study, with points indicating the day of sample collection. The bands indicate the duration of antibiotic exposure. Amoxicillin or amoxicillin-clavulanate are marked with red; other antibiotics are marked with blue

$R^2 = 0.030–0.042$, $P = 0.0015$ for all comparisons). The interaction term between breastfeeding and antibiotic exposure was not statistically significant for any comparison, suggesting that there was no microbiome response to antibiotics regardless of breastfeeding status. We also analyzed the overall gene ortholog composition between groups (Fig. 2C) and found no associations between gene ortholog composition and antibiotic exposure groups at any timepoint. However, we observed an association with breastfeeding at 4 and 12 months in comparisons for all exposure groups (Supplement Fig. 1B, $R^2 = 0.025–0.035$, $P = 0.0015$ for all comparisons).

To examine bacterial species abundance across the antibiotic exposure groups (Fig. 2D), we first considered breastfeeding effects. As expected [32], at 4 and 12 months, breastfeeding is associated with a higher relative abundance of *Bifidobacterium* species. In addition, breastfeeding is associated with a higher abundance of *Escherichia coli* and lower abundance of *Klebsiella pneumoniae*, *Akkermansia muciniphilia*, and several *Bacteroides* and *Clostridia* species. With respect to antibiotic exposure, we observed no statistically significant associations with bacterial species abundance for any exposure group at any timepoint (Fig. 2D, Supplement Table 2). The only association related to antibiotics was a lower abundance of *Bifidobacterium bifidum* at 12 months in the mid-exposure group ($P = 0.07$). The overall results were consistent when the tests were repeated with alternative statistical methods (Supplement Fig. 2). Even though the exposure group is defined by the initial antibiotic prescription, subjects were exposed to multiple courses over the first 2 years of life. To quantify the effect of repeat exposure on the microbiome, we identified the bacteria that correlated with the total number of antibiotics courses the subjects received at each timepoint. After correcting for breastfeeding status, *Bifidobacterium bifidum* was negatively correlated with the number of antibiotics courses at 12 months of age, suggesting a

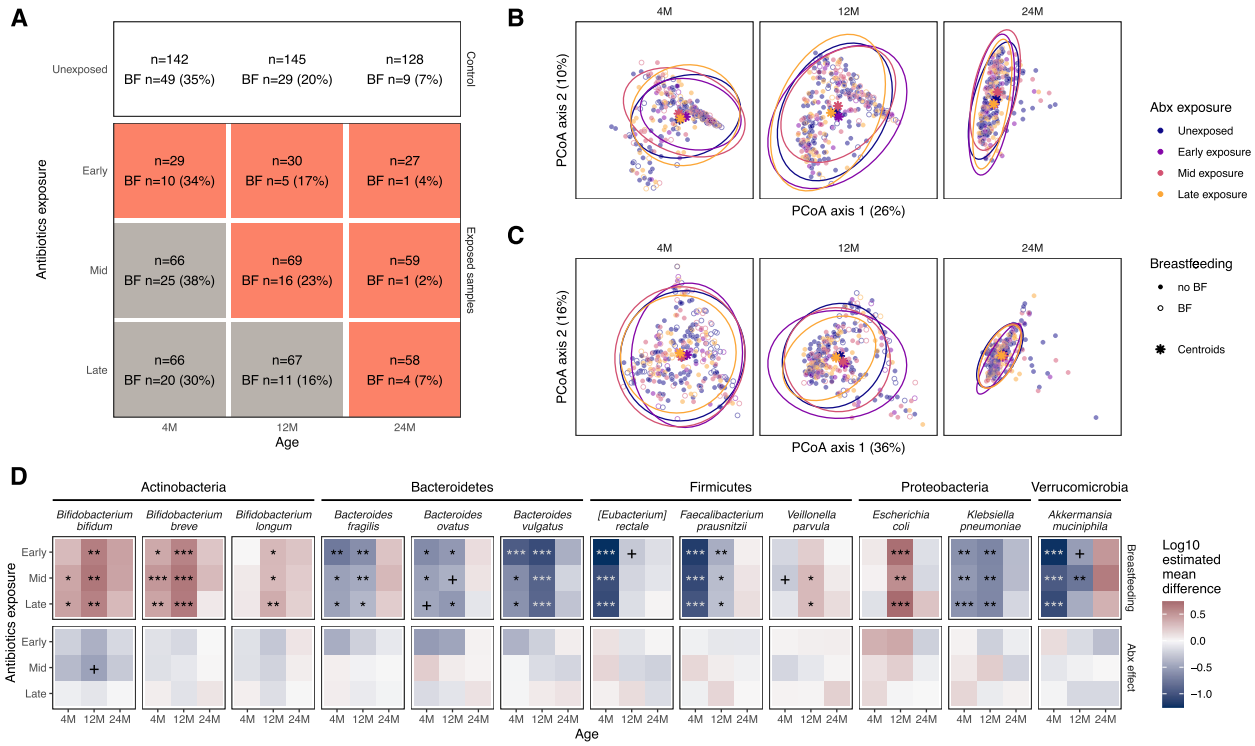

Fig. 2 Microbiome comparison in the main cohort. **A** The number of samples in the unexposed group and the antibiotics-exposed groups at each timepoint. The second line in each box denotes the number and percentage of subjects who were breastfed at the time of sample collection. At each timepoint, each exposure group was compared to the unexposed group. Gray boxes represent comparisons where the group is not yet exposed to antibiotics, and thus no difference is expected. **B** Principal coordinates (PCoA) of bacterial taxon abundances and **C** gene ortholog abundances, based on Bray–Curtis dissimilarity. The PCoA was carried out for all samples together, although samples from each timepoint are displayed separately. Ellipses represent 95% confidence interval of antibiotic exposure groups. **D** Comparison of relative abundance for selected taxa between exposed and unexposed infant groups. Out of the 26 taxa that were tested, the ones with the highest mean relative abundance in each phylum are shown. Breastfeeding and antibiotic exposure were added as fixed effects to the linear model with log-transformed relative abundances as the outcome. The first row of comparisons indicates the coefficients with respect to breastfeeding; the second row indicates the coefficients with respect to the exposure group compared to the unexposed group. Stars represent FDR-adjusted *p*-values. + $P<0.1$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

dose-dependent effect (Supplement Fig. 3). The same effect was not seen at 4 or 24 months of age. To isolate amoxicillin effects, we repeated the comparisons excluding the 69 subjects exposed to non-amoxicillin antibiotics and found that amoxicillin exposure was associated with decreased *B. bifidum* abundance only at 12 months and only in the mid-exposure group ($P=0.015$, Supplement Fig. 4). The inclusion of intrapartum antibiotics, delivery type, gestational age or the interaction between antibiotic use and breastfeeding did not alter the results of our analysis (Supplement Fig. 4).

The number of observed species (richness) in microbiome samples on average was 28% lower with breastfeeding at 4 months ($P<0.001$) and 10% at 12 months ($P=0.06$) and Shannon diversity was 18% lower only at 4 months ($P<0.001$) in comparisons for all exposure groups (Supplement Fig. 5A), as expected from prior studies. Richness in the early exposure group was 12% lower relative to the unexposed group ($P=0.05$), and Shannon diversity was 9% lower ($P=0.08$) at 12 months but was not different at 4 or 24 months. We observed no difference in alpha diversity between the mid-exposure or late-exposure groups and unexposed infants at any timepoint.

Overall, antibiotic exposure had a limited effect on the microbiome, compared to the strong and consistent associations with breastfeeding in these analyses.

Antibiotic exposure was associated with short-term microbiome perturbations that resolved over 1 month

To examine acute responses of the gut microbiome to antibiotic exposure in our short-term substudy (Fig. 1C), we analyzed microbiome community similarity based on taxonomic abundance for subject ID, baseline age, breastfeeding status, and time since antibiotic exposure (Fig. 3A). Intersubject variability had the largest effect size ($R^2=0.65$, $P=0.001$), followed by baseline age ($R^2=0.035$, $P=0.023$). Breastfeeding was

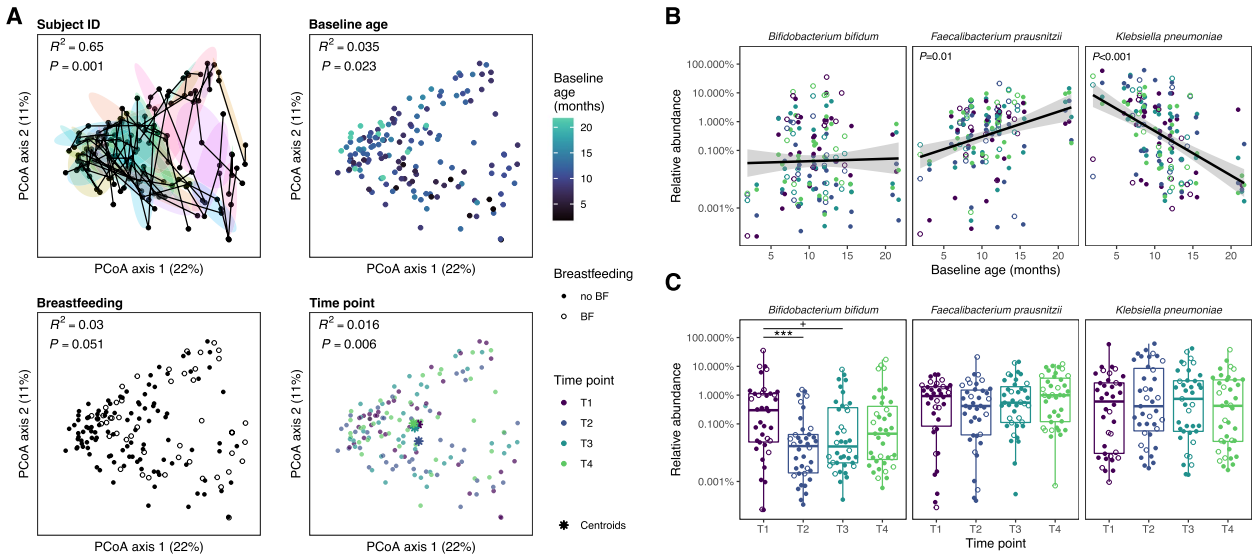

Fig. 3 Microbiome comparison in the nested short-term substudy. **A** Principal coordinates analysis (PCoA) of bacterial taxon abundances, based on Bray–Curtis dissimilarity. The sub-panels are annotated by subject, age at time of first stool sample following antibiotic exposure, breastfeeding status, and timepoint. In the top-left panel, samples collected from the same subject are connected with lines; ellipses represent the 50% confidence interval for each subject where each color represents an individual. **B** Relative abundance of taxa as a function of baseline age. **C** Relative abundance of taxa as a function of timepoints in the short-term substudy. The three taxa with the greatest effect size in response to antibiotics or age are presented

not significantly associated with taxonomic composition ($R^2=0.03$, $P=0.051$). However, we identified a difference in taxonomic composition according to time since antibiotic exposure ($R^2=0.016$, $P=0.006$). In pairwise comparisons between timepoints, the second timepoint (T2) differed from all other timepoints ($R^2=0.013$, 0.01, 0.015, $P=0.018$, 0.027, 0.007 for T1, T3, and T4, respectively); the third (T3) and fourth (T4) timepoints were not different from baseline ($R^2=0.0097$, 0.01, $P=0.097$, 0.055 respectively), suggesting a temporary perturbation in microbiome community composition at the second timepoint (T2) only. Shannon diversity increased at timepoints T3 ($P=0.04$) and T4 ($P=0.01$) compared to baseline (Supplement Fig. 5B). Richness was not different between any timepoints ($P>0.05$).

In the analysis of taxonomic abundance, among the 26 taxa tested, baseline age was associated with differences in four species, including an increase in *Faecalibacterium prausnitzii* ($P=0.01$) and a decrease in *Klebsiella pneumoniae* ($P<0.001$, Fig. 3B, Supplement Fig. 6A). Only *Bifidobacterium bifidum* was decreased at T2 and T3 relative to baseline ($P<0.001$ and $P=0.06$ respectively, Fig. 3C); however, the abundance did not differ between baseline and T4. On the other hand, the relative abundance of various *Bacteroides* species increased only at T3 compared to T1.

The first timepoint samples were collected 1.5 days after initiating antibiotics on average and do not always represent an antibiotics naïve state. Therefore, we compared the short-term substudy cohort to the unexposed group in the main cohort with samples matched for age and breastfeeding (Supplement Fig. 6B). Consistent with previous results, *Bifidobacterium bifidum* was lower at timepoints T2, T3, and T4 compared to the unexposed group, though the trend suggests that the levels were consistently increasing after T2. Furthermore, antibiotic exposure resulted in lower levels of *Veillonella parvula* at T1 and T2 which recovered at later timepoints.

Finally, we modeled the abundance of bacteria that correlate with time since initiating antibiotics to get a more sensitive estimate of time-dependent linear changes in the microbiome (Supplement Fig. 6C). Multiple bacteria such as *Veillonella parvula*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii,* and multiple *Bacteroides* species increased over the course of 1 month. These bacteria may be implicated in a pattern of short-term microbiome recovery after antibiotic exposure. No bacteria were consistently decreasing over the same time frame.

Antibiotic resistance gene abundance did not increase following antibiotic exposure

Considering the high prevalence of amoxicillin exposure in our cohorts, we focused on antibiotic resistance genes that confer resistance to the penam drug class, which includes amoxicillin. First, we evaluated the distribution of penam-class ARG prevalence and relative

abundance in the unexposed group of our main cohort (Fig. 4A). For the genes that were present in at least 50% of unexposed infants, we performed an analysis of relative abundance. This group of prevalent genes included the resistant penicillin-binding protein and resistant porin orthologs, all but one of the efflux pump gene orthologs, and six beta-lactamase gene orthologs. To establish a baseline for penam-class ARGs, we evaluated the relative abundance of each gene ortholog over time in the unexposed group. Most orthologs

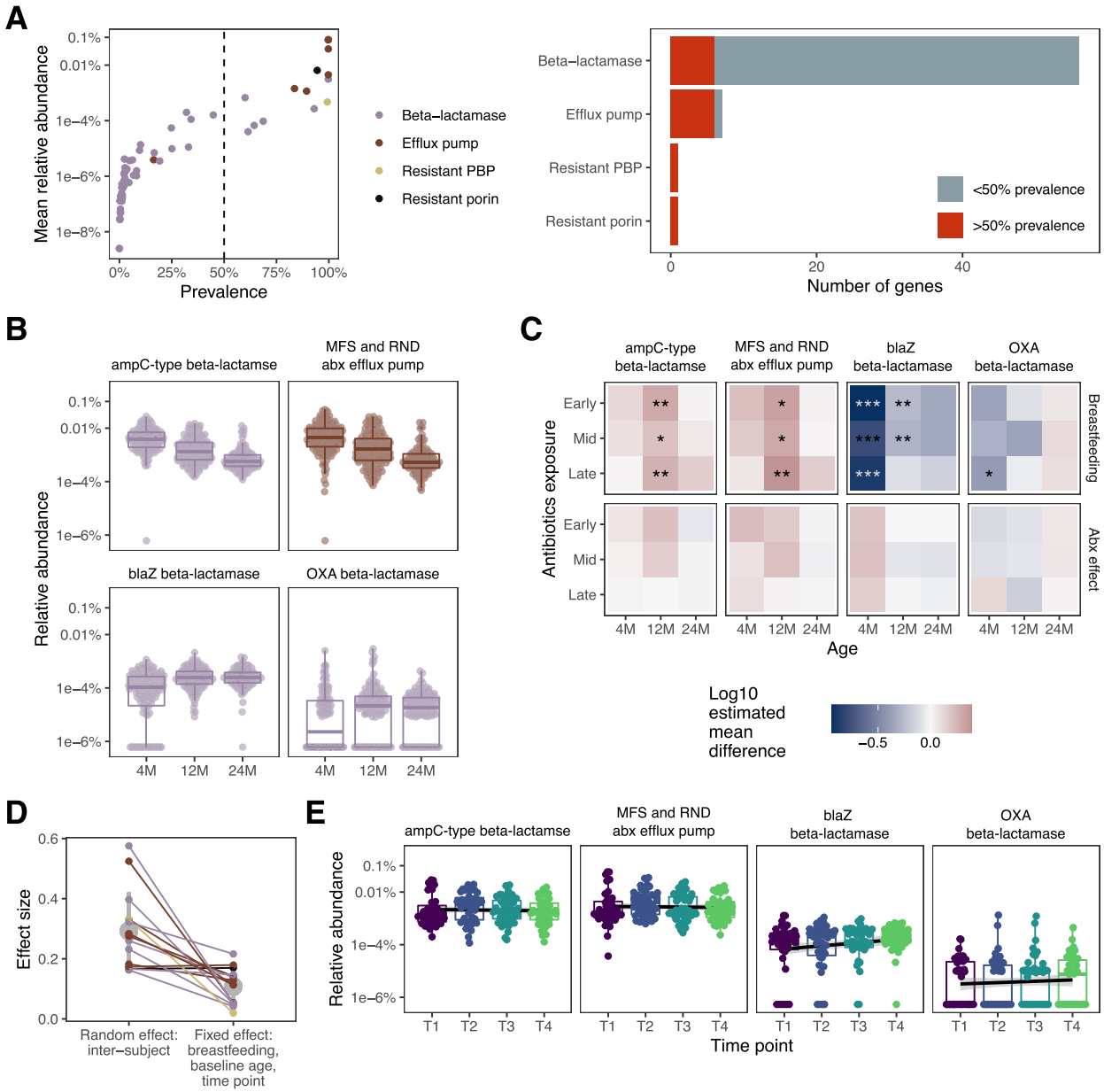

Fig. 4 Antibiotic-resistance gene (ARG) abundance. **A** The mean relative abundance vs. prevalence for genes conferring resistance to panam-class antibiotics in unexposed infants from our main cohort. The dashed line represents the prevalence cutoff for further analysis. The bar chart shows the total number of genes in each category and the percentage that surpassed the cutoff. **B** Select ARGs that increased or decreased with age in unexposed infants from the main cohort. **C** Comparison of ARG abundance between exposed and unexposed infants in the main cohort. Each grid represents a set of comparisons between antibiotic-exposed infant groups and the unexposed group, as in Fig. 2D. The top row indicates coefficients for breastfeeding and the second row indicates those for antibiotic exposure group. **D** Relative effect size of intersubject variability vs. that of all other factors (baseline age, breastfeeding, and timepoint) in the nested short-term substudy. The mean and standard deviation of the effect sizes are marked in gray. **E** Relative abundance of ARGs across the four timepoints of the short-term substudy

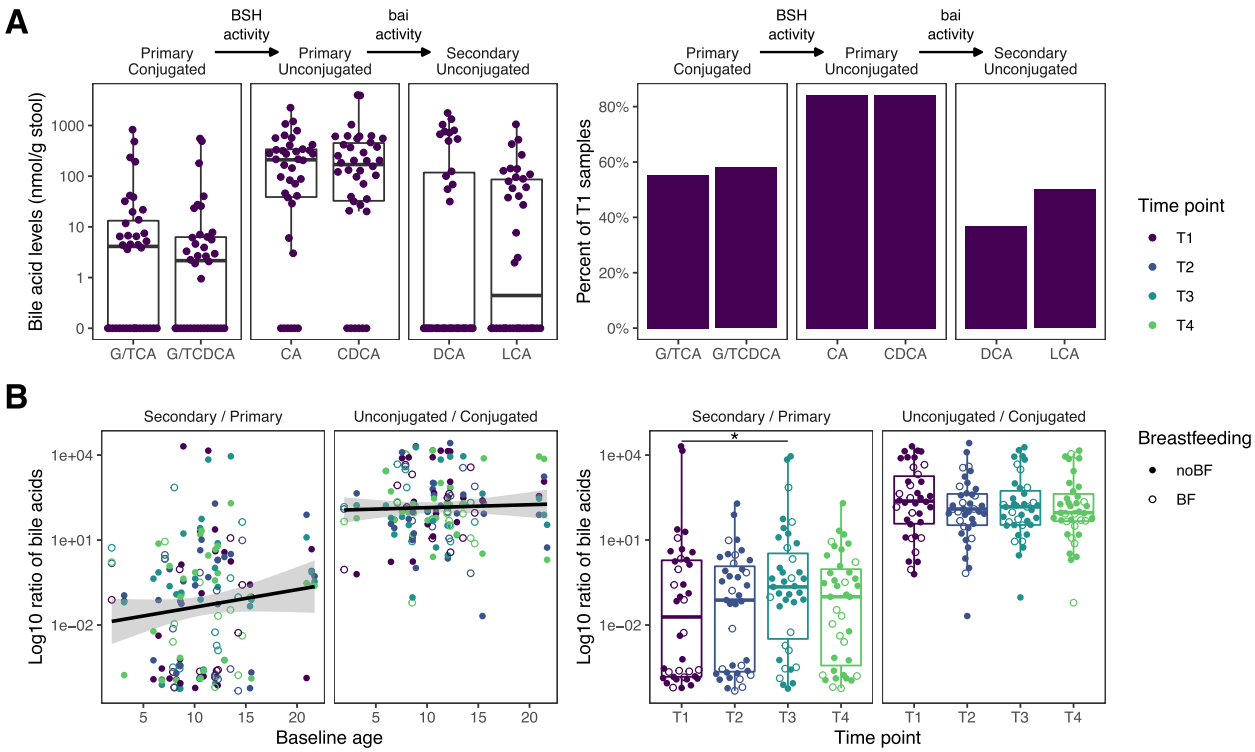

Fig. 5 Fecal bile acid composition. **A** Baseline concentration and prevalence of bile acid species in the short-term substudy. **B** Ratio of secondary to primary bile acids and unconjugated to conjugated bile acids. Colors represent the timepoints. Open circles represent subjects currently being breastfed and closed circles represent subjects who are not breastfed

decreased in abundance at 12 and 24 months, relative to 4 months (Fig. 4B and Supplement Fig. 7). Two orthologs increased with age: the blaZ and OXA beta-lactamases (Fig. 4B).

Next, we compared antibiotic-exposed to unexposed groups in our main cohort using the same approach described above (Fig. 4C, Supplement Fig. 8). No significant differences in ARG ortholog abundance were seen for any antibiotic exposure group; however, we observed associations between ARG abundance and breastfeeding status at 4 and 12 months. Furthermore, the ARGs with less than 50% prevalence showed no association with exposure groups with Fisher's exact test.

In our short-term substudy, we assessed the degree of intersubject variability in ARG abundance compared to baseline age, breastfeeding, and time since antibiotic exposure (Fig. 4D). In all but two of the genes tested, the between-subject variability was greater than the combined effects of all other variables tested. Thus, intersubject variability was the leading factor associated with ARG abundance in the weeks after antibiotic exposure. Moreover, ARG ortholog abundance was not different from baseline at any follow-up timepoints (Fig. 4E, Supplement Fig. 9). Furthermore, despite the association between *B. bifidum* abundance and antibiotic exposure at T2, the difference was not reflected in ARG ortholog abundance.

Antibiotic exposure did not inhibit microbiome modification of bile acids in the short-term substudy

In infancy, the gut microbiome gradually acquires the ability to deconjugate primary bile acids and transform them into secondary bile acids [33]. To determine if antibiotic exposure altered the succession of important metabolic functions, we first examined baseline bile acid concentrations. At baseline, microbial communities efficiently deconjugated primary bile acids in 92% of the samples (Fig. 5A). However, only 55% of the subjects had the functional capability to convert primary into secondary bile acids.

To assess the two sequential bile acid-modifying processes quantitatively, we analyzed the ratio of unconjugated to conjugated primary bile acids (quantifying deconjugation), and the ratio of secondary to primary bile acid concentrations (quantifying conversion to secondary bile acids). Following the same modeling approach used previously (Fig. 5B), we did not observe a significant effect of baseline age or breastfeeding on either of the bile acid ratios. Intersubject variability was the overriding factor in terms of effect size. The secondary to primary bile

acid ratio at T3 was higher relative to baseline ($P=0.01$), indicating an *increased* capability of the gut microbiome to convert primary to secondary bile acids. At T2 and T4, bile acid ratios were not different from baseline in our short-term substudy.

Discussion

We investigated the short-term and long-term effects of antibiotic exposure on the gut microbiome in a prospective longitudinal birth cohort of African American term infants followed to age 24 months, and in a short-term substudy to examine associations over 4 weeks. We observed a short-term decrease in the relative abundance of *Bifidobacterium bifidum* that resolved after 1 week. Long-term, we showed lower levels of *B. bifidum* at 12 months among subjects exposed to amoxicillin between age 4 and 12 months. This effect did not persist at 24 months. Antibiotic exposure was not associated with a long-term increase in antibiotic resistance genes in the gut microbiome, nor was it associated with differences in bile acid modification.

Amoxicillin was the most commonly prescribed antibiotic in our cohort. Amoxicillin is a narrow-spectrum beta-lactam antibiotic frequently used to treat common childhood infections, including otitis media [34]. Although prior infant microbiome studies have included subjects with amoxicillin exposure [14, 20], in most cases, antibiotic types were combined for analysis [8, 16] or the subjects encountered amoxicillin in combination with other antibiotics [13, 15, 19]. All prior studies that specifically examined amoxicillin's association with the microbiome in infants observed a decreased relative abundance of *Bifidobacterium*. However, the studies differed in taxa that were observed to increase in relative abundance: several studies indicated that *Enterobacteriaceae* increased [14–16], while others reported increased relative abundance of *Enterococcus* [13, 19]. The results of our taxonomic analysis are consistent with the identification of *Bifidobacterium*, particularly *B. bifidum*, as a species that is decreased in association with amoxicillin use in infants.

Previous studies of amoxicillin varied in the duration of antibiotic-associated perturbations; two studies observed that differences diminished over 2 weeks [13, 14], but Reyman et al. observed persistent differences 12 months after exposure [19]. We observed a modest, short-term perturbation lasting approximately 1 week and slightly lower levels of *Bifidobacterium* at 12 months only among subjects exclusively exposed to amoxicillin between 4 and 12 months of age. Notably, Reyman et al. administered amoxicillin intravenously in combination with cefotaxime [19], which may account for the prolonged effects on the gut microbiome. The pharmacokinetics and drug bioavailability of amoxicillin may explain its limited impact on the infant gut microbiome. Although amoxicillin is similar in structure to ampicillin, its relative bioavailability in bile is over 30 times lower [35].

The acquisition of antibiotic-resistant bacteria in the gut microbiome following antibiotic exposure is a concern, as these organisms may circulate or cause infections in the host. We investigated ARG abundance in a large, relatively healthy cohort of term infants predominantly exposed to amoxicillin, and therefore focused on penam-class ARGs. Even though these results were not validated with phenotypic culture experiments, we observed that short-term or long-term differences in ARG abundance were not associated with antibiotic exposure but were associated with breastfeeding. Others have observed associations between breastfeeding and ARG abundance in the gut microbiome [36]. Although one in vitro study of amoxicillin and gut microbiome reported an increase in ARG abundance [37], results were inferred from 16S rRNA sequencing rather than measured directly, as we have done. Also, the bioavailability of amoxicillin for microbiota exposure may be much greater in vitro than in vivo, where excretion into the intestinal tract is limited [35].

We examined bile acid concentrations in antibiotic-exposed infants to test for alterations in the fundamental biochemical activity of gut bacteria. Although these bacteria modify bile acids by a variety of mechanisms [38], we focused on the two predominant reactions: removal of glycine or taurine conjugates and 7α-dehyroxylation to form secondary bile acids. A diverse set of gut bacteria deconjugate bile acids [39]; here we probed the biochemical activity of a large component of the bacterial community. Conversely, only several low-abundance species carry out 7α-dehyroxylation[40]; thus, we probed the activity of low-abundance but biochemically important organisms. We found no antibiotic-associated differences in either process, underscoring our main result that exposure to common childhood antibiotics, predominantly amoxicillin, did not have major long-term consequences for gut microbiome development in infants.

This is a natural history study which limits our ability to control for all factors that shape the development of the gut microbiome in early life. While antibiotic exposure length was collected during study visits, often antibiotic dose was not known, limiting assessment of dose-dependent changes in the microbiome. Similarly, the amount of breastfeeding prior to stool collection could not be quantified. In the substudy, baseline samples were not always collected prior to the first antibiotic dose but within two days of exposure, hindering the evaluation of microbiome recovery. It is also possible that the antibiotics may have altered the absolute abundance of microbes

in the short-term substudy. Measurement of absolute abundance by quantitative real-time PCR with universal 16S primers would be a benefit to future studies.

Conclusions

While the widespread use of antibiotics in childhood is of concern given the potential impact on the gut microbiome, we found that microbiome associations with antibiotics in healthy term infants were minimal in comparison to factors such as breastfeeding and microbiome development with age. Long-term associations in our main analysis indicated lower levels of *Bifidobacterium bifidum* only at 12 months that was also dose dependent. Short-term associations were also linked to a transient decrease in *B. bifidum*. We did not find differences in antibiotic resistance gene abundance or in the capacity of the gut microbiome to modify bile acids following antibiotic use.

Abbreviations

ARG	Antibiotic resistance gene
IGram	Infant growth and microbiome
KEGG	Kyoto Encyclopedia of Genes and Genomes
CARD	Comprehensive Antibiotic Resistance Database

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01999-3.

Additional file 1: Supplementary material

Authors' contributions

BZ and KB had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. AJB and CT contributed equally to this work as first authors. AJB, BZ and KB designed the study. AJB, CT, ESF, JPZ, EF, JSG, PAD, AK, HL, MAE, GDW, BZ and KB collected, analyzed and interpreted the data. AJB, CT, BZ and KB drafted the manuscript. ESF, JPZ, EF, JSG, PAD, AK, HL, MAE, GDW reviewed the manuscript. CT, HL and KB conducted the statistical analysis. BZ obtained funding. ESF and EF provided administrative, technical and material support. GDW, BZ and KB supervised the study.

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Data availability

Sequence data from a portion of the cohort (antibiotic-unexposed infants at 12 months) were submitted to the NCBI Sequence Read Archive (accession PRJNA1042647). The rest of the data that were not published before are submitted under accession PRJNA1106565. These public submissions were limited to those individuals who consented to future use of their data.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Review Board (IRB) at Children's Hospital of Philadelphia (IRB 14–010833).

Consent for publication

The IGram study enrolled pregnant African American mothers and their newborn infants. The purpose of the research study was to learn more about the bacteria normally living in the child's gut, how it is transferred from mother to child, and whether it affects the child's growth in the first 3 years of life. The IGRAM data used in this publication are consistent with the stated purpose of the research. The Institutional Review Board-approved consent documents included language that allowed participants to indicate whether they would like to have their information included in future research. Subjects may participate in the original research without their information (even if de-identified) being included in future research.

Competing interests

Dr Elovitz receives salary support from NIAID, NICHD and NINR. She serves as a consultant with equity for Mirvie. No other disclosures were reported.

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