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Effects of Therapeutic Antibiotic Exposure on the Oropharyngeal and Fecal Microbiota in Infants With Cystic Fibrosis

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ABSTRACT

Background: Systemic antibiotics can impact all microbes inhabiting patients, regardless of the intended target organism(s). We studied the simultaneous effects on respiratory and fecal microbiomes of β-lactam antibiotics administered for respiratory symptoms in infants with cystic fibrosis (IWCF).

Objective: To compare the magnitude and duration of intended (respiratory) and unintended (fecal) antimicrobial action by analyzing oropharyngeal (OP) and fecal microbiota in IWCF.

Design: Shotgun metagenomic sequencing and qPCR were performed on OP and fecal samples collected longitudinally from 14 IWCF (ages 1–17 months) during ("On Antibiotics") and after ("Off Antibiotics") β-lactam therapy, and from 5 IWCF (3–16 months) never treated with antibiotics.

Results: Total bacterial loads (TBL) for On Antibiotics samples were lower than for both Never (OP and fecal) and Off Antibiotics samples (fecal only). α-diversities (within-sample) for OP On Antibiotics samples were lower than for Never and Off Antibiotics samples but did not differ between fecal sample groups. β-diversity (between-sample) differed between all OP sample groups and between fecal On and Never Antibiotics and Off and Never antibiotics samples; however, fecal On and Off Antibiotics sample β-diversities did not differ. Patterns of change in antibiotic resistance gene abundances reflected shifts in microbial community composition.

Conclusions: β-lactam antibiotic exposure was followed by marked alterations in both OP and fecal microbiota. While microbiota appeared to rebound after treatment in both sample types, our results suggest that fecal microbiota recovered less than OP. The clinical consequences of these findings should be studied in IWCF and other populations frequently treated with antibiotics.

Hillary S. Hayden and Maria T. Nelson contributed equally to this article.

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1 | Introduction

Cystic fibrosis (CF) is a multiorgan disease caused by mutations in the *CF Transmembrane Conductance Regulator* (CFTR) gene that alter the transport of fluid and ions across cell membranes. Complications from CF, including lung infections, begin early in life, and infants with cystic fibrosis (IWCF) frequently receive systemic antibiotics for respiratory symptoms. The benefits of antibiotics in people with CF (PWCF) have been well-documented, although evidence for their optimal use in IWCF is lacking. These treatments are generally chosen to target cultured respiratory pathogens; in IWCF, *Staphylococcus aureus* (*S. aureus*) and *Haemophilus influenzae* are the most commonly cultured conventional pathogens [1], and β-lactams such as amoxicillin/clavulanate are the antibiotics administered most frequently for these infections [2].

On the other hand, antibiotics can have unintended effects, including selecting for resistant bacteria [3], contributing to acute kidney injury [4, 5], bone marrow toxicity [6], ototoxicity [7], and hepatotoxicity [8, 9], and impacting nontargeted microbiota [10]. In addition, antibiotic treatments for respiratory symptoms could affect the microbiota in the gastrointestinal (GI) tract [11]. GI microbiota constituency contributes to the development of the immune system [12, 13], nutrient acquisition, and the respiratory microbiota [11]. Consequently, antibiotic-driven changes could affect susceptibility to early bacterial respiratory infections [14, 15], antibiotic resistance, GI function, and nutritional outcomes [16–18]. Understanding and minimizing these off-target effects is important for the long-term health of PWCF.

Sputum is the preferred sample for assessing CF respiratory tract microbiology, because this sample type is generally considered to provide an ideal balance of clinical utility (in reflecting lower airway microbiology) and convenience (because most PWCF historically were able to produce a sample [19]). However, with recent improvements in CF care and clinical outcomes, fewer PWCF now spontaneously produce sputum, and oropharyngeal swab (OP) samples are increasingly used for pathogen surveillance and to direct exacerbation therapy [20, 21]. While studies have demonstrated the effects of antibiotic therapy on CF sputum microbiomes [10, 22–24], only a few studies have examined the effects on CF OP microbiota. While many of these considered all antibiotic treatments together, regardless of antibiotic class [25, 26], others specifically examined the effects of β-lactams on the microbiota of multiple respiratory sample types, including OP swabs, sputum, and lavage, and generally in mixed populations of children and adults [27–29]. Given that IWCF are relatively naïve to antibiotics compared with older PWCF, and because we wished to compare effects in samples reflecting the respiratory and GI tracts, we studied the effects of β-lactam administered for respiratory exacerbations on the OP (swabs) and GI (fecal samples) microbiota in IWCF. Using shotgun metagenomic sequencing and quantitative PCR (qPCR), we characterized and compared the simultaneous effects on total bacterial loads (TBL) and microbiota constituencies in these two sample types, as well as treatment-emergent changes in the complement of antibiotic resistance genes in each space. We hypothesized that β-lactam exposure would be associated with transient changes in both the OP and fecal microbiota.

2 | Materials and Methods

2.1 | Study Design

Infants diagnosed with CF by sweat chloride and genetic testing were enrolled between 1 and 3 months of age at Riley Hospital for Children (Indianapolis, IN), Nationwide Children's Hospital (Columbus, OH), Cincinnati Children's Hospital (Cincinnati, OH), and American Family Children's Hospital (Madison, WI). IWCF were eligible if they had not received antibiotics from their CF Center for respiratory indications, though some may have received antibiotics for other indications, for example, neonatal sepsis prophylaxis. Eligible IWCF were enrolled when clinically well at routine clinic visits and were followed for ~12 months. We collected clinical characteristics and demographics, including gestational age, method of delivery, respiratory pathogens, smoke exposure, and oral intake (breast feeding, formula fed, taking solids). Mode of feeding (breast fed, formula fed, and/or taking solids) was not consistently collected. The use of pancreatic enzymes was used as a proxy for pancreatic insufficiency. None were exposed to CFTR modulators. A parent or guardian provided informed consent on their behalf.

The parents or caregivers of participants were instructed to collect fecal samples prior to clinic visits when well and after 24–48 h of antibiotics. OP swabs were collected in clinic by research staff at all CF clinic visits. Fecal samples and OP swabs were typically collected within 24 h of each other. Respiratory exacerbations were clinician-defined based on an increase in respiratory signs and symptoms, and treatments were prescribed by the clinical team. The study was approved by the Institutional Review Boards at Indiana University (IRB # 1607531974), Nationwide Children's Hospital (IRB # IRB16-00800), Cincinnati Children's (IRB # 2018-0721), and the University of Wisconsin (IRB # 2015-0011).

2.2 | Microbiome and Resistome Analyses

Full details regarding sample processing, DNA extraction, quantitative PCR, and shotgun metagenomic sequencing are provided in Supporting Information S1: Supplemental Methods. Taxonomic classification and relative species abundances of bacteria were obtained using MetaPhlAn3 [30, 31] with default settings. Species that failed to reach 1% relative abundance in any sample were removed to minimize sampling noise, and taxonomic proportions were rescaled to reflect the relative abundances of remaining taxa. For analysis of individual species, absolute abundance [10] was quantified by multiplying species relative abundance by TBL determined by 16S rRNA gene qPCR for each sample. Antimicrobial resistance gene (AMR) abundances were obtained by sequence read mapping to the Comprehensive Antibiotic Resistance gene Database (CARD [32]) and normalized using abundances of single copy genes with MUSiCC [33]. Normalized abundances for individual AMR genes were summed by AMR gene family for analysis (e.g., the normalized abundances of the β-lactamase-encoding genes TEM-1, TEM-2, TEM-3, etc. were summed as "TEM"). We removed gene families with < 1% prevalence.

2.3 | Statistical Analysis

All statistical analyses were performed in R (4.3.3). Alpha diversity was calculated from the relevant formula (i.e., Shannon index); Aitchison distance PCoA graphs were created using the ape, vegan, and compositions libraries. We evaluated the homogeneity of variance with the betadisper function. PERMANOVA was performed using the adonis2 function and the W_d* and $T_W{}^2$ tests were performed using R implementations (https://github.com/alekseyenko/WdStar and https://github.com/alekseyenko/Tw2). Our data consist of multiple samples from each individual over time. Because multiple samples from the same individual are not independent samples, for most hypothesis testing we used linear mixed effects models in our statistical analyses. However, when analyzing β-diversity, there is no established method that takes into account multiple samples per individual, and such analyses violate the assumptions of the PERMANOVA test so that p values are likely to be lower (more significant) than they should be. We, therefore, performed follow-up tests on individual taxa or genes with mixed effects models to reinforce our uncertain conclusions from the PERMANOVA. Specifically, we used the implementation of linear mixed effects models from the lme4 R package with the age of sample and antibiotic status (On, Off, or Never) as fixed effects and the participant as a random effect. Consistent with previous studies [34, 35], we found that age had a significant effect on microbiome composition (Supporting Information S1: Figure 1A, $p < 0.001$), and thus we included age as a fixed effect covariate in all models. We used the p value method from lmerTest and extracted p values between pairs of comparisons of antibiotic statuses using the emmeans package. Abundance values were $\log_{10}$ transformed prior to testing with the model. When testing lists of taxa or genes for significance, we used an FDR of 0.05 to control for multiple testing.

3 | Results

3.1 | Study Participants

A total of 19 IWCF provided OP and/or fecal samples. Cohort demographics are provided in Supporting Information S1: Table 1. All of the infants were non-Hispanic White; none were born preterm. All but one were pancreatic insufficient. A single participant had no copies of F508del. Fourteen infants received β-lactam antibiotics during the study and provided two or more OP samples; nine of these infants also provided three or more fecal samples (Supporting Information S1: Table 2). The remaining five IWCF never treated with antibiotics comprised the Never Antibiotics group and provided OP and fecal samples (four participants) or fecal samples alone (one participant). The average age on entering the study was 98.4 days for the 14 infants who received antibiotics during the study and 108.4 days for the Never Antibiotics group. β-lactam antibiotics that were used included amoxicillin, amoxicillin/clavulanate, ampicillin/sulbactam, cefdinir, and cephalexin.

3.2 | Study Samples

OP and fecal samples were collected during ("On Antibiotics") and after completion of ("Off Antibiotics") treatment with antibiotics from 14 and 9 IWCF, respectively, who received β-lactam antibiotics for one or more respiratory exacerbations during the study (some "Off Antibiotics" samples were collected after antibiotics administered before enrollment). Longitudinal control samples were also collected from five IWCF who reported no exposure to antibiotics ("Never Antibiotics"). We collected and analyzed a total of 74 OP swabs: 30 (7.5 samples/infant, range 5–10) from the Never Antibiotics group and 44 (3 samples/infant, range 2–9) from the infants given β-lactam antibiotics (Figure 1 and Supporting Information S1: Table 2).

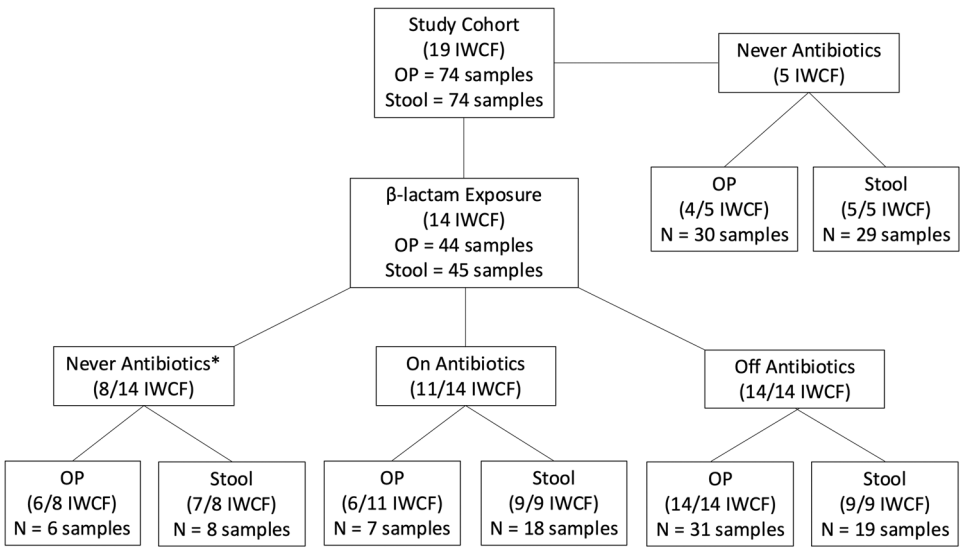

FIGURE 1 | Study schema. OP swab and stool samples were collected from IWCF never treated with antibiotics ($n = 5$) or IWCF who received β-lactam antibiotics during the study period ($n = 14$). Eight of the IWCF who received β-lactam antibiotics provided samples prior to receiving any antibiotics in their lifetime, and these samples were included in the Never Antibiotics category (here, marked with * for clarity). On Antibiotics samples from 11 of 14 IWCF were collected while infants were receiving β-lactam therapy for pulmonary exacerbations, and Off Antibiotics samples from all 14 IWCF were collected after antibiotic exposure.

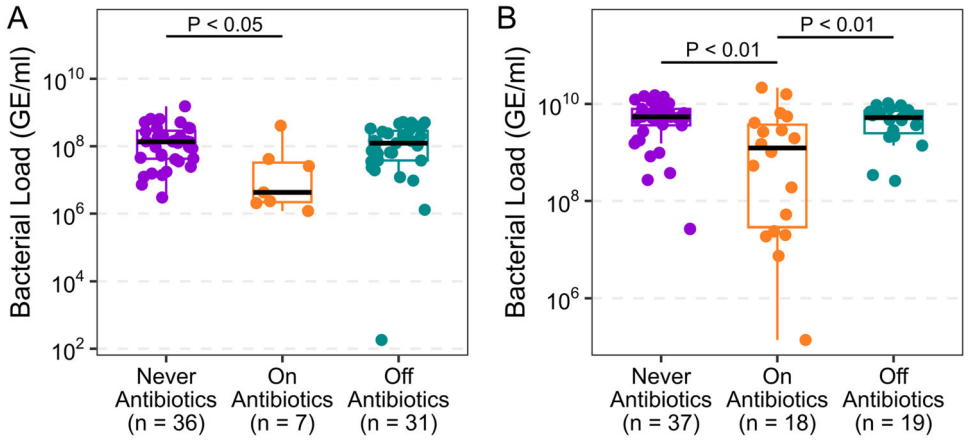

FIGURE 2 | Total bacterial loads in (A) OP and (B) fecal samples for each study group. Box center lines indicate medians, with hinges at 25th and 75th percentiles; whiskers extend to the minimum and maximum values. Dots indicate individual sample values. p values of < 0.05 (linear mixed-effects model) were considered significant. GE = genome equivalents. OP and fecal samples for individual infants displayed chronologically by age of collection are in Supporting Information S1: Figures 2 and 3. [Color figure can be viewed at wileyonlinelibrary.com]

Among the infants given β-lactam antibiotics, 8 of 14 had no exposure to antibiotics prior to this study, and 6 of these infants provided an OP swab before receiving β-lactam treatment. These 6 samples are included in OP Never Antibiotics, while 7 On Antibiotics OP samples were collected during treatment and 31 Off Antibiotics samples were collected after treatment (Figure 1 and Supporting Information S1: Table 2). We also collected and analyzed 74 fecal samples: 29 (5.8 samples/infant, range 4–7) from the Never Antibiotics group and 45 (5.0 samples/infant, range 0–9) from the infants given β-lactam antibiotics. Among the infants given β-lactam antibiotics, 7 provided a fecal sample before receiving β-lactam treatment (1 infant provided two fecal samples before treatment). These 8 samples are included in the fecal Never Antibiotics samples, while 18 On Antibiotics fecal samples were collected during treatment and 19 Off Antibiotics samples were collected after treatment (Figure 1 and Supporting Information S1: Table 2). The Off Antibiotics samples were collected a median of 17 (range 1–158) and 22 (range 2–94) days after the last treatment day of the most recent antibiotic course for OP and fecal samples, respectively. To determine whether Off Antibiotics samples collected only 1 or 2 days after antibiotic treatment reflected residual antibiotic effects, we analyzed the relationship between α-diversity and time after antibiotics. α-diversity steadily increased over time after the cessation of antibiotics for OP samples (Supporting Information S1: Figure 1B, $p < 0.01$), indicating the importance of considering postantibiotic duration in our categories. We therefore tested the effect of defining Off Antibiotics as either ≥ 7 or ≥ 14 days after treatment. We observed only one significant difference in α-diversity for any sample/category comparison between these alternate thresholds, which was between On and Never Antibiotics for fecal samples; no other significant result changed from those reported below. Therefore, we included all postantibiotic samples in the Off Antibiotic category for subsequent analyses.

3.3 | Total Bacterial Loads (TBL)

For OP swabs, TBL in On Antibiotics samples were significantly lower than in the Never Antibiotics group ($p < 0.05$), while TBL in Off Antibiotics samples were not significantly different from either On Antibiotics or Never Antibiotics ($p > 0.1$) (Figure 2A). Fecal TBL in On Antibiotics samples were significantly lower than in both Off Antibiotics and Never Antibiotics ($p < 0.01$), but TBL did not differ between Off Antibiotics and Never Antibiotics ($p > 0.1$) (Figure 2B). For many infants, OP and fecal TBL were conspicuously lower On Antibiotics than both Off Antibiotics and Never Antibiotics samples when viewed chronologically (Supporting Information S1: Figures 2 and 3). These results indicate that the number of bacterial cells in OP and fecal samples decreased during β-lactam antibiotic treatment, and the decrease was often greater in fecal than in OP samples for individual infants. For most infants, OP and fecal TBL appeared to rebound after antibiotic treatment.

3.4 | Alpha (Within-Sample) Diversity

We measured OP and fecal sample microbiota α-diversity using the Shannon Diversity Index (SDI). For OP samples, α-diversity in On Antibiotics samples was significantly lower than in both Off Antibiotics and Never Antibiotics groups ($p < 0.001$ and $p < 10^{-5}$, respectively), but α-diversity did not differ significantly between Off Antibiotics and Never Antibiotics ($p > 0.05$) (Figure 3A,B). Conversely, for fecal samples, α-diversity did not differ significantly between any groups ($p > 0.05$), although average α-diversity appeared to be lower in On Antibiotics samples than in both Off Antibiotics and Never Antibiotics samples (Figure 4A,B). As noted above, when we included samples collected within 7 days of completing antibiotic treatment in the On Antibiotics group, we observed a significant difference in α-diversity between On and Never Antibiotics for fecal samples, indicating the reduction in α-diversity persists for at least a week after stopping antibiotics, as can be observed at the individual level in Figures 3B and 4B. Therefore, similar to the TBL results, α-diversity appeared to decrease during β-lactam antibiotic exposure and rebound after treatment in both feces and OP samples; however, the diversity decrease was often greater in OP than in fecal samples.

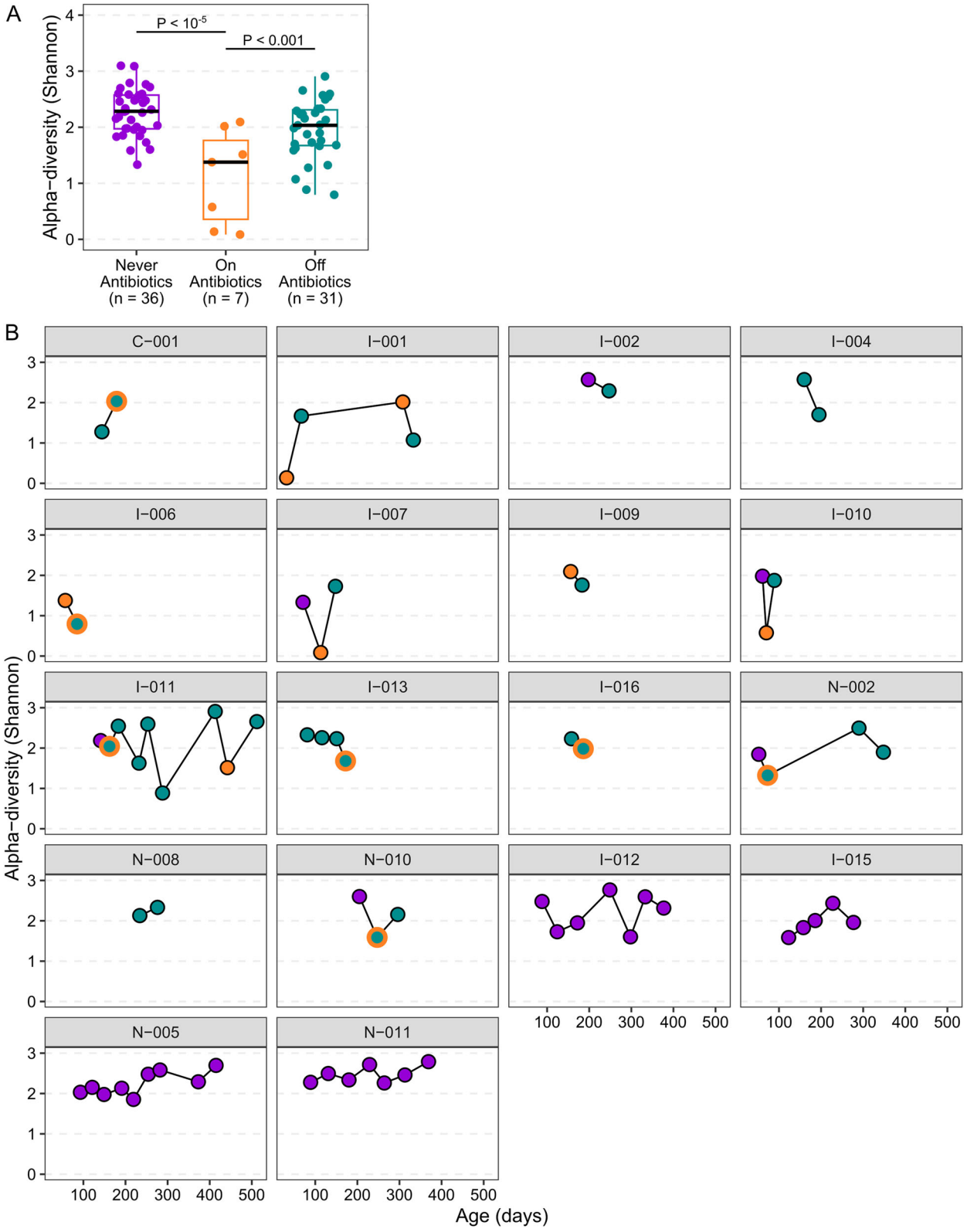

FIGURE 3 | Alpha (α)-diversity using the Shannon Diversity Index for OP samples. (A) OP samples are grouped by antibiotic sample type. Box center lines indicate medians, with hinges at 25th and 75th percentiles; whiskers extend to the minimum and maximum values. Dots indicate individual sample values. *p* values of < 0.05 (linear mixed-effects model) were considered significant. (B) OP samples for individual infants are displayed chronologically by age of collection. Samples are colored as in (A) with Never Antibiotics = purple, On Antibiotics = orange, and Off Antibiotics = green. Green points with a thick orange border were collected 7 days or less after cessation of antibiotic treatment. [Color figure can be viewed at wileyonlinelibrary.com]

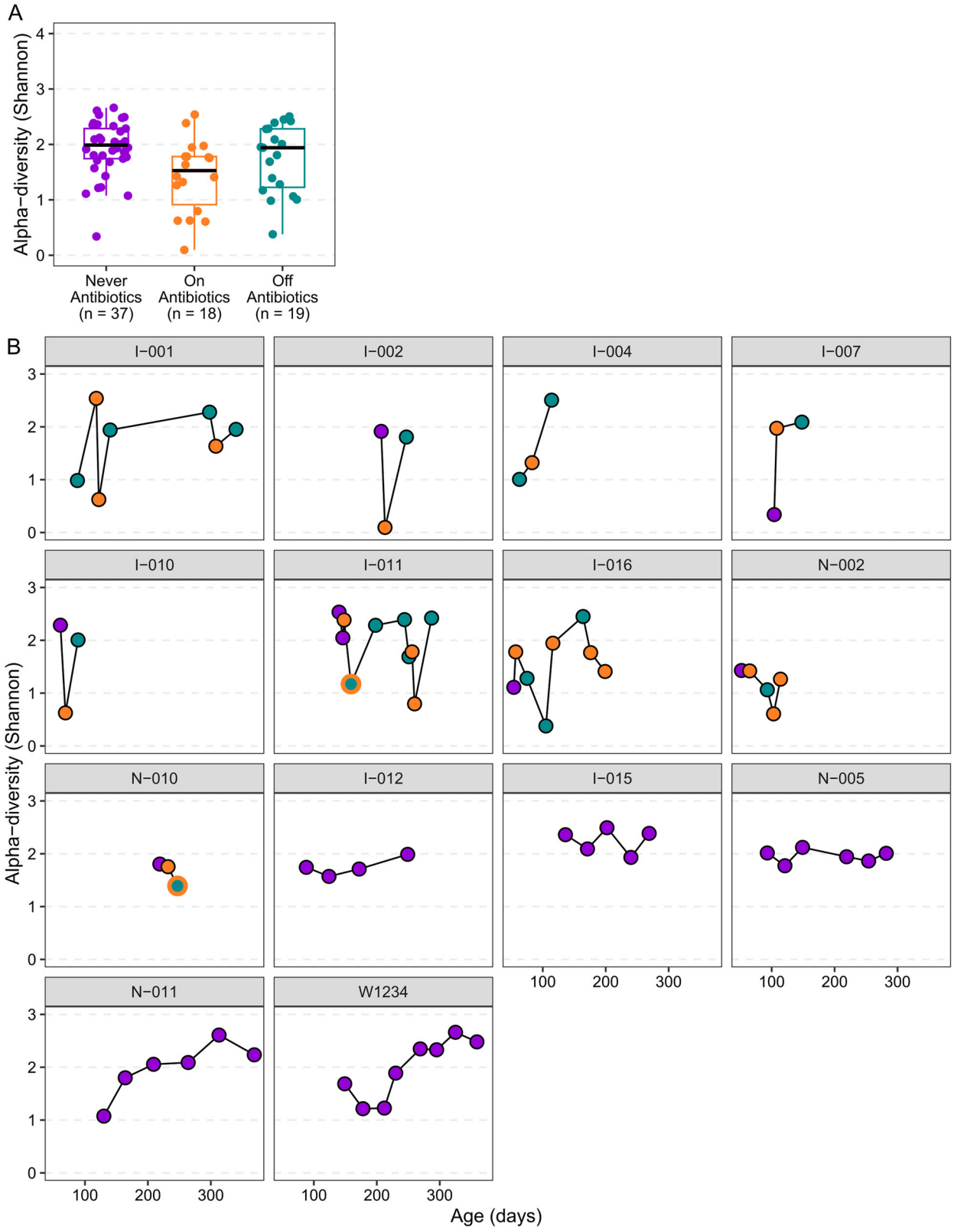

FIGURE 4 | Alpha (α)-diversity using the Shannon Diversity Index for fecal samples. (A) Fecal samples grouped by antibiotic sample type. Box center lines indicate medians, with hinges at 25th and 75th percentiles; whiskers extend to the minimum and maximum values. Dots indicate individual sample values. *P* values of < 0.05 (linear mixed-effects model) were considered significant. (B) Fecal samples for individual infants displayed chronologically by age of collection. Samples are colored as in (A) with Never Antibiotics = purple, On Antibiotics = orange, and Off Antibiotics = green. Green points with a thick orange border were collected 7 days or less after cessation of antibiotic treatment. [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Beta (Between-Sample) Diversity

3.5.1 | Oropharyngeal Sample Microbiota

Principal coordinates analysis (PCoA) of the relative abundances of species in OP samples indicated that the microbiota in On Antibiotics, Off Antibiotics, and Never Antibiotics samples were significantly different from each other ($p < 0.001$ using PERMANOVA, Figure 5A) and identified the major taxonomic contributors to these differences, including species of *Prevotella, Veillonella*, *Streptococcus*, *Neisseria*, and *Porphyromonas*, among others (Figure 5B). However, Homogeneity of Variance analysis, which tests for differences in microbiota variances between groups, was significant for OP samples ($p < 0.01$). Because the validity of PERMANOVA p values assumes equal variability across groups, we compared the between-group differences using the W_d*-test [36], which also demonstrated between-group differences in relative abundances, $p < 0.01$. Using the T_W^2 test [37], we found statistical differences between all pairwise comparisons of our three study groups (Never vs. Off Antibiotics, $p < 0.05$, Never vs. On and Off vs. On Antibiotics, $p < 0.01$) (Supporting Information S1: Figure 4). Predictably, differences in microbiota composition were more evident across chronological OP samples from infants who received β-lactam antibiotics during the study than infants in the Never Antibiotics group (Figure 5C).

PCoA shows overall differences in microbial community composition among sample types, and the species drivers of those differences, but cannot say whether bacterial species increase or decrease during antibiotic treatment. To assess this, we analyzed the absolute abundances of individual species among the three groups of OP samples. Compared to the Never Antibiotics group, the average absolute abundances of three species (*Veillonella atypica* (*V. atypica*), *Campylobacter concisus* (*C. concisus*), and *Actinomyces graevenitzii* (*A. graevenitzii*)), none considered typical CF pathogens, in On Antibiotics samples were significantly lower (FDR < 0.05, Supporting Information S2: Table 3). No species differed significantly either between Never Antibiotics and Off Antibiotics or between On Antibiotics and Off Antibiotics. *V. atypica* and *C. concisus* are found in the human oral cavity and intestinal tract. *A. graevenitzii* is a relatively rare *Actinomyces* species that has been isolated from oral and respiratory sites associated with clinical actinomycosis [38]. Of conventional CF pathogens, *S. aureus* was detected in a single Off Antibiotics sample following β-lactam exposure in one infant, and *Pseudomonas aeruginosa* (*P. aeruginosa*) was not detected in any sample.

3.6 | Fecal Sample Microbiota

Similar to OP samples, PCoA analysis found that the fecal sample microbiota in the three study groups were distinct (Figure 6A; PERMANOVA and W_d*-test $p < 0.001$; Homogeneity of Variance $p > 0.1$). The major taxonomic contributors to these differences included species of *Lactobacillus, Rothia*, *Streptococcus*, *Klebsiella, Intestinibacter, Ruminococcus, and Bifidobacterium* among others (Figure 6B). In pairwise comparisons, Never Antibiotics sample microbiota were significantly different from both On Antibiotics and Off Antibiotics samples, $p < 0.05$ T_W^2 test (Supporting Information S1: Figure 5). However, in contrast with the OP samples, the difference between On and Off Antibiotics microbiota did not reach significance ($p > 0.05$). Similar to OP samples, differences in microbiota composition were more evident across chronological samples from infants who received β-lactam antibiotics during the study than infants in the Never Antibiotics group (Figure 6C). When analyzing individual species, we found that compared to the On Antibiotics group, the average absolute abundances of three species were lower than in the Never Antibiotics group (*Veillonella parvula* (*V. parvula*)), Off Antibiotics group (*Streptococcus parasanguinis* (*S. parasanguinis*)), or both (*V. atypica*) (FDR < 0.05, E-table 3). Like *V. atypica, V. parvula, and S. parasanguinis* are commonly found in the human oral cavity and intestinal tract.

Taken together, these TBL and microbiota diversity results indicate that β-lactam antibiotic exposure was followed by changes in both OP and fecal bacterial microbiota. Although the compositions of these communities rebounded at least partially after treatment, the effects of antibiotic exposure may have persisted in both spaces, as suggested by the significant differences in β-diversity between OP and fecal Off Antibiotics and Never Antibiotics samples, but similar fecal sample Off Antibiotics and On Antibiotics β-diversity. Because our study design includes nonindependent samples, and the significance of the differences we observed both between OP and fecal Off compared to Never Antibiotics was borderline ($p < 0.05$), additional studies are required to validate these results.

3.7 | Resistome

Using shotgun metagenomic sequencing, we also determined whether β-lactam treatment was associated with changes in OP and fecal resistomes [39]—the AMR gene complements—in each sample type. We hypothesized that these treatments would be followed by increased abundances of microbial genes encoding β-lactam resistance in each space. Abundances for individual AMR genes were summed by AMR gene family for analysis; a total of 490 and 523 CARD gene families were identified in OP and fecal samples, respectively. For OP and fecal samples, W_d*-test ($p < 0.01$) and PERMANOVA ($p < 0.001$), respectively, indicated significant differences in AMR gene family abundances among study groups (Supporting Information S1: Figure 6). In OP samples, we found statistical differences for all pairwise comparisons of our three study groups (T_W^2 test, all comparisons, $p < 0.05$). In contrast, in fecal samples, while On Antibiotics samples were different from the Never Antibiotics group (T_W^2 test, $p < 0.05$), the difference between On and Off Antibiotics and Off and Never Antibiotics samples did not reach significance (T_W^2 test, $p > 0.1$).

We investigated whether individual AMR gene families differed significantly in abundance among our 3 study groups and identified 67 AMR gene families that differed among OP sample groups and none for fecal samples (Supporting Information S2: Table 3 and Supporting Information S1: Figure 7). In OP samples, these gene families encoded resistance to many different antibiotic drug classes, with 20 (30%) gene families known to target β-lactam drugs. The abundance of 61 AMR gene families

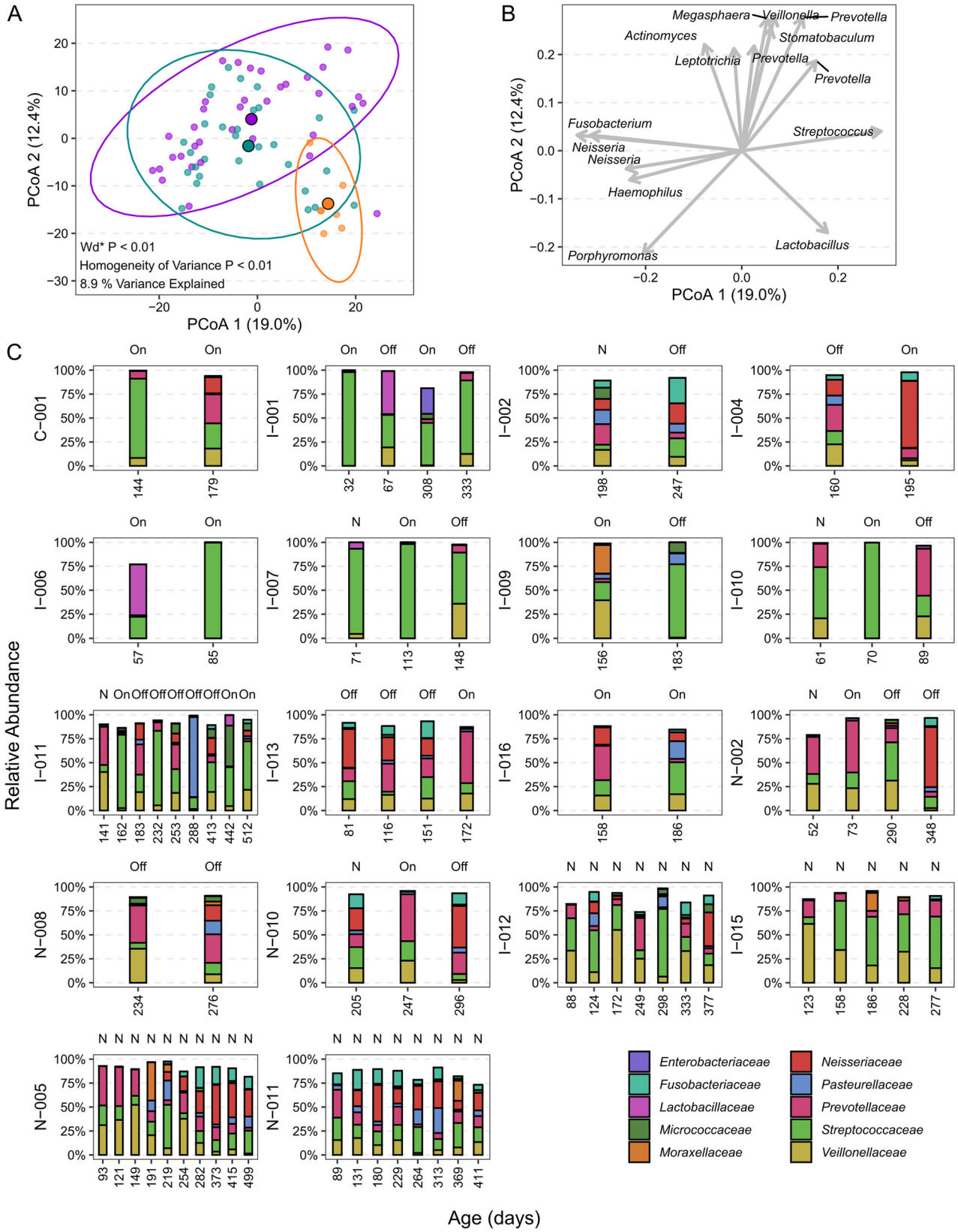

FIGURE 5 | Beta (β)-diversity of OP samples. (A) Principal coordinates analysis using the Aitchison dissimilarity metric of taxonomic relative abundances. Large dots represent the centroid of each group, with ellipses encircling 95% of individual samples. Colors indicate antibiotic sample type with Never Antibiotics = purple, On Antibiotics = orange, and Off Antibiotics = green. (B) Biplots demonstrating the taxa most responsible for the taxonomic difference among all sample types. Biplots were generated at species level; genus names are listed for illustration clarity. Length of vectors indicates the extent to which taxa contribute to inter-sample dissimilarity. (C) Bar charts of family level relative abundances for each infant at each sample point are displayed chronologically by age. Only families that reach 25% abundance in at least one sample are shown. On = On Antibiotics, Off = Off Antibiotics, and NA = Never Antibiotics. [Color figure can be viewed at wileyonlinelibrary.com]

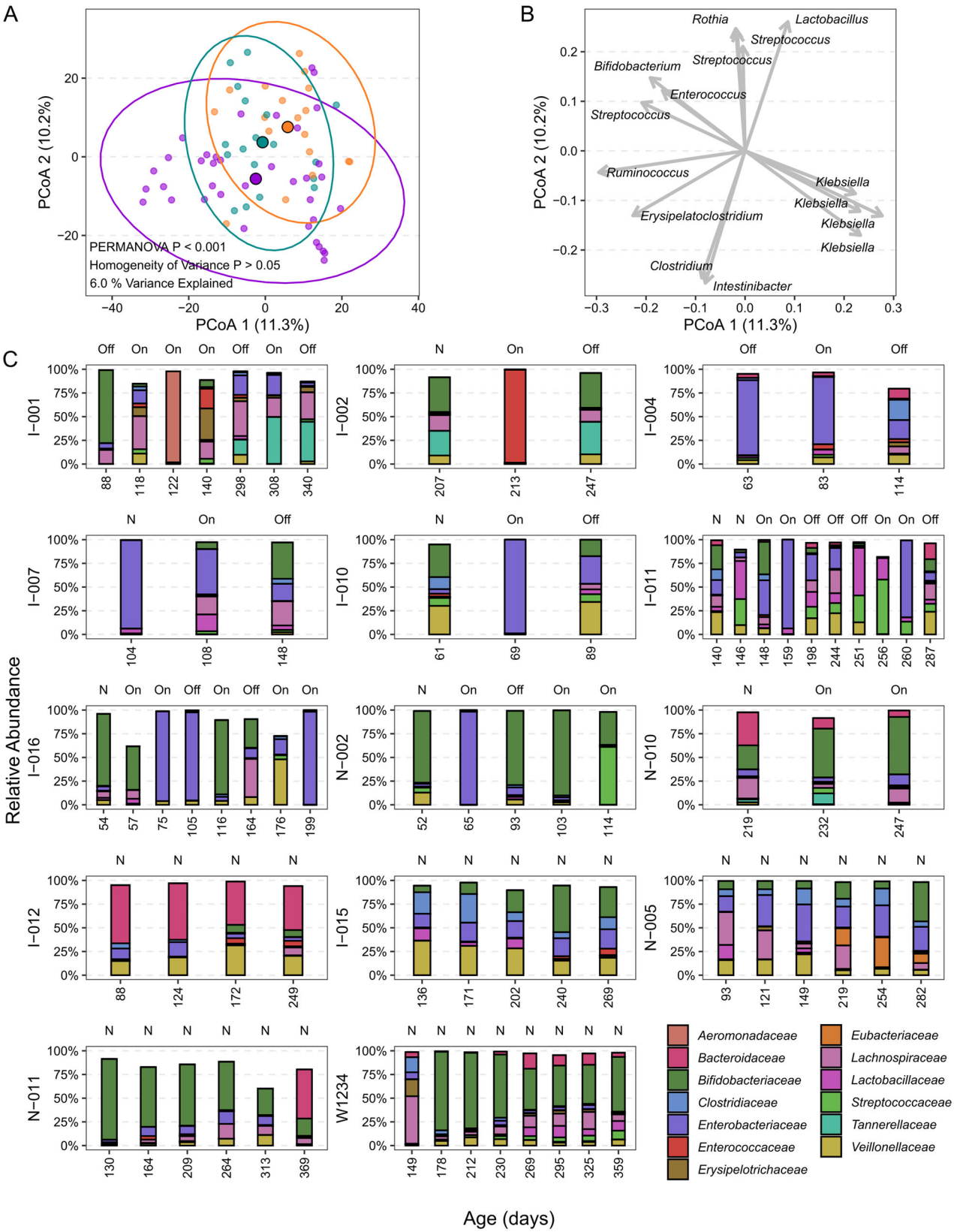

FIGURE 6 | Beta (β)-diversity of fecal samples. (A) Principal coordinates analysis using the Aitchison dissimilarity metric of taxonomic relative abundances. Large dots represent the centroid of each group, with ellipses encircling 95% of individual samples. Colors indicate antibiotic sample type with Never Antibiotics = purple, On Antibiotics = orange, and Off Antibiotics = green. (B) Biplots demonstrating the taxa most responsible for the taxonomic difference among all sample types. Biplots were generated at species level; genus names are listed for illustration clarity. Length of vectors indicates the extent to which taxa contribute to inter-sample dissimilarity. (C) Bar charts of family level relative abundances for each infant at each sample point are displayed chronologically by age. Only families that reach 25% abundance in at least one sample are shown. On = On Antibiotics, Off = Off Antibiotics, and NA = Never Antibiotics. [Color figure can be viewed at wileyonlinelibrary.com]

was significantly different in On compared to Never Antibiotics, and for most (92%) the abundance was lower in On Antibiotics samples. Of the 5 AMR gene families with higher abundance in the On Antibiotics group, only 1 encoded a known β-lactam resistance determinant, the SRT β-lactamase family whose gene members inactivate cephalosporins. The abundance of 55 AMR gene families was significantly different in On compared to Off Antibiotics, and for most (84%) the abundance was lower in On Antibiotics samples. Of the 9 AMR gene families with higher abundance in the On Antibiotics group, 5 encoded known β-lactam resistance determinants (*cmeR, mecB, MuxC, smeC*, and SRT). In the comparison between Off and Never Antibiotics, a single AMR gene family reached significance; *mepR*, a multidrug and toxic compound extrusion (MATE) transporter of glycylcycline and tetracycline compounds, was significantly higher in Off Antibiotics (Supporting Information S2: Table 3 and Supporting Information S1: Figure 7).

We tested the effects of defining Off Antibiotics as ≥ 7 and ≥ 14 days after treatment on differences between groups in the resistome, and the number of significant AMR gene families in OP decreased from 67 to 7 and 6, respectively (Supporting Information S2: Table 3). All had lower abundances in On compared to Off or Never Antibiotics. Two AMR gene families reached significance in fecal samples for the 14-day cutoff only (Supporting Information S2: Table 3). None of the AMR gene families at these alternate cutoffs encoded β-lactam resistance determinants for either sample type.

These findings suggest that β-lactam antibiotic exposure was associated with alterations in AMR gene family abundances, particularly in OP samples. Most AMR gene families, including those targeting β-lactams, decreased in abundance during antibiotic treatment, suggesting, contrary to our hypothesis, that changes in sample resistomes with and following β-lactam treatment primarily reflected shifts in microbial community composition, rather than solely reflecting selection for cells encoding β-lactam resistance genes. Many species' abundance decreased during antibiotic treatment, and consequently, antibiotic resistance genes encoded in these genomes also decreased. Similarly, our observation of fewer significant differences in AMR gene families when defining Off Antibiotics as both ≥ 7 and ≥ 14 days after treatment suggests a partial community recovery. Therefore, these results suggest that the taxonomic changes we observed with treatment cannot be explained solely by selection for taxa carrying known β-lactam resistance genes.

4 | Discussion

We identified relatively large residual changes in CF infant fecal microbiomes compared with OP microbiomes after treatment with β-lactams, the class of antibiotics used most often in CF respiratory exacerbations [2]. The magnitudes of the changes in both sample types with treatment were at least comparable, despite the intentional selection of these treatments to target pathogens identified by OP swab cultures, which serve as proxies for lower-airway microbiology [21, 40]. While the clinical consequences of the off-target treatment effects on the GI microbiome observed here remain to be defined, these effects were characterized by a relative depletion in fecal abundances of many health-associated taxa, as well as marked decreases in antibiotic resistance gene complements in OP samples well beyond those conferring resistance to β-lactams, underscoring the importance of considering the unintended effects of antibiotic therapies for these and other chronic diseases.

These results confirm, clarify, and expand findings from other work on the effects of antibiotics on both fecal and respiratory microbiomes by directly comparing them. Most studies to date of antibiotic impact on CF respiratory microbiomes analyzed sputum, necessarily focusing on older PWCF. For example, Stressman et al. found [23] that antibiotics for respiratory exacerbations were associated with changes in sputum microbiota among 14 PWCF during treatment, with a return to pretreatment microbiota configurations at 1 month; similarly transient changes in sputum bacterial loads and microbiota with IV antibiotics were identified by Smith et al. [24] examining 23 adults with CF. Zhao et al. [41] studied over 400 sputum samples from 66 PWCF who received antibiotics to compare the effects of antibiotic class, disease severity, sample timing, and other variables on the magnitude of change in microbiota diversity, finding relatively large effects of sample timing relative to treatment and of antibiotic class, two variables that were relatively constant in our study design.

By comparison, relatively little work has focused on the effects of antibiotics on IWCF or on the microbiota in OP samples, the most common respiratory sample in children and the growing proportion of adults with CF who do not reliably expectorate [1]. While OP samples do not always accurately reflect lower airway microbiology, we focused on them here as convenient respiratory samples to compare with fecal samples, which are convenient and imperfect reflections of the GI tract. Given their convenience, previous work studied the effects of antibiotics on the microbiota of CF OP samples. For example, Pittman et al. [26] compared the microbiota defined by 16S rRNA gene sequencing among both OP and bronchoalveolar lavage (BAL) samples collected from 42 IWCF in Australia and the US, finding lower diversity and bacterial loads in OP samples from infants receiving continuous prophylactic antistaphylococcal therapy, but not in those who had received intermittent antibiotic treatments (interestingly, differences in diversity were also found in BAL with prophylactic antibiotics, but not in TBL). The timing of sampling during that study relative to antibiotic exposure was not described. Similarly, Harris et al. [25] recently defined the OP microbiota of 205 IWCF during the first year of life using 16S sequencing, finding only small differences in TBL and diversity in those exposed to antibiotics; sample collection timing for that study also did not allow for a rigorous or specific analysis of the effects of antibiotics. While that study identified relatively small effects of intermittent antibiotic treatments on OP microbiota during infancy compared with those we identified here, those studies were not designed with that focus in mind. In addition, several recent studies by the same group used metagenomic analyses [28, 29] and 16S rRNA sequencing [27] to define the effects of β-lactams on the microbiota of multiple respiratory sample types (including OP swabs) from adults and children beyond infancy, demonstrating marked changes of respiratory microbiota with these treatments, which our findings support.

A growing body of literature has identified a fecal dysbiosis in PWCF relative to those without, including in IWCF who were antibiotic-naïve [35, 42], that has been linked with a variety of adverse health outcomes (reviewed in Caley et al. [18] and Price and O'Toole [43]), though whether the relationship is causal is not yet determined. Many recent studies [16, 17, 44, 45] have also focused on the effects of antibiotics, both continuous and intermittent, on CF fecal microbiota, with varied findings depending on type, duration, and intensity of antibiotic exposure. However, as described in recent reviews [18, 43, 46], most such studies found a relationship between antibiotic treatment and lower fecal diversity, with one study of adult PWCF identifying a significant negative correlation between number of IV antibiotic courses in the prior year and fecal microbiota diversity [44]. These aggregate results support our finding that antibiotic courses administered for respiratory exacerbations are followed by long-lasting (more than 2 weeks) decreases in fecal TBL and diversity, in this case, focused on a single antibiotic class. Amoxicillin–clavulanate, which has a relatively broad spectrum of coverage including anaerobes, was the most common antibiotic used during this study.

While this work did not analyze the relationships between fecal microbiota and clinical outcomes, evidence suggests that there is an association between fecal dysbiosis and adverse clinical consequences. For example, measures of fecal microbiome constituency and diversity have been shown to correlate with measures of intestinal inflammation [42], motility [17], and fat malabsorption [42, 47], in addition to stunted body growth [35, 48] and diagnosis with CF-related liver disease [49]. However, because these prior studies were observational, whether fecal dysbioses were causally related to these clinical outcomes could not be established; for example, differences in GI microbiomes and clinical outcomes may have different causes. Similarly, these questions are not addressed by our study; for example, the clinical effects of microbiological changes during antibiotic treatment and those after treatment cessation remain undefined. Nevertheless, the significant relationships observed between CF fecal dysbioses with a variety of poor outcomes underscore the importance of studying the impact of otherwise beneficial therapies on GI microbiology and disease, particularly as life expectancy of PWCF continues to increase with transformative therapies such as CFTR modulators [1].

There is no standard definition of a respiratory exacerbation, especially in infants and young children with CF. Consequently, the use of antibiotics varies considerably among (and within) CF Centers. The use of OP swabs as respiratory samples in this study reflects common practice for surveillance of respiratory microbiology in people who cannot expectorate. While these samples are convenient to collect, their accuracy for reflecting the microbiota either of the lower respiratory tract or sinuses [21, 40], both of which are of clinical importance in CF care, has been repeatedly shown to be poor [50, 51]. For example, OP swabs have relatively high negative predictive value but low positive predictive value for the detection of the pathogen *P. aeruginosa* in concurrent BAL samples [21, 40]. The use of OP swabs in this study reflected their common use in CF testing [19] and provided an opportunity to study the effects of antibiotics on these increasingly common samples, informing the interpretation of OP samples obtained during and after antibiotic treatment for exacerbations by defining the types and duration of the microbiological effects of treatment. However, the results here do not necessarily reflect the dynamics of antibiotic effects on either lower respiratory tract or sinus microbiota in PWCF. Notably, our study used shotgun metagenomic sequencing, rather than 16S sequencing; metagenomics has previously been used only rarely to analyze CF OP microbiota [28, 29, 52, 53] but provides the opportunity to analyze bacterial genetic features, such as antibiotic resistance gene content, as we performed here.

The metagenomic analysis of known antibiotic resistance determinants led to two key findings: first, the differences in microbiota in samples collected during antibiotics compared to controls in the Never Antibiotics group could not be explained solely by selection for cells encoding known β-lactam resistance determinants (although such selection could have played some role). Therefore, the mechanisms responsible for persistence or depletion of specific taxa during β-lactam treatment are not apparent from our results (notably, our data allowed us to rigorously compare the abundances, but not coding, of genes, precluding an analysis of mutations in drug targets or other mechanisms of drug resistance). Second, treatment-emergent changes involved genes conferring resistance to many non-β-lactam antibiotics that are also used in CF care. The consequences of these and other off-target antibiotic effects could thereby include altered responses to unrelated classes of antibiotics from those used for the current treatment. These observations and their potential consequences warrant further study.

Our study had a number of limitations. Our sample size, both in terms of subjects and samples, was limited, as was the observation period. In some cases, fecal and OP samples were not paired due to missing or uncollected samples. We did not directly compare metagenomic results with clinical respiratory samples obtained simultaneously. Given the limitations of CF pathogen detection from OP swabs, undetected CF pathogens may be present, so CF clinicians often choose antibiotics empirically based on past culture results and clinical experience. We were unable to investigate the effects of potential confounders, including diet and medications such as acid blockers, on our results due to missing or variably reported data. For these and other reasons, our findings should be validated in a larger, longer study with rigorous sample and data collection.

In conclusion, we found that β-lactam antibiotic treatments for CF respiratory exacerbations were followed by marked changes in both fecal and OP microbiota in IWCF. These changes led to alterations in known antibiotic resistance gene complements in OP samples that could impact the effects of subsequent antibiotic treatments. The clinical consequences of these off-target effects require further study, especially in the era of highly effective CF therapies.

Author Contributions

Hillary S. Hayden: writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, project administration, data curation. **Maria T. Nelson:** conceptualization,

methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Sydney E. Ross:** investigation, methodology, validation, writing – review and editing, resources. **Adrian J. Verster:** methodology, visualization, writing – review and editing, formal analysis, data curation. **Drake C. Bouzek:** visualization, writing – review and editing, formal analysis, data curation. **Alex Eng:** methodology, validation, writing – review and editing, formal analysis, project administration. **Adam Waalkes:** methodology, validation, writing – review and editing, formal analysis, project administration. **Kelsi Penewit:** methodology, validation, writing – review and editing, formal analysis, project administration. **Benjamin T. Kopp:** conceptualization, investigation, methodology, writing – review and editing, project administration, supervision. **Christopher Siracusa:** conceptualization, investigation, methodology, writing – review and editing, project administration, supervision. **Michael J. Rock:** investigation, writing – review and editing, resources. **Stephen J. Salipante:** methodology, validation, visualization, writing – review and editing, software, formal analysis, project administration, resources, supervision. **Lucas R. Hoffman:** conceptualization, investigation, funding acquisition, writing – original draft, methodology, writing – review and editing, project administration, supervision, resources, data curation. **Don B. Sanders:** conceptualization, investigation, funding acquisition, writing – original draft, methodology, writing – review and editing, project administration, data curation, supervision, resources.

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Ethics Statement

The study was approved by the Institutional Review Boards at Indiana University (IRB #1607531974), Nationwide Children's Hospital (IRB # IRB16-00800), Cincinnati Children's (IRB #2018-0721), and the University of Wisconsin (2015-0011).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All sequence read data generated for this research have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information under BioProject ID: PRJNA1015704.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.