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Impact of antibiotics on off-target infant gut microbiota and resistance genes in cohort studies

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BACKGROUND: Young children are frequently exposed to antibiotics, with the potential for collateral consequences to the gut microbiome. The impact of antibiotic exposures to off-target microbes (i.e., bacteria not targeted by treatment) and antibiotic resistance genes (ARGs) is poorly understood.
METHODS: We used metagenomic sequencing data from paired stool samples collected prior to antibiotic exposure and at 1 year from over 200 infants and a difference-in-differences approach to assess the relationship between subsequent exposures and the abundance or compositional diversity of microbes and ARGs while adjusting for covariates.
RESULTS: By 1 year, the abundance of multiple species and ARGs differed by antibiotic exposure. Compared to infants never exposed to antibiotics, *Bacteroides vulgatus* relative abundance increased by 1.72% (95% CI: 0.19, 3.24) while *Bacteroides fragilis* decreased by 1.56% (95% CI: −4.32, 1.21). *Bifidobacterium* species also exhibited opposing trends. ARGs associated with exposure included class A beta-lactamase gene *CfxA6*. Among infants attending day care, *Escherichia coli* and ARG abundance were both positively associated with antibiotic use.
CONCLUSION: Novel findings, including the importance of day care attendance, were identified through considering microbiome data at baseline and post-intervention. Thus, our study design and approach have important implications for future studies evaluating the unintended impacts of antibiotics.

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IMPACT:

- The impact of antibiotic exposure to off-target microbes and antibiotic resistance genes in the gut is poorly defined.
- We quantified these impacts in two cohort studies using a difference-in-differences approach. Novel to microbiome studies, we used pre/post-antibiotic data to emulate a randomized controlled trial.
- Compared to infants unexposed to antibiotics between baseline and 1 year, the relative abundance of multiple off-target species and antibiotic resistance genes was altered.
- Infants who attended day care and were exposed to antibiotics within the first year had a higher abundance of *Escherichia coli* and antibiotic resistance genes; a novel finding warranting further investigation.

INTRODUCTION

Infants and young children are exposed to more antibiotics than any other age group[1,2] with an estimated 30–40% of children receiving at least one course of antibiotics within their first year of life.[3–7] Although most of these antibiotics are prescribed for acute respiratory tract infections including acute otitis media,[8,9] an unintended consequence of antibiotic use is its impact on microbes other than the targeted pathogen.[10,11] Understanding antibiotics' impacts on off-target or "bystander" microbes in the infant gut, in particular, is significant for two main public health

reasons.[11] First, systemic antibiotics can result in microbial dysbiosis as the developing infant gut is sensitive to perturbation.[12] Encompassing both taxonomic and metabolic changes, antibiotics and resulting dysbiosis have been associated with negative health outcomes in young children including overweight/obesity,[13–16] asthma,[7,17] and celiac disease.[18] Second, antibiotic exposures can lead to antimicrobial resistance. This is a particular concern in the human gut, a known reservoir for antimicrobial resistance genes (ARGs).[19,20] Indeed, multiple studies have found that antibiotic exposures lead to the proliferation of

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ARGs that confer resistance to the antibiotic prescribed and to other antibiotics.[21–24]

Mitigation of antimicrobial resistance is a global health priority as the incidence of infections requiring second or third-line broad-spectrum antibiotics for treatment is on the rise,[25] leading to further risk of antibiotic-resistant microbial infections. Integrated strategies that consider the microbiome are increasingly becoming useful for improving the surveillance of ARGs and providing clinical recommendations to prevent antimicrobial-resistant infections.[26] Commensal bacteria can harbor ARGs and carriage prevalence of potentially pathogenic microbes can lead to antimicrobial-resistant infections. Thus, there is an urgent need to conduct epidemiologic studies and build quantitative models to understand how antibiotics affect off-target microbes, antimicrobial resistance, and unintended health outcomes.

The goal of the current study was to quantify the population-level effects of antibiotics on the abundance and diversity of off-target microbes and ARGs. Specifically, we use pre/post-antibiotic data to estimate average population-level changes to individual microbes, ARGs, and compositional diversity metrics emulating a randomized controlled trial. This difference-in-differences approach is frequently used in health services and policy research but has not been previously applied to microbiome studies.[27] The advantage of this design is that it uses baseline information (i.e., data from before the intervention) to account for intra-subject variation over time and a comparison group to assess differences among those exposed and unexposed to an intervention. This approach is equivalent to assessing the interaction effect between the exposed group and the post-intervention time point.[28] As antibiotics are often prescribed for respiratory illnesses and ear infections unnecessarily,[8,9,29] this study offers insight that can support antibiotic stewardship practices in light of the effect of antibiotics on commensal gut microbes and ARGs.

METHODS
Study cohorts
The New Hampshire Birth Cohort Study (NHBCS) is an ongoing prospective cohort study of over 2250 pregnant women and their young children from New Hampshire and Vermont. A detailed description of the cohort has been published,[30–32] but, in brief, enrollment began in 2009 to study the effects of environmental exposures on pregnant women and young children. Extensive data are available for the first year of life and include delivery and pediatric medical records, as well as interview data for 4, 8, and 12 months post-delivery. Stool samples have been collected and undergone shotgun sequencing from a subset of children throughout the first year of life but predominantly at the 6-week and 1-year timepoints. Pregnant women and their children were recruited using the NHBCS's current Dartmouth Institutional Review Board-approved procedures by the Center for the Protection of Human Subjects.

The DIABIMMUNE Study is a cohort of infants in Finland, Estonia, and Russia focused on the hygiene or microbiota hypothesis and its potential role in the development of autoimmune diseases.[33] We used data from infants in a DIABIMMUNE sub-study that aimed to examine the role antibiotics play in microbiome development over the first 3 years of life.[23] The sub-study included 39 infants from Finland having either 0 or at least 9 antibiotic exposures within their first 3 years of life. Children in this cohort had stool samples collected throughout their early life starting at about 2 months of age. We accessed quality-controlled shotgun sequencing FASTQ files and covariate data through their publicly available website.[34]

Antibiotic exposure classification
NHBCS. We were interested in systemic antibiotic exposures between the 6-week and 1-year timepoints (to match available stool samples) with a goal of studying both exposure (yes/no) and the frequency of antibiotic courses given to all infants. A unique advantage of the NHBCS is that antibiotic prescriptions, including indication, are captured in both medical records and caregiver questionnaires administered at 4, 8, and 12 months of age.

As outlined in Supplementary Fig. S1, not all infants with stool samples available had full medical record or interview data over the first year of life. Therefore, we considered two sub-cohorts for assessing antibiotic exposure. The first cohort, the NHBCS Antibiotic Exposure Cohort, classifies antibiotic exposure from medical record and/or interview data. This cohort was used to assess differences between antibiotic exposure versus non-exposure. In this dataset, we have overall less precise knowledge of the timing of antibiotics (i.e., the exact day of antibiotic prescription) but were able to maximize the sample size. Our second cohort, the NHBCS Antibiotic Frequency Cohort, only uses antibiotic prescription data from medical records enabling us to know the number and exact timing of antibiotics prescribed. The schema, sensitivity analysis, assumptions, and sample sizes for these two sub-cohorts are available in the Supplementary Notes.

DIABIMMUNE Study. To increase the external validity of this study and include subjects that were known to have a relatively high number of antibiotic exposures, infants from the DIABIMMUNE Study cohort were included. Antibiotic exposures in the DIABIMMUNE Study were collected through parental reporting of antibiotic use, duration, and reason for use as described previously.[33] Infants from the DIABIMMUNE Study were added to NHBCS sub-cohorts to make combined cohorts, referred to respectively as the Antibiotic Exposure and Antibiotic Frequency Cohorts (Supplementary Fig. S1).

Stool microbiome assessment
Details on stool collection and metagenomic DNA shotgun sequencing have previously been described for the NHBCS cohort[31,35] and the DIABIMMUNE Study group.[23,36] For all samples, taxonomy down to the species level was assigned using MetaPhlAn3[37] and quantified using ShortBRED[38] with ARGs derived from the Comprehensive Antibiotic Resistance Database (CARD) v3.1.1.[39] In CARD, model names are sometimes annotated by microbes that they have been identified in, but this may not necessarily reflect the microbial origin. Information on the ARGs profiled is available in Supplementary Table S1. Additional details are discussed in the Supplementary Notes.

Statistical analyses
All statistical analyses were performed using R version 3.6.0,[40] with additional details on overall ARG abundance and correlation analysis in the Supplementary Notes.

Assessing individual off-target microbes and antibiotic resistance genes. The difference-in-differences approach is frequently used in health services and policy research to estimate the effect of change due to an intervention or policy on a population when a randomized controlled trial is not possible or ethical.[27] Assumptions required for the difference-in-differences approach[27,28] were assessed prior to use.

The difference-in-difference estimate was performed using Microbiome Multivariable Associations with Linear Models (MaAsLin2).[41] Our general formula was:

$$\text{Abundance} \sim (\text{intercept}) + \text{sample age(in days)} + \text{antibiotic exposure}$$
$$+ \text{antibiotic exposure} \times \text{sampling interval (baseline vs 1 year)}$$
$$+ \text{covariates} + (1|\text{infant})$$

Where abundance is the relative abundance of the microbe or ARG in each sample, sample age is the time in days of the sample collection, antibiotic exposure is a dichotomous indicator of if the infant was exposed or not exposed to an antibiotic ever, and sampling interval is a binary variable used to denote that the measurement was from baseline (~ 6 weeks or 2 months) or 1-year. Antibiotic exposure × sampling interval represents the difference-in-difference estimate or interaction between exposure and time interval. We considered intercept random effects at the infant level (1|infant).

RESULTS
Study groups
We used rigorous classification rules (Supplementary Notes) to retrospectively assess antibiotic exposure and frequency in 238 infants in the NHBCS that had shotgun sequencing data from stool samples collected at both ~6-week and ~1-year timepoints

(Supplementary Fig. S1). Ultimately, we assigned 183 infants from the NHBCS as exposed or unexposed to antibiotics for a specific condition based on interview and/or medical record data (the NHBCS Antibiotic Exposure Cohort). A group of 99 infants could be classified by antibiotic frequency based on medical record data (NHBCS Antibiotic Frequency Cohort). Sensitivity analyses were used to evaluate exposure misclassification between medical record and interview data, but we found high concordance between the two methods (Supplementary Notes). In addition to infants participating in the NHBCS, we were able to classify the antibiotic exposure and frequency profiles of 33 infants with stool samples collected at approximately 2 months and 1 year from the DIABIMMUNE Study.[23]

Descriptive characteristics of infants and samples
Comparisons between the DIABIMMUNE and NHBCS Antibiotic Exposure and Antibiotic Frequency sub-cohorts indicated that the proportions of infants that were female and that were born full-term were similar (Table 1, χ^2 p value > 0.1). Infants from the DIABIMMUNE Study were more likely than infants from at least one NHBCS sub-cohort to be vaginally delivered (χ^2 p value < 0.1). The average baseline age and days breastfeeding of infants from the DIABIMMUNE Study was approximately 20 days greater than that of infants from the NHBCS (Kruskal–Wallis p value < 0.1).

The majority of antibiotic exposures occurred after 6 months of age and for otitis media (Fig. 1). As expected based on the inclusion criteria that DIABIMMUNE Study infants would have high exposure to antibiotics over the first 3 years of life, infants in the DIABIMMUNE Study were more frequently exposed to antibiotics and, when exposed, were given more antibiotics than infants in the NHBCS. Amoxicillin-type antibiotics (e.g., amoxicillin, amoxicillin with clavulanate) were the most commonly prescribed antibiotics among all infants. The alpha diversity of samples did vary by antibiotic exposure both before and after exposure (Supplementary Notes).

Abundance of off-target microbes and antibiotic resistance genes
Since all antibiotic exposures were for non-gut-associated infections, we classified all microbes as off-target. Looking specifically at antibiotic-exposed versus unexposed infants from the NHBCS and the DIABIMMUNE Studies (Fig. 2a and Supplementary Table S2), we found the relative abundance change for two microbes was significantly different than zero. In particular, the relative abundance of *Bacteroides vulgatus* across the two time-points increased by 1.72% (95% CI: 0.19, 3.24) more than it would have if the infant was never exposed to an antibiotic, while the relative abundance of *Actinomyces odontolyticus* decreased by 0.008% (95% CI: 0.015, −0.001). Relative abundance changes based on antibiotic exposure went in opposing directions for *Bacteroides* and *Bifidobacterium* species. While *B. vulgatus* abundance increased, *Bacteroides fragilis* decreased by 1.42% (95% CI: −3.06, 0.23). Similarly, *Bifidobacterium bifidum* relative abundance increased 1.41% (95% CI: −0.44, 3.27) more than it would have if the infant was never exposed, but *Bifidobacterium longum* and *Bifidobacterium breve* decreased by 1.56% (95% CI: −4.32, 1.21) and 1.14% (95% CI: −3.55, 1.27) respectively.

As day care attendance increases an infant's exposure to infections[42–44] and is time variant (see Supplementary Notes), we stratified NHBCS infants in the Antibiotic Exposure Cohort by day care attendance by 1 year to attempt to separate the effect of day care attendance and antibiotic exposure on off-target microbes (Fig. 2b–d). Some microbe associations were consistent in both day care strata (i.e., no evidence of effect modification). However, among infants who attended day care, the relative abundance of *Escherichia coli* and *Veillonella parvula* was considerably higher among infants exposed to antibiotics compared to those who were unexposed, suggesting a synergistic interaction between

antibiotic exposure and day care attendance towards the abundance of certain microbes.

We also assessed the impact of antibiotic exposure on changes in ARGs over the follow-up period (Fig. 3). The most significant change occurred for the Antibiotic Resistance Ontology marker (ARO): 3003097 *CfxA6*, which increased on average by 15.26 reads per kilobase of reference sequence per million samples reads (RPKM) (95% CI: 5.33, 25.20) more than if the infant was not exposed to antibiotics (Fig. 3a and Supplementary Table S3). Similar results were found among infants only in the NHBCS (Fig. 3b). Results stratified by day care attendance were discordant for many ARGs. Among infants that did not attend day care by 1 year (Fig. 3c), *CfxA6* was increased among infants exposed to antibiotics, whereas a different trend in ARG abundance change was identified among infants that attended day care by 1 year. While *CfxA6* was no longer associated with antibiotic exposure, many other ARGs were positively associated such as *mdtN* (ARO: 3003548), *tetM* (ARO: 3000186), and *mdtO* (ARO: 3003549) (Fig. 3d).

To investigate this further, we looked at overall ARG abundance in the same infants (Supplementary Table S4). In the Antibiotic Exposure Cohort, there was no statistically significant mean ARG difference by antibiotic exposure between timepoints (516.16 RPKM increase in overall RPKM load among infants exposed to an antibiotic, 95% CI: −904.61, 1936.92) after adjustment for covariates. However, among infants who attended day care in the NHBCS, ARGs increased on average by 2305.54 RPKM (95% CI: −379.80, 4990.87) more in infants exposed to antibiotics than those who were not. For all analyses, while only a few ARGs changed significantly across the two timepoints, we found that the majority of the estimated mean differences for individual ARGs was greater than 0 suggesting a greater net RPKM trend increase among infants exposed to antibiotics.

To jointly evaluate associations noted in the off-target microbe and ARG analyses, we conducted a block correlation analysis using Hierarchical All-against-All association (HAllA) testing. Across the 41 species and 55 ARGs analyzed, 111 pairs and 47 clusters were identified as having a statistically significant correlation (Benjamini–Hochberg q value < 0.05) among infants unexposed to antibiotics and 60 pairs and 19 clusters for exposed infants with 49 pairwise associations overlapping (Supplementary Tables S5 and S6). Across infants both unexposed and exposed to antibiotics, *E. coli* was highly associated positively and negatively with many ARGs (Fig. 4). Results further stratified by day care attendance were similar (Supplementary Fig. S2).

DISCUSSION
Overall findings
We evaluated the off-target effects of antibiotic exposures on the infant gut microbiome and resistome among a large number of infants from the United States and Finland. Using metagenomic sequencing of pre/post-antibiotic stool samples combined with detailed covariate information from two separate cohorts, we identified and quantified the population-level impact antibiotics have on off-target microbes and ARGs within the infant gut. We found that even with a broad categorization of antibiotic exposures between approximately 6 weeks and 1 year of life, antibiotics were associated with changes to off-target microbe and ARG abundance.

In the NHBCS Antibiotic Exposure Cohort, we found that 34.4% of infants were exposed to antibiotics between approximately 6 weeks and 1 year of life with 58 out of the 63 (92.1%) antibiotic exposures prescribed for otitis media. This largely correlates with other studies from similar demographic groups that have estimated approximately 30–40% of infants are exposed to antibiotics in the first year.[3–7] Current protocols in the United States recommend antibiotics for most otitis media and lower

Table 1. Descriptive overview of infants in the study.

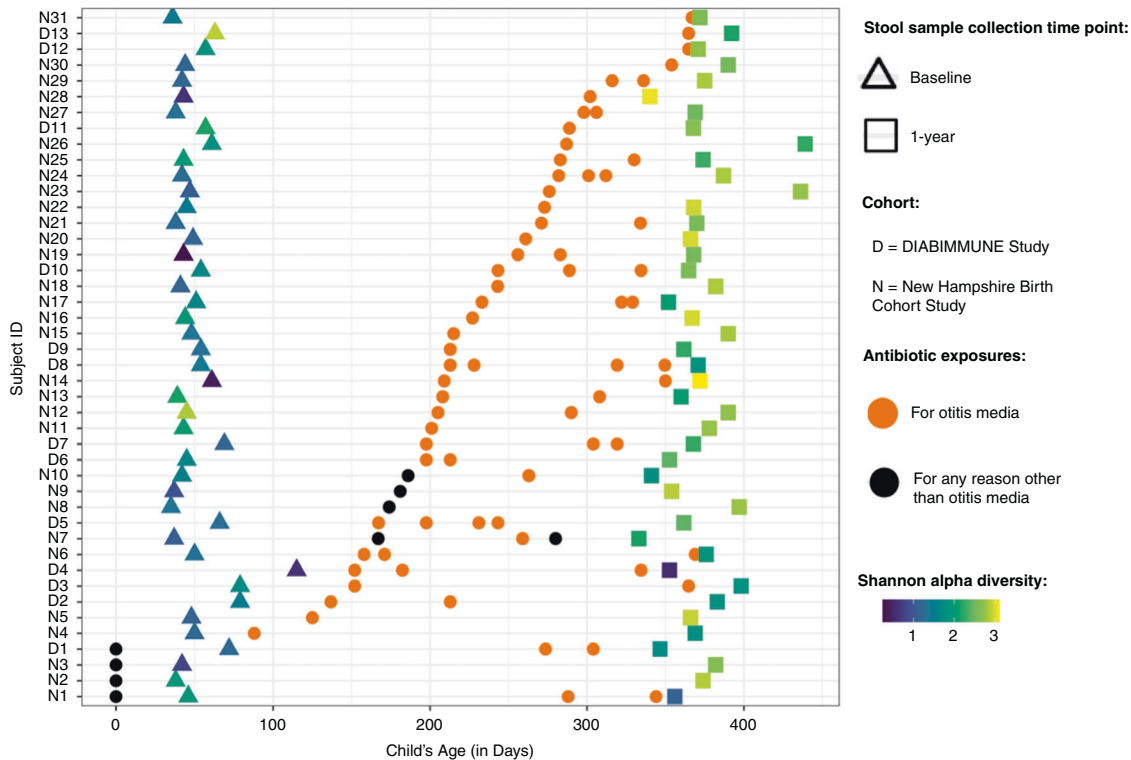|	New Hampshire Birth Cohort Study (NHBCS) Antibiotic Exposure Cohort (n = 183)	NHBCS Antibiotic Frequency Cohort (n = 99)	DIABIMMUNE Study (n = 33)
Antibiotic classification derivation	Combination of medical record review and parental recall	Medical record review	Parental recall
Mean (SD) sample age in days	Baseline: 46 (15)	Baseline: 45 (16)	Baseline: 64 (15)
	1 year: 371 (29)	1 year: 376 (29)	1 year: 366.6 (15)
Female	70 (38.3%)	40 (40.4%)	14 (42.4%)
Vaginal delivery	131 (71.6%)	67 (67.7%)	29 (87.9%)
Mean (SD) days breastfeeding by sample collection	Baseline[a]: 43.0 (17.7)	Baseline[a]: 41.8 (18.9)	Baseline: 64 (15)
	1 year: 247 (133)	1 year: 238 (140)	1 year: 281 (99)
Attend day care by 1 year	Yes: 76 (41.5%)	Yes: 38 (38.4%)	N/A
	No: 91 (49.7%)	No: 45 (45.5%)	
	Missing: 16 (8.7%)	Missing: 16 (16.2%)	
Full-term (at least 37 weeks' gestation)	171 (93.4%)	91 (92.0%)	31 (94.0%)
Parity	0: 89 (48.6%)	0: 44 (44.4%)	N/A
	1: 62 (33.9%)	1: 41 (41.4%)	
	2+: 26 (14.2%)	2+: 13 (13.1%)	
	Missing: 6 (3.3%)	Missing: 1 (1.0%)	
Maternal education level	High school or equivalent: 16 (8.7%)	High school or equivalent: 9 (9.1%)	N/A
	Junior college, technical school, or some college: 26 (14.2%)	Junior college, technical school, or some college: 15 (15.2%)	
	College: 67 (36.6%)	College: 38 (38.4%)	
	Post-graduate: 71 (38.8%)	Post-graduate: 36 (36.4%)	
	Missing: 3 (1.6%)	Missing: 1 (1.0%)	
Antibiotic exposure immediately following birth	6 (3.3%)	3 (3.0%)	1 (3.0%)
	Missing: 3 (1.6%)		
Number of antibiotic prescriptions between 6 weeks and 1 year	N/A	For otitis media: 42	For otitis media: 36
		Not otitis media[b]: 5	Not otitis media: 0
Children with antibiotic exposure between 6 weeks and 1 year	For otitis media: 58 (31.7%)	For otitis media: 27 (27.3%)	For otitis media or any condition: 13 (39.4%)
	For any condition[c]: 63 (34.4%)	For any condition: 29 (29.3%)	
Number of antibiotics for otitis media given to child	N/A	0: 72	0: 20
		1: 15	1: 3
		2+: 12	2+: 10
Number of antibiotics for any reason given to child between baseline and 1-year time point	N/A	0: 70	0: 20
		1: 15	1: 3
		2+: 14	2+: 10
Number of prescriptions by antibiotic type between baseline and 1-year time point	N/A	Amoxicillin-type: 37	Amoxicillin-type: 20
		Cephalosporin-type: 8	Cephalosporin-type: 7
		Azithromycin: 2	Azithromycin: 5
			Trimetoprime and sulfadiazine: 3
			Unknown: 1

[a]One infant included in both NHBCS sub-cohorts did not have available breastfeeding information at baseline.
[b]Other reasons included: upper respiratory infection (2), pneumonia (1), bronchitis (1), and reaction to pneumococcal vaccine (1).
[c]Other reasons included: upper respiratory infection (2), eye infection (1), pneumonia (1), and strep throat (1).

respiratory tract infections but not for upper respiratory infections.[45,46] Due to challenges in diagnosis, gastrointestinal consequences of antibiotics, and concern for the growing threat of antimicrobial resistance, many country-level antibiotic stewardship protocols encourage watchful waiting.[47] Accordingly, randomized controlled trials[29,48] and population-level cross-sectional studies[49,50] have examined delayed, shortened, or no antibiotic therapy for uncomplicated otitis media and respiratory infections.[29,48] While these studies are critical, cohort studies with combined covariate and microbiome data also yield valuable evidence in support of antibiotic stewardship practices because they reflect current treatment practices. Thus, while this study's main goal was to provide a population-level overview of the impact of antibiotics to infant gut microbiota and the resistome, it

Fig. 1 Descriptive overview of antibiotic exposures among New Hampshire Birth Cohort and DIABIMMUNE Study infants exposed to antibiotics in the Antibiotic Frequency Cohort. Figure only includes antibiotic exposures occurring before the 1-year stool sample collection. Samples are ordered by first antibiotic exposure. Baseline samples were collected at approximately 6 weeks for New Hampshire Birth Cohort Study participants and 2 months for DIABIMMUNE Study infants. Age of antibiotic exposure reflects the start day of antibiotic exposure. Infants with antibiotic exposures prior to the baseline microbiome collection received them immediately following birth (age 0 days).

also provides public health professionals and clinicians with quantifiable results that may be applicable to antibiotic steward-ship recommendations.

Antibiotic exposure differentially impacts commensal microbes

Commensal microbes have many roles in the infant gut microbiome that include: (1) degrading polysaccharides into useable sugars and short-chain fatty acids for other gut microbes[51,52] and (2) inhibiting pathogen colonization directly through nutrient competition and bacteria-bacteria attacks[53] as well as indirectly by stimulating the development of cells necessary for innate and adaptive immunity.[54] Thus, decreased presence or abundance due to antibiotic exposure is a major concern. However, previous studies inconsistently identify particular commensal microbial abundances that have increased or decreased as a result of antibiotic exposure and often only profile results at the genus level.[55] While the majority of our results did not indicate statistically significant differences between antibiotic-exposed and unexposed infants, our results justified the need to evaluate differences below the genus level especially for *Bifidobacterium* and *Bacteroides*.

Bifidobacterium species exhibited conflicting trends in our study. The relative abundance of two species, *B. longum* and *B. breve*, consistently decreased in the antibiotic-exposed population. However, *B. bifidum* increased in relative abundance after antibiotic exposure. This trend suggests that antibiotic exposure may lead to long-term selection changes to favor certain species of *Bifidobacterium*. Limited studies have specifically assessed *Bifidobacterium* species,[55] but a prior study in 1.5-year-old children found that *B. bifidum* concentration decreased by 23% in amoxicillin exposed children compared to 54.5% in unexposed children while *B. longum* concentration remained consistent immediately following exposure.[56] Differences identified between

studies may be a result of differences in population demographics including the age of the children as well as the time period post antibiotic exposure.

We consistently identified that *B. vulgatus* relative abundance increased in antibiotic-exposed children but *B. fragilis* decreased. These results are interesting in context to the DIABIMMUNE Study that identified *B. fragilis* as a "single-colonization species" and *B. vulgatus* as a "multiple-colonization species" based on the dominance of one vs. multiple strains across time.[23] Species with multiple strains in an environment are thought to contribute to a more resilient microbiome.[57,58] While we did not assess strain diversity and at least one previous study suggests these species may have a similar sensitivity to amoxicillin-based antibiotics,[59] our results underscore the need to consider differential sensitivity to antibiotics as a result of strain-level diversity, differences in biogeographical niches,[60] and variation in ARG carriage by species. Regarding ARG carriage, the CARD continually tracks the prevalence of antibiotic resistance phenotypic profiles in reference sequences for many non-pathogenic microbes. According to the CARD prevalence 3.0.9 update, there is wide variability in ARG phenotypes between commensals from the same and different genera.[39] These data, in combination with the results from this study and others assessing antimicrobial susceptibility in bacteria often considered off-target,[61,62] provide further evidence that intra- and inter-species variation is important to consider.

Describing differential patterns of antimicrobial resistance abundance

Multiple studies have assessed how antibiotic exposure after the neonatal period impact the resistome[21–23,63–67] but have been conducted in predominantly preterm infants[21,22] or contained relatively small cohorts (<30 infants) of antibiotic-exposed infants.[23,63–66] We found two distinct patterns in our study, but,
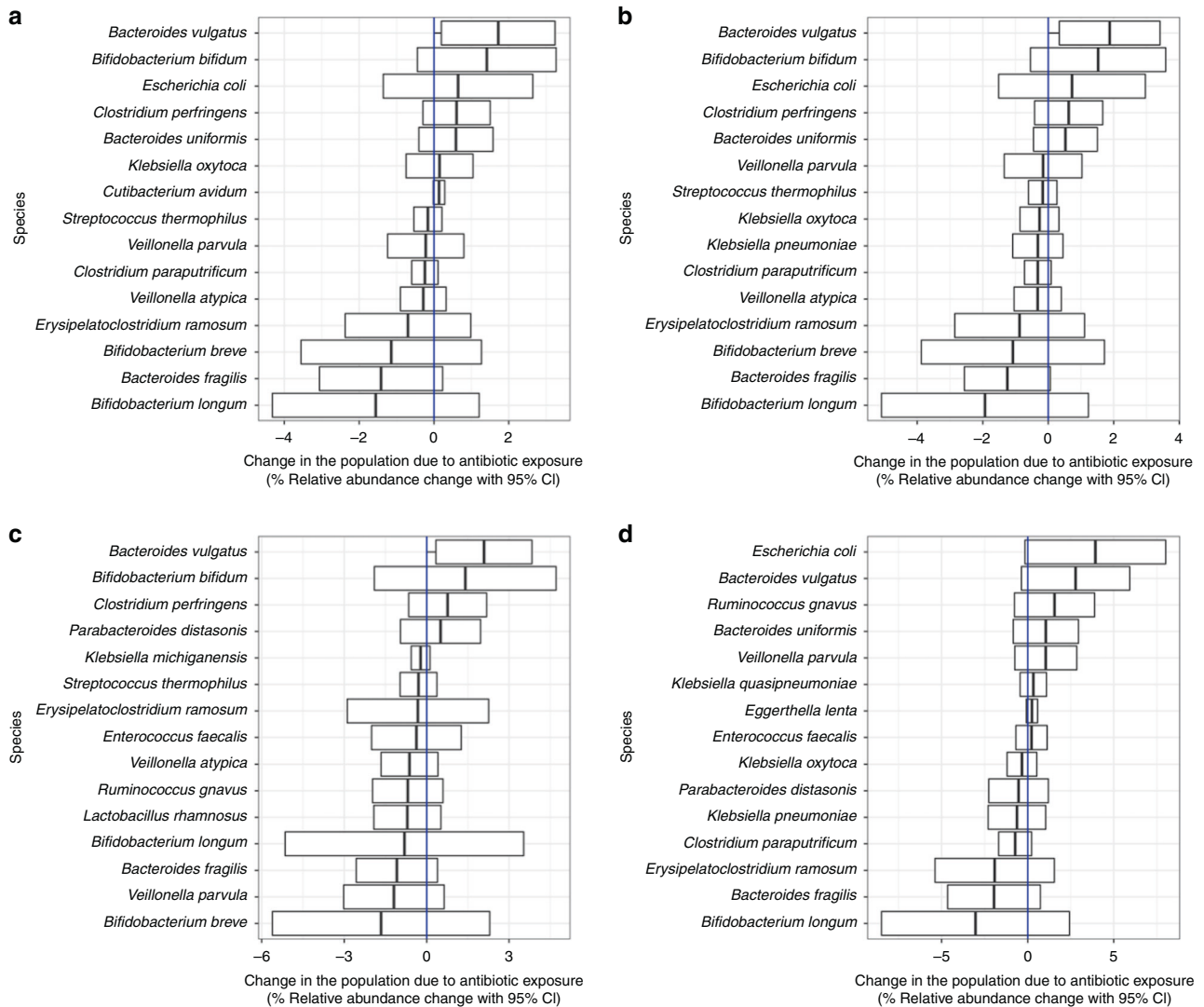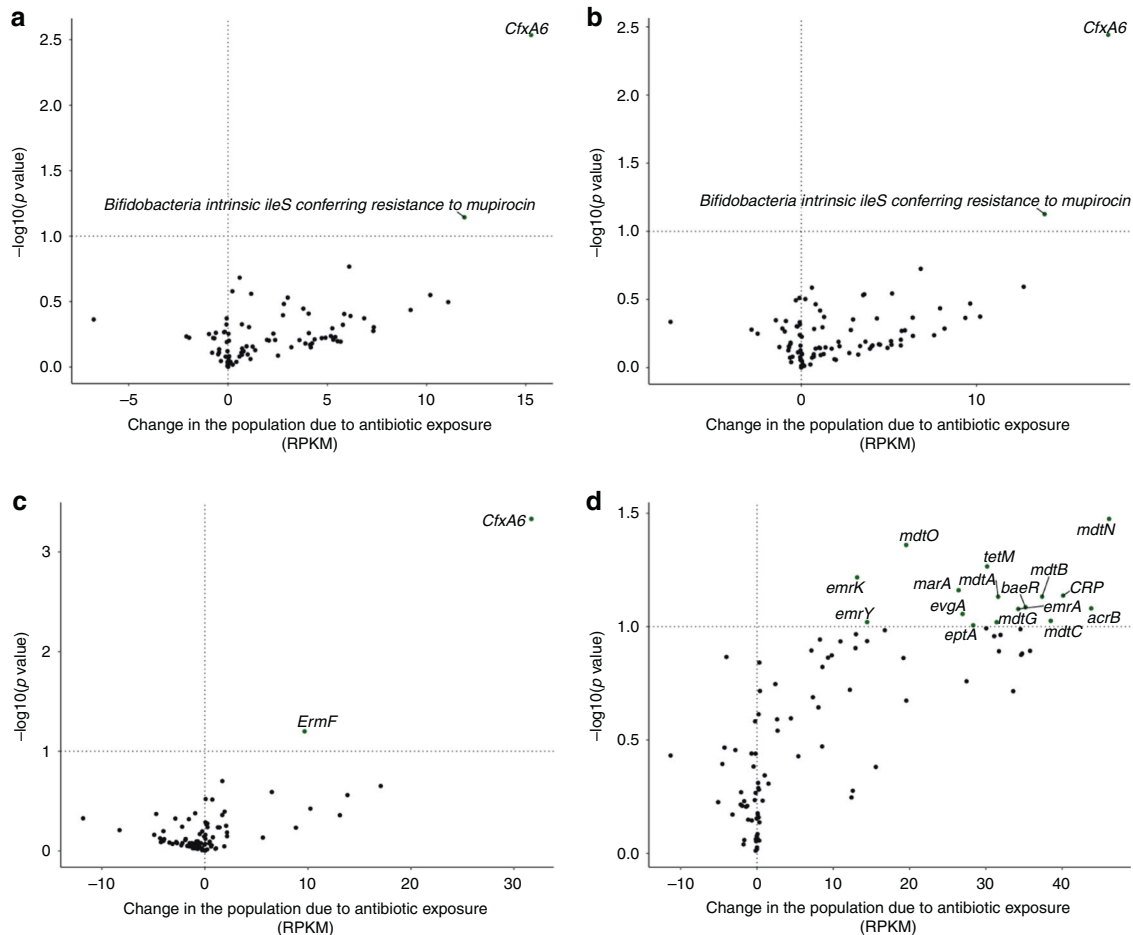
Fig. 2 Off-target species changes in the population due to any antibiotic exposure. a Change in the population due to antibiotic exposure in the Antibiotic Exposure Cohort ($n = 216$). The difference-in-difference model for the relative abundance change between exposed and unexposed infants across the two timepoints was adjusted for sample age, duration of breastfeeding, delivery mode, sex, gestational age, antibiotic use immediately following birth, study cohort, and a random effect for each subject. **b** Change in the population due to antibiotic exposure in NHBCS infants ($n = 183$). **c** Change in the population due to antibiotic exposure in NHBCS infants that did not attend day care by 1 year ($n = 91$). **d** Change in the population due to antibiotic exposure in NHBCS infants that did attend day care by 1 year ($n = 76$). **b** was adjusted for the same variables as **a** with the exception of study cohort and addition of day care attendance by 1 year. **c** and **d** models were adjusted for the same variables as **b** except for day care attendance by 1 year. For all plots, the 15 species with the greatest absolute change are shown. Species are ordered by the point estimate for the relative abundance change.

regardless of antibiotic exposure, found that *E. coli* was the microbe most frequently correlated with ARGs (Fig. 4 and Supplementary Fig. S2).

In our Antibiotic Exposure Cohort, only ARO: 3003730 *Bifido-bacteria intrinsic ileS* conferring resistance to mupirocin and *CfxA6* were statistically significantly increased among antibiotic-exposed infants (Fig. 3a). According to the current version of CARD resistomes (v3.0.9), ARO: 3003730, works through antibiotic target alteration and has only been found in whole-genome sequences of *B. longum*.[39] As we found that *B. longum* abundance decreased when this ARG increased (Fig. 2), this provides some evidence that this ARG may be associated with multiple species and requires follow-up studies. While we could not identify specific species hosts for *CfxA6*, three studies[21,23,68] also identified *CfxA6* beta-lactamase gene and one study[67] identified *CfxA2* (another *CfxA* class A beta-lactamase gene[69]) as ARG markers of interest. *CfxA* genes work via antibiotic inactivation[39] and have

been identified in a wide range of microbes from Bacillota (formally the phylum Firmicutes) and Bacteroidota (Bacteroidetes) including *Bacteroides*, *Prevotella*, and *Capnocytophaga* with point mutations differing between *CfxA2* and *CfxA6*.[67,69–71] The DIA-BIMMUNE Study found the *CfxA6* gene marker persisted in children long after antibiotic exposure.[23] Although some of these infants were included in our study, we independently found that the gene increased in abundance in infants in the NHBCS only (Fig. 3b). The most comparable study to ours demonstrated in 662 Danish children that antibiotic exposures during the first year of life impacted the gut resistome of healthy infants.[67] In particular, they found time since antibiotic exposure and number of antibiotics influential to overall ARG abundance. In addition, they identified specific ARGs including *CfxA2* and *APH(3')-IIIa* to be increased among infants exposed to a beta-lactam inhibitor compared to infants not exposed to any antibiotic. For *CfxA2*, they found that it was also increased among infants given another

Fig. 3 Volcano plots demonstrating abundance of antibiotic resistance gene changes in the population due to any antibiotic exposure in RPKM. Line marks p value < 0.1. **a** Change in the population due to antibiotic exposure in the Antibiotic Exposure Cohort ($n = 216$). The difference-in-difference model for the relative abundance change between exposed and unexposed infants across the two timepoints was adjusted for sample age, breastfeeding duration, delivery mode, sex, gestational age, antibiotic use immediately following birth, study cohort, and a random effect for each subject. **b** Change in the population due to antibiotic exposure in NHBCS infants ($n = 183$). **c** Change in the population due to antibiotic exposure in NHBCS infants that did not attend day care by 1 year ($n = 91$). **d** Change in the population due to antibiotic exposure in NHBCS infants that did attend day care by 1 year ($n = 76$). **b** was adjusted for the same variables as **a** with the exception of study cohort and addition of day care attendance by 1 year. **c** and **d** models were adjusted for the same variables as **b** except for day care attendance by one year. Only antibiotic resistance genes with a baseline prevalence greater than 20% within each population are included in the plot. RPKM reads per kilobase of reference sequence per million samples reads.

antibiotic compared to no antibiotic exposure. Although they did not have resistome measurements taken before antibiotic exposures to establish baseline ARG abundances, the results of our study aligned with these results and those presented previously in our cohort.[35] In addition, a study of healthy adults exposed to a 7-day treatment of cefprozil found specific point mutations in *CfxA6* were enriched in multiple participants[68] and another study identified *CfxA6* to be an important discriminator of gestational age.[21] Given all this data, further detailed investigations of the *CfxA6* gene at the sequence level are warranted.

Among infants who attended day care by 1 year, we found that multiple ARGs and the overall resistance load were comparatively much higher among infants exposed to antibiotics (Fig. 3d). *E. coli* relative abundance increased in antibiotic-exposed infants (Fig. 2d) suggesting a synergistic interaction between antibiotic exposure and day care attendance. Although we were unable to identify other studies that assessed both antibiotic exposure and day care attendance, given *E. coli*'s role in horizontal gene transfer[72,73] and previous studies in the NHBCS and others noting their association with harboring antibiotic resistance genes,[21,35,67,74,75] the corresponding changes to resistome composition aligned with changes to *E. coli* relative abundance.

Strengths and limitations
While this study is one of the largest to consider the population-level impact of antibiotic exposures to the infant gut over a follow-up period, we note some important limitations. Our main limitation was the precise timing and dosage of antibiotic exposures in the NHBCS Antibiotic Exposure Cohort. To mitigate this, we utilized stool samples from the DIABIMMUNE Study as a validation cohort since it obtained both precise timing and dose of antibiotic exposures (Supplementary Notes). We also used exposure data with more precision on a smaller sample group of infants via a medical record review to assess timing and type of antibiotic exposure. Although statistical tools have used mixed-effects models to assess microbiome composition longitudinally,[41,76] the difference-in-differences approach offers a straight-forward interpretation of the effect of an intervention in a population through its strong counterfactual estimate of the effect of eliminating unnecessary antibiotic exposures on the infant gut microbiome. Moreover, the epidemiologic framework provides the flexibility for researchers to estimate the difference-in-differences using a variety of transformations, statistical packages, and tools. A major consideration, however, in formally applying the difference-in-difference approach to microbiome analyses is fully addressing inherent difference-in-
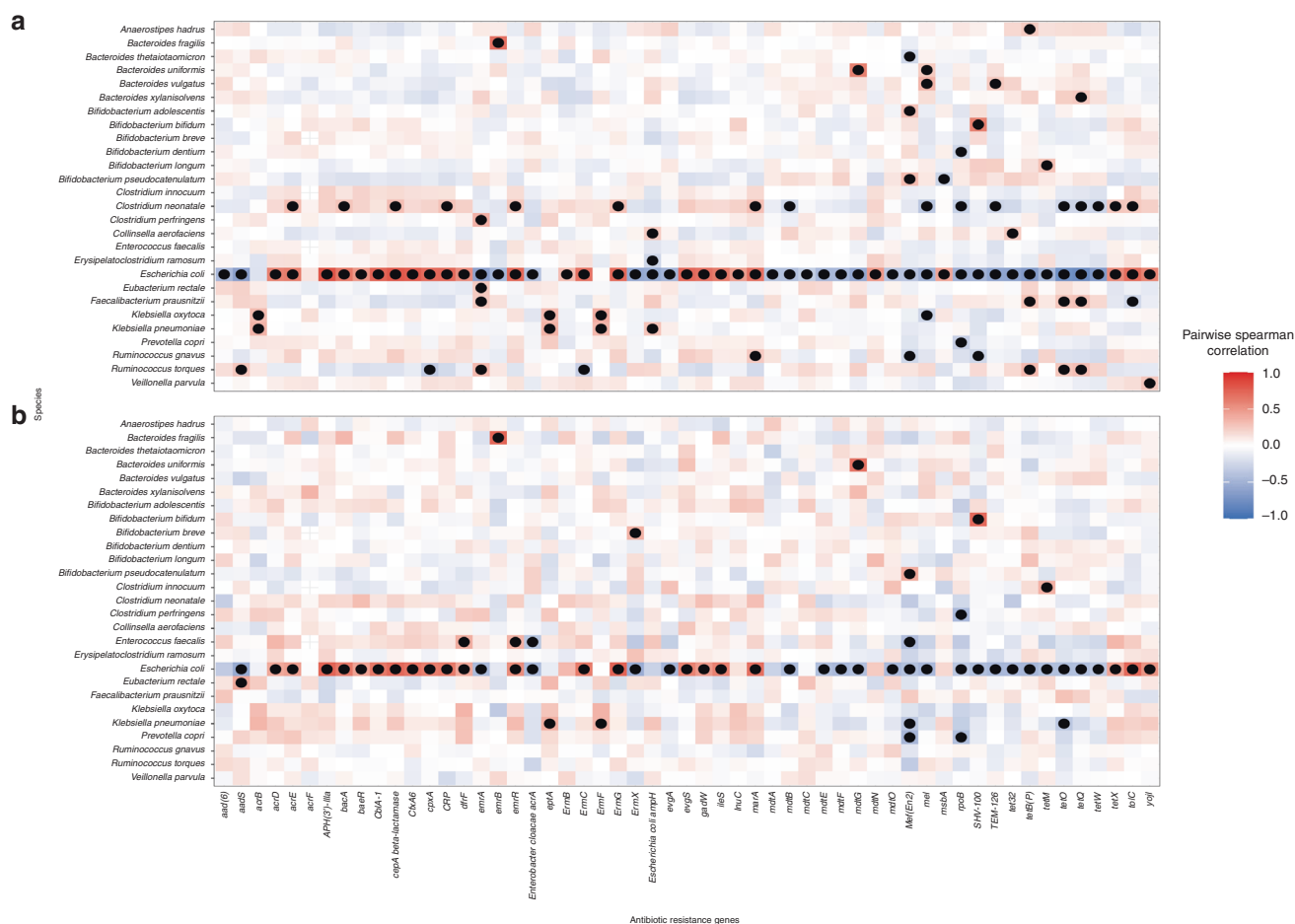
Fig. 4 Pairwise spearman correlation between species and antibiotic resistance genes by antibiotic exposure. a Infants not exposed (*n* = 140) and **b** exposed to antibiotics (*n* = 76). HAllA was used to determine pairwise associations that were statistically significant. Only species and antibiotic resistance genes with at least one statistically significant pairwise association were included in the plot. Dots represent statistically significant associations (Benjamini–Hochberg adjusted *p* value < 0.05). Due to length of gene-associated model names, *ileS* is used as an abbreviation for *Bifidobacteria* intrinsic *ileS* conferring resistance to mupirocin and *rpoB* is used for *Bifidobacterium rpoB* conferring resistance to rifampicin.

difference assumptions.[27,28] For instance, in this analysis, we were unable to fully account for the common trend assumption due to only having two timepoints to assess metagenomic data. However, in the process of validating our assumptions, sensitivity analysis revealed that antibiotic exposure did differentially impact some microbes and ARGs in infants depending on their day care status; a novel finding. Lastly, an important caveat to much microbiome data is inherent limitations in accurately reflecting the composition of gut microbiota and ARGs using stool samples. Thus, while we did identify the relative abundance of microbes and ARGs changing in our differential abundance analyses, we cannot rule out that the change would affect the absolute abundance, rule out unmeasured confounding, nor fully predict changes in function or the gut itself.[77] Regardless, misclassification likely would be non-differential by antibiotic exposure and we still can conclude that the infant gut microbiome of antibiotic-exposed and unexposed infants is different.

CONCLUSION
Our results support the notion that microbiota and the resistome should be considered when weighing the costs and benefits of antibiotic interventions. Moreover, we apply an established framework used to emulate a randomized controlled trial to the microbiome space. This enabled quantification of the magnitude of the population-level effects of antibiotic exposure in the context of other pressures impacting the developing microbiome in early life. We imagine that this novel application can be used to evaluate the potential impact of new or current antibiotic prescription practices on gut microbiota and residual impacts on ARGs.

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The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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ADDITIONAL INFORMATION

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