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MAJOR ARTICLE

# Gastrointestinal Microbiome Disruption and Antibiotic-Associated Diarrhea in Children Receiving Antibiotic Therapy for Community-Acquired Pneumonia

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Antibiotic-associated diarrhea (AAD) is a common side effect of antibiotics. We examined the gastrointestinal microbiota in children treated with β-lactams for community-acquired pneumonia. Data were from 66 children (n = 198 samples), aged 6–71 months, enrolled in the SCOUT-CAP trial (NCT02891915). AAD was defined as ≥1 day of diarrhea. Stool samples were collected on study days 1, 6–10, and 19–25. Samples were analyzed using 16S ribosomal RNA gene sequencing to identify associations between patient characteristics, microbiota characteristics, and AAD (yes/no). Nineteen (29%) children developed AAD. Microbiota compositional profiles differed between AAD groups (permutational multivariate analysis of variance, $P < .03$) and across visits ($P < .001$). Children with higher baseline relative abundances of 2 *Bacteroides* species were less likely to experience AAD. Higher baseline abundance of Lachnospiraceae and amino acid biosynthesis pathways were associated with AAD. Children in the AAD group experienced prolonged dysbiosis ($P < .05$). Specific gastrointestinal microbiota profiles are associated with AAD in children.

**Keywords.** antibiotic-associated diarrhea; microbiota; children; community-acquired pneumonia.

Antibiotic-associated diarrhea (AAD) is the most common side effect of antibiotic use and occurs in up to 30% of individuals [1]. AAD is often defined as 1–3 loose stools in a 24- to 48-hour window following exposure to antibiotics [2, 3]. AAD contributes significantly to nonadherence to antibiotic therapy [4].

Antibiotics are an important cause of dysbiosis in the gastrointestinal (GI) microbiota [5, 6]. Antibiotic-induced perturbations include reductions in microbial diversity and altered metabolic pathways [6, 7]. Consequentially, AAD is often associated with reduced colonization resistance, which can lead to colonization and/or overgrowth of pathogenic microbes and long-term changes in microbiota structure [6, 8].

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The pathogenesis of AAD differs in children and adults [3]. *Clostridioides difficile* is a leading etiology of AAD in adults [2, 3], but it only accounts for 15%–30% of cases [9]. *Clostridioides difficile* testing is not routinely recommended in infants due to the high prevalence of asymptomatic *C. difficile* carriage [10]. Adult AAD cases have been associated with pathogens such as *Clostridium perfringens* and *Staphylococcus aureus* in addition to *C. difficile* [11]. Pediatric AAD symptoms may be caused by other infectious agents and also by disruption of homeostasis in the GI microbiota [3]. Antibiotic-induced changes in the GI microbiota may lead to loss of anaerobes that produce short-chain fatty acids and disruption of carbohydrate and bile metabolism with concomitant osmotic imbalance [12].

Studies examining relationships between antibiotics, microbiota disruption, and *C. difficile* have often focused on adults and/or evaluated the microbiota at a single time point [12, 13]. We leveraged samples and data from the Short-Course Outpatient Therapy for Community-Acquired Pneumonia in children (SCOUT-CAP) study to prospectively examine relationships between the GI microbiota, the duration of antibiotic treatment, and AAD in children [14]. SCOUT-CAP is

a multisite, randomized placebo-controlled study that compared short (5 days) vs standard (10 days) strategies of β-lactam therapy for pediatric community-acquired pneumonia in children 6 months to <6 years of age (ClinicalTrials.gov identifier NCT02891915) [14]. Our goals were to (1) describe the GI microbiota in children with and without AAD; (2) identify taxa associated with development of AAD; and (3) compare microbiota characteristics in children with and without AAD after the cessation of antibiotic treatment.

## MATERIALS AND METHODS

### Study Design and Participants

Subjects were included if they consented and enrolled in SCOUT-CAP and consented to the future use collection of stool samples. The institutional review boards of Yale University and Duke University approved the future use study. Study protocols for SCOUT-CAP are described elsewhere [14]. Children were approached and enrolled on days 3–6 of their initially prescribed β-lactam therapy. Participants were randomized 1:1 to a strategy of 5 days of matching placebo (short course) or 5 additional days of their initially prescribed prestudy antibiotic (standard course). Stool samples were collected at 3 time points: stool sample (SS) 1 (study day 1, which occurred on day 3–5 of β-lactam therapy), SS-2 (study day 6–10), and SS-3 at the end of the study (study day 19–25). SS-1 served as a comparable baseline for all study participants; participants received antibiotics for a similar length of time regardless of their strategy group assignment. SS-3 samples were used to represent the postantibiotic therapy state.

Of 380 children enrolled in SCOUT-CAP, 131 contributed stool samples. This study was restricted to 66 children who contributed stool samples for all 3 visits (198 stool samples in total). Participants were classified into 2 groups based on whether they experienced AAD during the study. Children were classified as experiencing AAD if they had ≥1 day of diarrhea during the study. The no AAD group were children who did not experience any diarrhea during the study.

### Fecal Specimen Processing and Sequencing

DNA was extracted from stool samples using the PureLink Microbiome DNA Purification Kit (Invitrogen, Carlsbad, California) and quantified using the Quant-iT Assay Kit (Invitrogen). From the extracted DNA, the hypervariable V4 region of the 16S ribosomal RNA (rRNA) gene was polymerase chain reaction amplified [15] and sequenced on the Illumina MiSeq platform using a paired-end 250 bp protocol with a PhiX control at the Yale Center for Genome Analysis.

### Bioinformatics Analysis

We used Btrim software to demultiplex the sequence reads and for trimming, sorting, and filtering low-quality sequence reads [16]. The quality-filtering process also included removing adaptors followed by dereplication and removing chimeras using USEARCH. We used USEARCH software to calculate α- and β-diversity indices and for taxonomic classification. Sequences were clustered into operational taxonomic units (OTUs) at a threshold value of 97% using the UPARSE-OTU algorithm and normalized to 10 000 reads per sample to create the OTU table [17].

### Statistical Analysis

Statistical analyses were conducted using R version 4.0.2 software. We first summarized baseline demographic and clinical characteristics by the AAD outcome (yes/no). To infer differences in characteristics between groups, the Wilcoxon rank-sum test was used for continuous data and $\chi^2$ test for categorical data (Fisher exact test when appropriate). Visualization plots were created using the ggplot2 R package [18] and downstream compositional microbiome analyses were done using phyloseq [19], vegan [20], and microbiome [21] R packages.

We used the Bray–Curtis distance metric, which considers both presence and abundance of features, to calculate β-diversity dissimilarity indices [22]. The calculated distance matrix was used to create ordination plots, stratified by AAD outcome groups and color coded to visualize temporal clustering, using nonmetric multidimensional scaling (NMDS) [23, 24]. The temporal clustering and microbiome dissimilarity of samples are represented by different colors and the degree of overlap across ellipses. Profile comparisons were then tested for statistical significance using permutational multivariate analysis of variance (PERMANOVA) iterated over 10 000 permutations [25]. The α-diversity indices calculated for each stool sample were Shannon (natural log) and inverse Simpson (1/Simpson) [26]. For each visit, we compared α-diversity levels between outcome groups at an $\alpha = .05$. An interaction term was added in the model to assess for potential effect modification. Given a significant *P* value for heterogeneity, we stratified by outcome groups and assessed the longitudinal trend of α-diversity using a linear mixed-effects model (lme4 R package [27]). In the model, subject ID was used as a random effect to account for repeated α-diversity measures per individual.

The high dimensionality of microbiome data presents a challenge for the reproducible elucidation of differentially abundant biomarkers associated with health and disease [28]. The best methods for differential abundance (DA) analysis are often debated; best practices include the use of 1 or more methods to gauge concordance between results and to counteract false positives [29]. We used 3 different DA analysis methods to identify potential discriminant taxa: random forest classification (randomForest R package) [30, 31], linear discriminant analysis effect size (LEfSe) [32], and analysis of compositions of microbiome with bias correction (ANCOM-BC) [33]. Prior to DA testing, we applied uniform filtering to remove all-zero OTUs and selected OTUs with a minimum prevalence of 10%

[34]. This process yielded 256 OTUs of 1676 total OTUs identified; the 1420 filtered OTUs were collectively categorized as "other" OTUs. The random forest approach ranks important OTUs based on their ability to discriminate between outcomes (ie, the AAD and no AAD groups). We used the default parameters of the "rfcv" function (with ntree = 2000) to estimate the minimum number of OTUs required for the best prediction of AAD status. Additionally, we performed 5-fold cross-validation to compensate for possible overfitting. This cross-validation function selects the best number of variables for the model based on the predicted model performance evaluated by the increase of mean-squared error for each number of OTUs tested. This resulted in the inclusion of 64 OTUs to train the final model. We then used a validated selection method, the varSelRF package, to quantify and select the most important OTUs that minimize out-of-bag errors [35]. Collectively, the final classification model selected the 4 most important OTUs. In LEfSe analysis, the linear discriminant analysis (LDA) score ($\log_{10}$ scale) of 3.0 was set as a threshold to identify differentially abundant taxa for each of the outcome groups. In the ANCOM-BC analysis, *P* values were corrected for multiple comparisons using the Benjamini–Hochberg procedure (*Q* value) with a *Q* value threshold of .05 [36].

We evaluated the independent association between baseline (ie, SS-1) microbiota composition (abundance of selected OTUs and α-diversity measures) and AAD (yes/no) in series of multiple logistic regression models (odds ratios with 95% confidence intervals). Covariates (age, sex, race, ethnicity, antibiotic type, SCOUT-CAP strategy group assignment, and antibiotic therapy duration) were assessed for confounding. A difference between adjusted and crude odds ratio >10% was considered a significant confounder [37]. We controlled for the SCOUT-CAP treatment strategy group (5-day vs 10-day) assignment regardless of its significance.

We used the PICRUSt2 pipeline to compare functional differences in the gut microbiota in the AAD and no AAD groups [38]. Pathways were filtered prior to DA analysis by removing all-zero pathways and a minimum prevalence threshold of 10%. DA analysis on PICRUSt2 predicted functional pathways was conducted using ANCOM-BC [33]. *P* values were adjusted for false discovery rate using the Benjamini–Hochberg procedure (*Q* values) with a threshold of .05 [36, 39].

## RESULTS

Participant characteristics are shown in Table 1. Characteristics differed from those of the 380 children in the SCOUT-CAP trial by age and race. Children in the current study tended to be younger; 31 (47%) were 6–23 months of age compared to 111 (29%) in SCOUT-CAP [14]. Fifty-two (79%) children in the current study were white compared with 234 (62%) who identified as white in SCOUT-CAP [14]. Among the study population, 19 (29%) experienced at least 1 diarrhea episode. The remaining 47 (71%) did not experience any diarrhea episodes. AAD vs no AAD groups were similar with the exception that the proportion of males was higher in the AAD group (Fisher exact test, $P = .03$). The proportion of children randomized to a short (5 days) vs standard (10 days) β-lactam treatment strategy did not significantly differ between groups.

**Table 1. Characteristics of the Study Population**[a]

| Characteristic | AAD (n = 19) | No AAD (n = 47) | *P* Value[b] |
|---|---|---|---|
| Age, mo, median (IQR) | 24 (18.5–34.5) | 25 (16.5–37.5) | .67 |
| Sex | | | .03 |
| Male | 15 (79) | 23 (49) | |
| Female | 4 (21) | 24 (51) | |
| Race | | | .56 |
| Asian | 0 (0) | 2 (4) | |
| Black | 3 (16) | 5 (11) | |
| White | 14 (74) | 38 (81) | |
| Multiple | 2 (10) | 2 (4) | |
| Ethnicity | | | .20 |
| Non-Hispanic or Latino | 17 (90) | 41 (87) | |
| Hispanic or Latino | 1 (5) | 6 (13) | |
| Not reported | 1 (5) | 0 (0) | |
| Strategy group | | | .94 |
| Short-course strategy | 8 (42) | 22 (47) | |
| Standard-course strategy | 11 (58) | 25 (53) | |
| Antibiotic therapy, d, median (IQR) | 9.0 (5.0–9.5) | 9.0 (5.0–10.0) | .75 |
| Diarrhea duration, d, median (IQR) | 3.0 (2.0–5.5) | … | |

Abbreviations: AAD, antibiotic-associated diarrhea; IQR, interquartile range.

[a]Values are represented as No. (column %) unless otherwise indicated.

[b]*P* values were calculated by Wilcoxon rank-sum test for continuous data, and $\chi^2$ test for categorical data (unless any value was <5, then Fisher exact test was used).

The median duration of diarrhea was 3 days. Figure 1 shows the timing of diarrhea events experienced by the AAD group. Diarrhea occurred sporadically throughout the study ranging from enrollment to 20 days postenrollment.

#### Microbiota Profiles in AAD and No AAD Groups

Stool samples were collected at 3 time points, SS-1, SS-2, and SS-3; an NMDS ordination plot of Bray–Curtis distance in stool samples is shown in Figure 2. The lack of overlap in ellipses, observed in the AAD group, suggests that these samples are more dissimilar and less stable over time in comparison to samples from children in the no AAD group. These observations are supported by statistical analyses, which identified a statistically significant difference in the compositional profile of the GI microbiota by outcome (ie, AAD yes/no) (PERMANOVA, $P = .03$) and also across stool samples (PERMANOVA, $P < .001$). The observed differences were not due to heterogeneity in variances (multivariate homogeneity of groups dispersions, $P > .05$), which indicates that the observed differences were due to differences in the compositional profile of the microbiota.

#### Temporal Comparison of α-Diversity Measures Between Outcomes

Next, we investigated temporal trends in α-diversity. Figure 3A shows median α-diversity indices stratified by AAD (yes/no) at each stool sample collection. There were no significant differences in Shannon diversity (Supplementary Figure 1) or inverse Simpson indices ($P > .05$) at baseline (SS-1) or at SS-2. However, significant differences in α-diversity were observed between the AAD and no AAD groups at the end of the study, SS-3. We performed effect modification analyses to test for differences in longitudinal trends in α-diversity between the outcome groups. A significant $P$ value for heterogeneity confirmed the presence of effect modification (interaction term, $P < .01$). To further visualize the observed heterogeneity, we performed subgroup analyses. Figure 3B shows the longitudinal trends in α-diversity separately for each of the AAD outcomes. Collectively, these data suggest that the longitudinal trends in α-diversity differed significantly between the AAD groups (Figure 3B), and AAD-experiencing children had a prolonged dysbiosis reflected by significantly lower levels of α-diversity at SS-3.

#### Identification of Key Taxa Associated With the Development of AAD

Next, we determined whether the relative abundance of specific OTUs differed between the AAD and no AAD groups at baseline (ie, SS-1). Based on the results of the 5-fold cross-validation method, 64 OTUs were included in the final random forest classification model (Supplementary Figure 2). The final model

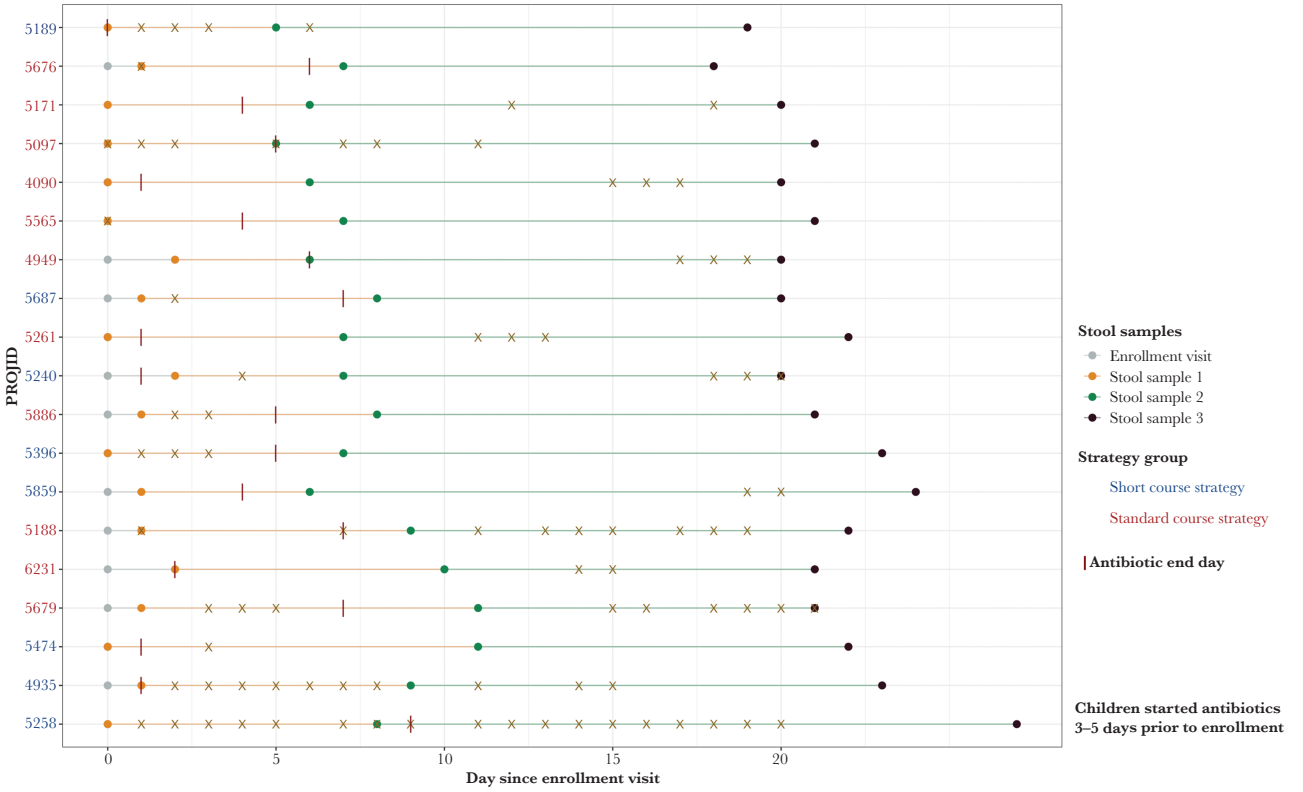


**Figure 1.** Study timeline of children experiencing antibiotic-associated diarrhea (AAD). Timeline showing 57 samples (circles) taken from 19 children who experienced AAD. Each PROJID represents a patient from the group and each circle represents stool sample collection. Every diarrheal episode experienced during study period is marked with a "X" and end of antibiotic therapy is represented with a red line (|).

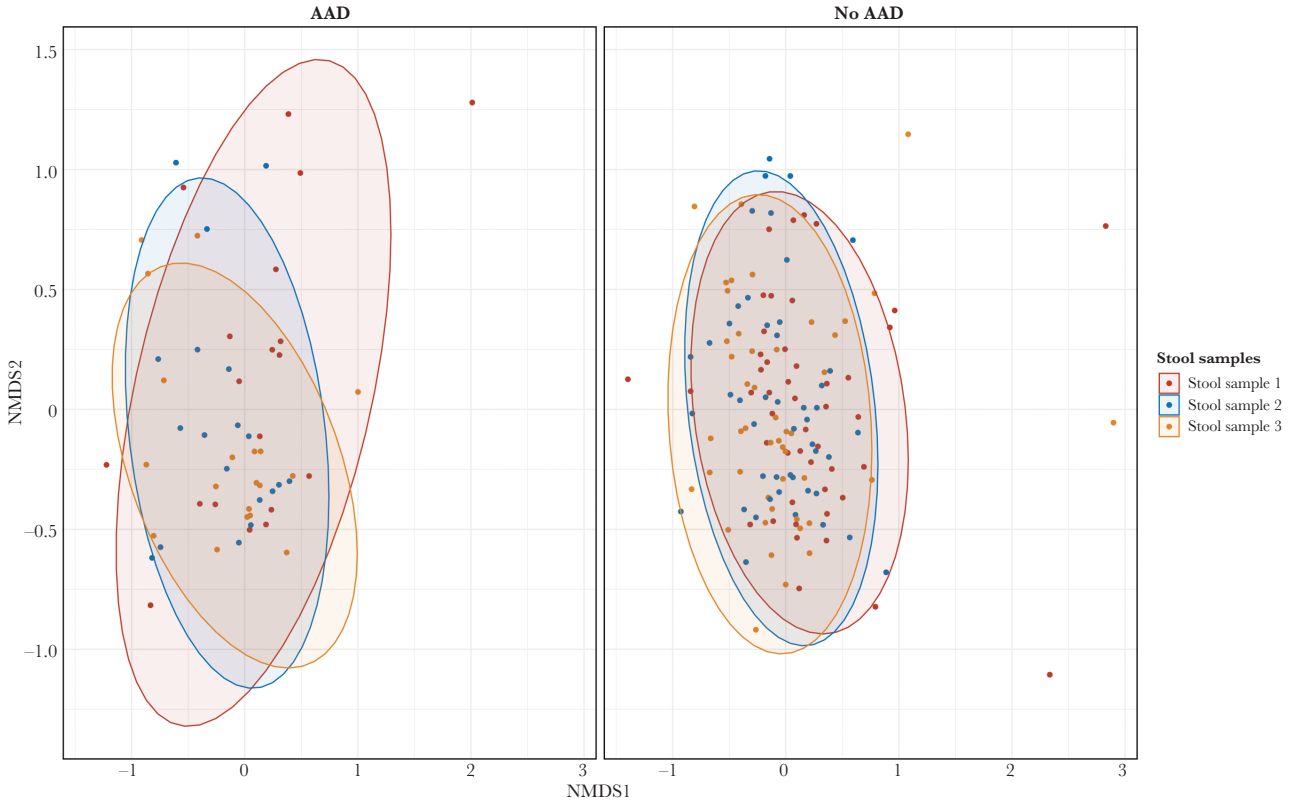


**Figure 2.** Overall microbiota profile: gastrointestinal microbiota comparison between children with and without antibiotic-associated diarrhea (AAD). Nonmetric multidimensional scaling (NMDS) ordination plot of Bray–Curtis β-diversity at the operational taxonomic unit level for each outcome group. NMDS plots are used to ordinate multidimensional microbiome data condensed into 2 dimensions. NMDS1 and NMDS2 represent the 2 axes in this ordination plot and each dot represents a sample colored by stool sample collection number.

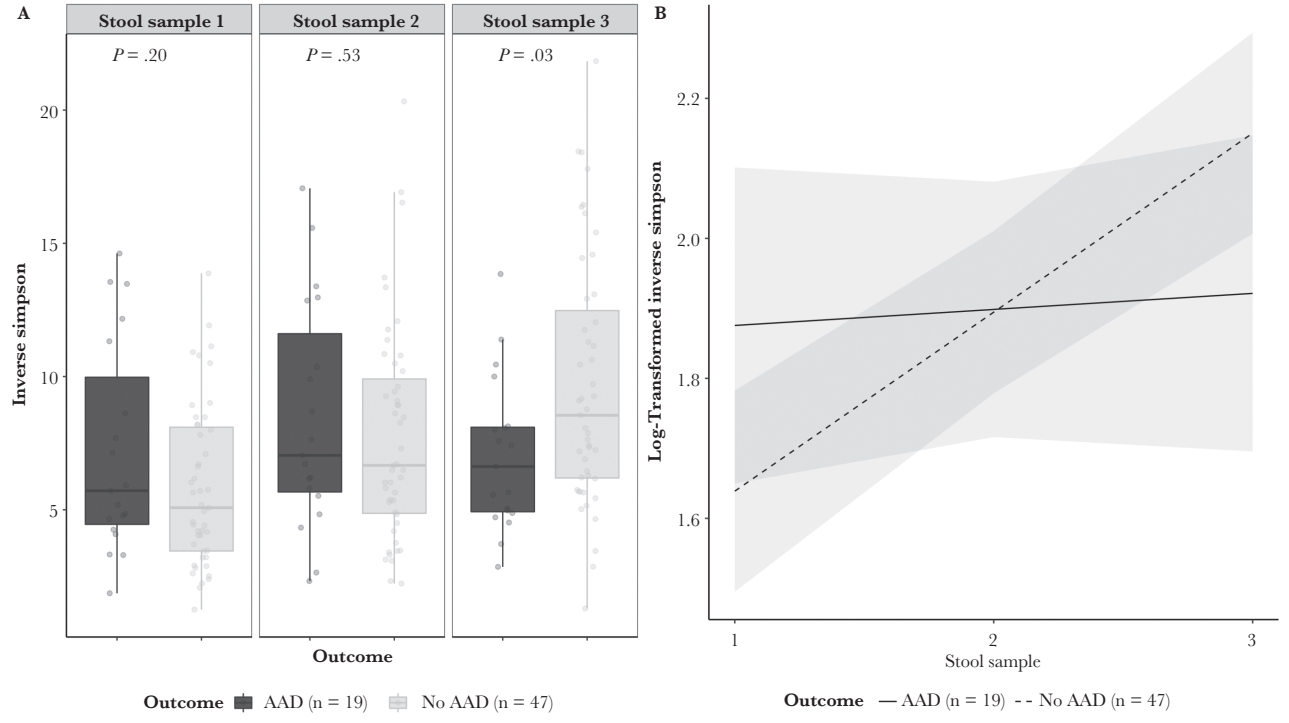


**Figure 3.** 16S α-diversity by antibiotic-associated diarrhea (AAD) outcome and visit. *A*, Inverse Simpson index comparison at each stool sample collection. Each grid represents a collection. Statistical significance of differences between groups were determined using a 2-sided Wilcoxon rank-sum test. *B*, Subgroup analyses of longitudinal α-diversity trend separated by AAD outcomes. The AAD group is designated by a solid line and the no AAD group is shown with a dashed line.

was trained using these 64 most relevant OTUs (Figure 4) and we identified the top 4 AAD (yes/no) distinguishing OTUs; *Bacteroides* species (OTU806), *Bacteroides fragilis* (OTU28), *Blautia* species (OTU36), and Lachnospiraceae (OTU1357) (Supplementary Figure 3). LEfSe analysis identified 8 OTUs whose relative abundance differed when comparing AAD outcome groups (Supplementary Figure 4). The relative abundances of 5 OTUs were higher in the AAD group. These taxa were *Escherichia fergusonii* (OTU9), *Finegoldia magna* (OTU326), *Megamonas funiformis* (OTU53), Lachnospiraceae (OTU1357), and *Erysipelotrichales* (OTU39). Taxa of the phylum Firmicute (OTU368), *Bacteroides* species (OTU806), and *B. fragilis* (OTU28) were more abundant in the no AAD group. Last, we conducted an ANCOM-BC DA analysis (Supplementary Figure 5). Two OTUs, *B. fragilis* (OTU28) and *Bifidobacterium* (OTU739), were identified as differentially abundant between AAD groups; both OTUs were positively associated with the no AAD group.

The ANCOM-BC method is conservative and produces consistent results across studies [29]. Therefore, all ANCOM-BC identified taxa were included in the downstream analyses. To counteract the possibility of overlooking true positives, we included OTUs concordantly identified by both random forest and LEfSe. The final list of potential discriminatory OTUs included for downstream analyses were *Bacteroides* species (OTU806) and *B. fragilis* (OTU28), Lachnospiraceae (OTU1357), and *Bifidobacterium* (OTU739).

In Figure 5, we show the temporal trends of the identified OTUs by outcome groups. At baseline, higher levels of Lachnospiraceae were associated with the AAD group.

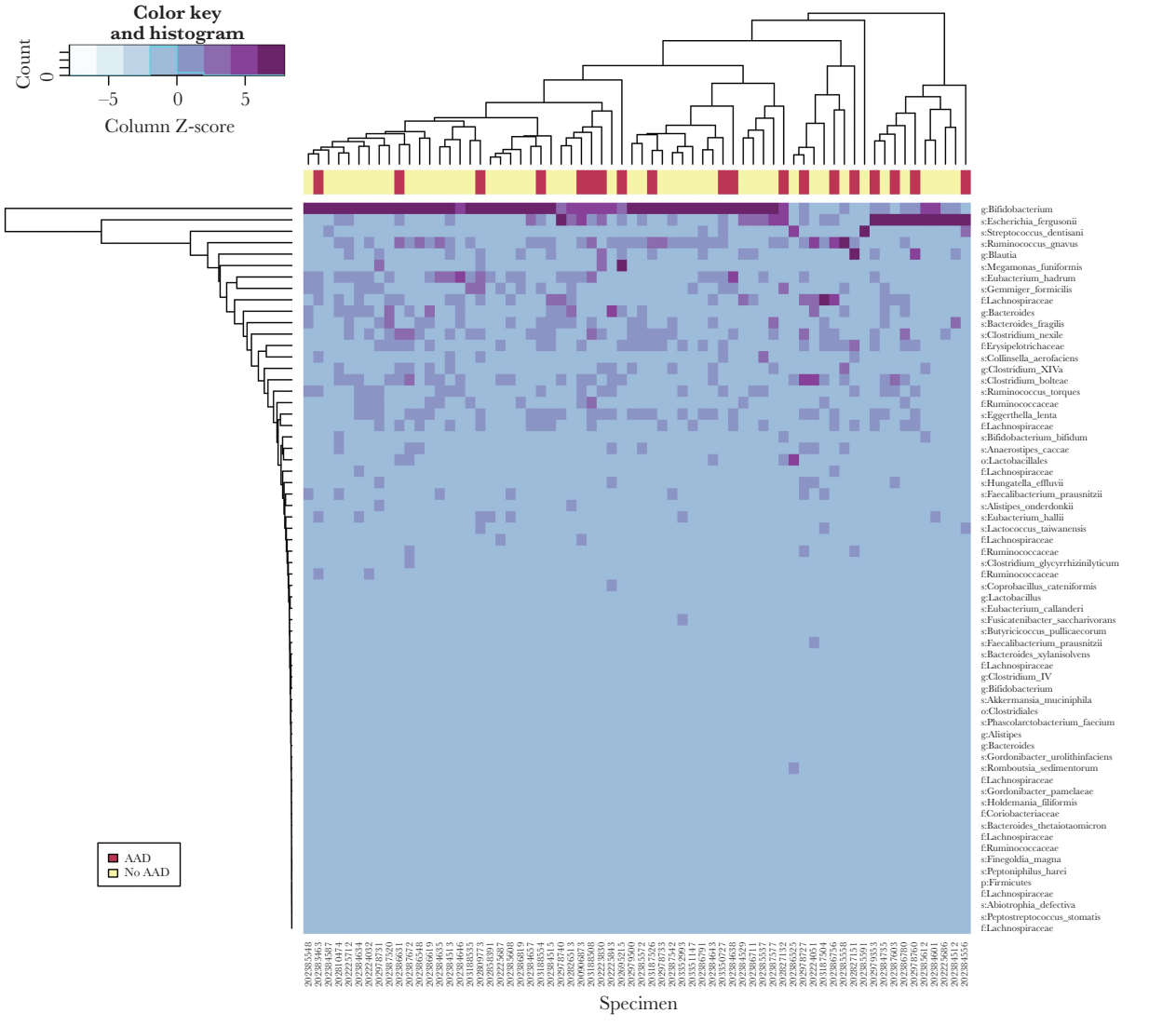


**Figure 4.** Heatmap comparing the $\log_2$ transformed relative abundances of top 64 most relevant operational taxonomic units (OTUs) at stool sample 1. Antibiotic associated diarrhea (AAD) groups are shown in the color bar and the corresponding color key is shown in the lower-left corner.

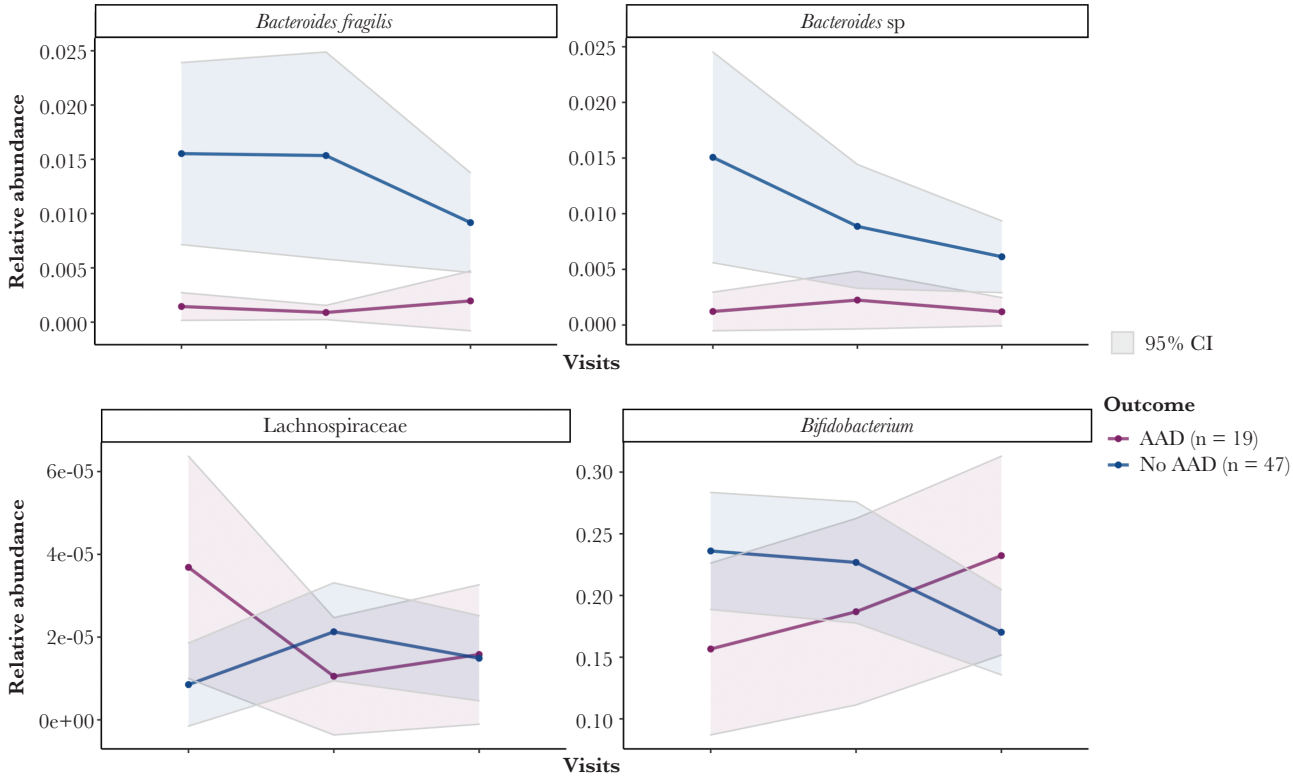


**Figure 5.** Longitudinal trends of four important operational taxonomic units (OTUs) identified to distinguish between the antibiotic-associated diarrhea groups at baseline enrollment visit. Abbreviations: AAD, antibiotic-associated diarrhea; CI, confidence interval.

Elevated levels of all other OTUs were associated with the no AAD group.

#### Logistic Model Building Using Diversity and Taxonomic Information

At baseline SS-1, logistic regression models were used to evaluate potential associations between diversity, abundance of specific taxa, and the development of AAD. Table 2 shows the odds ratio predicting AAD per increment unit increase in relative abundance and α-diversity. At baseline, the relative abundance of 3 taxa discriminated between the outcome groups. Higher relative abundances of *Bacteroides* OTUs (OTU806 and OTU28) were associated with lower odds of AAD, and Lachnospiraceae (OTU1357) were associated with higher odds of AAD. The 2 baseline α-diversity measures and *Bifidobacterium* species (OTU739) were not significantly associated with AAD.

#### PICRUSt2 Comparison of Functional Capacity

We performed functional pathway profiling to accompany our taxonomic comparisons at each time point using 335 identified pathways. DA analysis of functional pathways identified 5 statistically significant PICRUSt2-predicted MetaCyc pathways at the baseline SS-1 (Supplementary Figure 6). Children in the AAD group had higher relative abundances of NAD salvage pathway II ($Q = .04$), thiazole biosynthesis II pathway ($Q = .04$), biosynthesis superpathways of polyamine ($Q = .04$), L-phenylalanine ($Q = .01$), and L-tyrosine ($Q = .01$). None of the pathways were differentially abundant at later timepoints in the SS-2 and SS-3 samples.

## DISCUSSION

Our study examined longitudinal trends in the GI microbiota in children who did and did not experience AAD. All participants were prescribed β-lactam therapy for the treatment of community-acquired pneumonia. Comparisons of the baseline sample SS-1 identified both taxonomic and functional features associated with AAD. Higher relative abundances of *Bacteroides* OTUs were inversely associated with AAD, which suggests a protective role. Additionally, the GI microbiome of the AAD group showed elevated levels of amino acid biosynthesis pathways at SS-1. The α-diversity levels at SS-1 were not a significant predictor of AAD. Analysis of SS-3 indicated that children in the AAD group had evidence of greater microbiome disruption at the end of the study (ie, SS-3).

Microbiome datasets are considered high-dimensional data due to the large number of OTUs detected in the samples. Having fewer subjects than OTUs often introduces substantial sparsity (multiple zeroes due to absence of taxa in most samples), and dimension reduction helps overcome this challenge [28, 40]. We used 3 different dimensionality reduction methods. Across multiple microbial composition analyses, 2 members of the genus *Bacteroides*, including the species *B.*

**Table 2. Odds Ratios and 95% Confidence Intervals From Separate Logistic Regression Models Predicting Antibiotic-Associated Diarrhea Outcome for Each of the Important Baseline Operational Taxonomic Units and α-Diversity Measures**

| OTU | Range | Adjusted OR (95% CI)[a] | Increment[b] |
|---|---|---|---|
| *Bacteroides fragilis* | 0.00–1451.00 | **0.37 (.11–.79)** | 50 |
| *Bacteroides* species | 0.00–1976.00 | **0.59 (.24–.90)** | 50 |
| *Bifidobacterium* species | 0.00–5823.00 | 0.98 (.96–1.01) | 50 |
| Lachnospiraceae | 0.00–2.00 | **3.76 (1.06–13.28)** | 1 |
| Shannon diversity (natural log) | 0.57–3.22 | 1.05 (.95–1.15) | 0.1 |
| Inverse Simpson | 1.25–14.62 | 1.13 (.96–1.33) | 1 |

Abbreviations: CI, confidence interval; OR, odds ratio; OTU, operational taxonomic unit.

[a]OR (95% CI) per increment unit increase. Values in bold represent statistically significant values. ORs are adjusted for treatment strategy group assignment. No other covariates tested were considered a confounder.

[b]Coefficient multiplier. ORs reflect the change in odds per increment unit increase in predictor variables.

*fragilis*, were consistently identified as differentially abundant in the 2 AAD outcome groups. The logistic regression model further confirmed this observation; higher relative abundance of *Bacteroides* species and *B. fragilis* was positively associated with the no AAD outcome group across all time points (Figure 5).

These data are consistent with the literature. *Bacteroides* species are associated with increased microbiome diversity and stability [41]. The protective role of *B. fragilis* in AAD has been demonstrated in a murine model of AAD; oral administration of nonenterotoxigenic *B. fragilis* resulted in a reduction in diarrhea symptoms [42]. Our data are also consistent with a 16S rRNA gene profiling study in adults that compared stool samples from participants with non–*C. difficile* diarrhea to healthy controls; controls had higher levels of *Bacteroides* [13]. *Bacteroides* species play an important role in gut homeostasis [43]. For example, *B. fragilis* secretes polysaccharide A, which has been shown to activate and train immune responses, help maintain gut homeostasis, and enhance host–microbiota communication in the intestinal epithelium [44, 45].

The relative abundances of *Bifidobacterium* species and Lachnospiraceae were also associated with AAD. However, there was less robust evidence for these OTU groups when compared to *Bacteroides* species. DA analyses suggested that *Bifidobacterium* species play a protective role, and these taxa are found in the healthy microbiome [2, 13]. However, baseline levels of *Bifidobacterium* were not significantly related to AAD in our logistic regression models. Lachnospiraceae species had higher relative abundances in the AAD group. Relative abundance of *Blautia*, a species of the family Lachnospiraceae, was shown to be higher in patients with non–*C. difficile* diarrhea compared to healthy controls [13] and also among mice with AAD [46]. The relative abundances of *Bifidobacterium* and Lachnospiraceae were not consistently higher in one group or the other over time (Figure 5). The fluctuation of relative abundances over time is a common challenge in longitudinal microbiome studies and is due to relationships between an individual species' relative abundance and the composition of the microbiota [22]. Since relative abundances sum to 1, the relative abundance of an individual species is negatively correlated with an increase in the abundance of other species.

We supplemented the taxonomic data with predictions of functional capacity. Both functional pathways with the highest significance ($Q = .01$) belong to the proteinogenic amino acid biosynthesis pathway class. This finding was consistent with data from murine models, which showed that mice with experimental AAD had elevated levels of amino acid metabolism [47, 48].

Administration of antibiotics is associated with lowered microbial diversity [49]. Our data suggest that AAD-experiencing children had prolonged dysbiosis in the gut microbiota reflected by low levels of α-diversity. Heterogeneity in the longitudinal trends of α-diversity between the outcome groups (Figure 3B) suggested that the gut microbiome of children who do not experience AAD rebounded more quickly from the disrupted baseline state after cessation of antibiotic therapy. Although initial α-diversity levels at SS-1 and SS-2 were similar between groups, α-diversity levels at SS-3 differed significantly between the outcome groups. The observed differences at SS-3 support the findings of a previous study that noted spontaneous recovery after 21 days postcessation [50].

The children with AAD experienced a range of patterns of diarrhea (Figure 1). Some children experienced diarrhea while on antibiotics and others experienced diarrhea afterward. Moreover, some experienced episodes of short duration and others experienced prolonged diarrhea. Supplementary Figure 7 shows relationships between the abundance of individual taxa and the timing of diarrhea in children with AAD. The small number of samples precluded meaningful statistical comparisons among the various subgroups.

Strengths of our study include the temporal evaluation of pediatric microbiome markers during and after β-lactam therapy. Our study also had several limitations. We restricted our study to subjects with stool samples across all 3 time points. This reduced our sample size and may have resulted in the overrepresentation of diarrhea cases. Our analysis

had a total of 19 (29%) subjects in the AAD group; in comparison, 31 (24%) of 131 subjects who contributed at least 1 stool sample in the SCOUT-CAP study experienced diarrhea. Consequentially, this small sample size may have contributed to type II statistical error. Finally, we did not have stool samples prior to the start of antibiotic treatment, which may have further strengthened the temporal comparisons. However, the SS-1 samples served as a comparable baseline for both outcome groups.

We identified differences in the gastrointestinal microbiome of children who did and did not experience AAD during their β-lactam therapy for community-acquired pneumonia. Baseline levels of several potentially protective taxa and levels of amino acid biosynthesis pathways distinguished children who did and did not experience AAD. Further studies are needed to investigate whether similar trends are observed across different antibiotic types. The identified potentially protective taxa may inform the development of preventive approaches for AAD. Moreover, taxa associated with risk of AAD could be studied further as biomarkers of risk of disease.

### Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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