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Early exposure to antibiotics in the neonatal intensive care unit alters the taxonomic and functional infant gut microbiome

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Abstract

Introduction—The infant gut microbiome is thought to play a key role in developing metabolic and immunologic pathways. Antibiotics have been shown to disrupt the human microbiome, but the impact they have on infants during this key window of development remains poorly understood. Through this study we further characterize the effect antibiotics have on the gut microbiome of infants by looking at metagenomic sequencing data over time.

Materials and Methods—Stool samples were collected on infants from a large tertiary care neonatal intensive care unit. After DNA extraction, metagenomics libraries were generated and sequenced. Taxonomic and functional analyses were then performed. Further directed specimen sequencing for fungal species was also performed.

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Author Contributions

JMB, FL, and GMA conceptualized the study. JMB, CC, HP, TC, and NF enrolled subjects and assisted with specimen processing. JMB, SZ, and LH performed sequencing and other experiments involved in this study. FL performed formal analysis of the data. JMB and FL wrote the original draft of the paper and created all of the figures. SC and PSP assisted throughout with the study design and final manuscript preparation. GMA provided leadership and oversight. All authors read, edited, and approved the final manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

Statement of Ethics

The parents or guardians of the research subjects have given their written informed consent. The study protocol has been approved by CHLA's committee on human research.

Supplementary Material

Supp. Table 1

Supp. Fig. 1

Results—A total of 51 stool samples from 25 infants were analyzed: 7 infants were on antibiotics during at least one of their collection time points. Antibiotics given at birth altered the microbiome (PERMANOVA R^2= 0.044, p=0.002) but later courses did not (R^2= 0.023, p= 0.114). Longitudinal samples collected while off antibiotics were more similar than those collected during a transition on or off antibiotics (mean Bray-Curtis distance 0.29 vs. 0.63, Wilcoxon p=0.06). Functional analysis revealed four microbial pathways that were disrupted by antibiotics given at-birth (p<0.1, folate synthesis, glycerolipid metabolism, fatty acid biosynthesis, and glycolysis). No functional changes associated with current antibiotic use were identified. In a limited sample set, we saw little evidence of fungal involvement in the overall infant microbiome.

Conclusion—Through this study we have further characterized the role antibiotics have in the development of the infant microbiome. Antibiotics given at birth were associated with alterations in the microbiome and had significant impact on the functional pathways involved in folate synthesis and multiple metabolic pathways. Later courses of antibiotics led to stochastic dysbiosis and a significant decrease in *Escherichia coli*. Further characterization of the infant mycobiome is still needed.

Keywords

Bifidobacterium; Escherichia coli; metagenomics; mycobiome; FishTaco

Introduction

The infant gut microbiome is thought to play a key role in developing and establishing metabolic and immunologic pathways [1–5]. Multiple perinatal factors and interventions have now been identified to alter the infant gut microbiome, but much of this process remains poorly understood [6–10]. Some of these factors include mode of delivery, prematurity, and feeding all of which have been associated with long-term effects [7,11,12]. These factors represent exposures and ongoing introduction of exogenous microbes. Antibiotics have a more direct and deleterious effect on host bacterial populations leading to a dysbiosis of the infant microbiome.

Because infants are at high risk for infections, they are frequently exposed to antibiotics. Even before infants are born, maternal antibiotic usage has been associated with alterations in the infant microbiome [13,14]. Antibiotics given in the perinatal period have been further associated with alterations of the infant's microbiome, especially in preterm infants [8,11,15–17]. Antibiotic use has further been associated with increased risk of yeast infections in neonates [18]. Our objective was to further characterize changes in the infants' developing bacterial communities, functional shifts created by these changes, and changes in the fungal microbiome, when antibiotics were given at birth or later on during hospitalization. In order to accomplish this, we performed shotgun metagenomic sequencing on stool from a cohort of infants followed over time in a tertiary care neonatal intensive care unit (NICU).

Materials and Methods

Study Population

All neonates admitted to the Children's Hospital Los Angeles (CHLA) NICU between October 2014 and May 2015 were eligible for enrollment. There were no specific exclusion criteria. Families were approached and consented at, or near, the time of admission. The CHLA institutional review board approved this study.

Clinical Data and Sample Collection

Once enrolled in the study, sequential stool samples were collected from diapers approximately 1 week apart and immediately placed in a −20 degrees Celsius freezer. They were then aliquoted to freezer vials, labeled, and frozen at −80 degrees Celsius. Relevant clinical data was abstracted from the electronic medical record for each collection time point. Study data were filed and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at the University of Southern California [19]. Current antibiotic use was defined as antibiotics being given on the same day as sample collection. Feeding at birth was defined as the primary nutrition during the first 48 hours of life. Antibiotics given at birth was defined as antibiotics given within the first 48 hours of life. Ampicillin and gentamicin were the most frequent antibiotics administered, typically for short prophylactic courses. No more than 4 sample/time points were incorporated into analysis per infant.

DNA Extraction

DNA was extracted from stool samples using a QIAcube workflow (Qiagen, Hilden, Germany). The frozen stool samples were homogenized in PSP (Stratec, Berlin, Germany) stool stabilization buffer first. Then we used the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) for extraction following the manufacture's protocol, substituting Lysing Matrix E tubes (MP Biomedicals, Burlingame, California, USA) for the provided beads. Extracted DNA was stored in elution buffer at −80 degrees Celsius [20].

Shotgun Metagenomics

Libraries were generated using the Nextera XT DNA Library Preparation Kit (Illumina) and sequenced on a Nextseq 500 platform (Illumina) using 2×150bp chemistry. A total of 64 fecal samples from 29 subjects were sequenced to an average depth of 2,249,434 ± 1,429,818 read pairs per sample. Adapter trimming and quality filtering were performed using trim galore, host sequences were removed using kneadData, and taxonomic classification was performed with Kraken (v0.15-beta). A minimum cutoff of 81,513 classified reads was identified as indicative of a robust microbial community and samples with fewer than this number were removed prior to further analysis. A total of 51 samples from 25 subjects were retained. Diversity and ordination analyses were performed using the 'phyloseq' R package (version 1.20.0). Linear mixed effects models ('lmerTest' R package version 2.0–33) were used to identify specific bacterial taxa associated with current and at-birth antibiotics exposure, with a model specification of taxa_relative_abundance ~ on_antibiotics + antibiotics_at_birth + age_in_days + (1 | subject_id). To increase robustness

and limit the testing burden, only taxa with a relative abundance greater than 1% in at least 10% of the samples were tested. All p-values were corrected for multiple testing using the Benjamini-Hochberg FDR method and an adjusted p-value < 0.1 was considered significant. Functional profiling was performed using HUMAnN2, and FishTaco (version 1.1.1) was used to identify taxonomic drivers of functional shifts in the infant gut microbiome [21].

Mycobiome profiling

Mycobiome profiling was performed using a previously published protocol [22] for amplification of the fungal ITS region of the 18S gene [23]. These amplicons were sequenced on a MiSeq desktop sequencer (Illumina, San Diego, California, USA) using 300 cycle v2 chemistry. A custom protocol yielding 250 bases on the reverse read was used for the purposes of other libraries in the pool. Negative and positive mock controls were run in parallel. Reverse reads were de-multiplexed and split into individual files using QIIME 1.9.1 [24]. Divisive amplicon denoising algorithm version 2 (DADA2) was used for error correction, exact sequence inference, and chimera removal [25]. All statistical analyses, including calculation of alpha and beta diversity metrics and taxonomic compositions, were performed using the ‘phyloseq’ package in the R software environment (version 3.3.2) [26]. Association testing between fungal abundances (filtered for at least 3 samples with at least 1% relative abundance) and clinical covariates was performed using zero-inflated negative binomial regression or standard negative binomial regression as appropriate (‘pscl’ R package). All p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method and significance was assessed at q=0.1.

Results

Study demographics

A total of 51 stool samples from 25 infants met all criteria for analysis (Table 1). Of these, 7 infants were on antibiotics during at least one of their collection time points and 18 of the infants were not on antibiotics during any of the collections. In comparing these two groups, we saw no differences in other factors known to affect microbiome such as delivery mode, intrapartum maternal antibiotics, gestational age, and age at time of sampling.

Antibiotic exposure contributes to differences in the infant gut microbiome

Overall, we observed several distinct bacterial profiles including *Bacteroides/Bifidobacterium*-dominant, *Escherichia*-dominant, and *Klebsiella*-dominant phylotypes (Fig. 1a). Principal coordinates analysis (PCoA) using Bray-Curtis distances revealed a similar separation of sample compositions associated with specific bacterial taxa (Fig. 1b). Permutational multivariate analysis of variance (PERMANOVA) identified sex, age, intrapartum antibiotics, gestational age at time of delivery, mode of delivery, birth weight, antibiotics given at birth, feeding at birth, and gastroschisis as small, but statistically significant independent drivers of variation (Supp. Table 1). Surprisingly, the current use of antibiotics was not a significant independent contributor to variation (R2= 0.023, p= 0.114). Although the use of antibiotics did not drive directed changes in the infant gut microbiome, it did appear to decrease stability of the infant gut microbiome (e.g. longer lines connecting red points on Fig 1b). Indeed, we observed a mean Bray-Curtis distance of 0.626 versus

0.289 between samples encompassing a transition either on or off antibiotics versus samples without a transition (Wilcoxon p=0.062, Fig. 1c). This suggests stochastic instability of the gut microbiome associated with current exposure to antibiotics.

To identify specific bacterial taxa impacted by at-birth and current antibiotics exposure, we utilized linear mixed effects models (Fig. 2). Intriguingly, both *Bifidobacterium breve* and *Escherichia coli* trended toward being decreased in samples with at-birth antibiotics exposure (p=0.18), although the difference was not statistically significant after multiple testing correction. The current use of antibiotics was associated with a significant decrease in the proportion of *Escherichia coli* (p=0.03).

At-birth antibiotic exposure alters the functional gut microbiome

Eighteen of the 25 subjects received a course of antibiotics at birth. Given the relatively minimal differences in bacterial composition observed in association with antibiotics at time of sampling, we wondered if antibiotic exposure at birth might instead drive large-scale differences in microbially-encoded function irrespective of other exposures. To this end, we utilized a novel computational framework (FishTaco) to identify taxonomic drivers of functional shifts in the infant gut microbiome associated with at-birth and current antibiotics [21]. Four microbial pathways were significantly disrupted by antibiotics given at-birth (p<0.1, Fig. 3). Increased folate biosynthesis was primarily driven by decreases in *Bifidobacterium breve* and *Escherichia coli* when antibiotics were given at birth. In contrast, increases in *Klebsiella* species and *Bacteroides* species drove increases in glycerolipid metabolism, fatty acid biosynthesis, and glycolysis. Decreases in *Bifidobacterium breve* were further associated with increases in these metabolic pathways. Functional analysis revealed no significant changes associated with current antibiotics use, again demonstrating the stochastic effect of this intervention.

Minimal impact of antibiotics on the mycobiome

Although we had hypothesized that antibiotic use would impact the fungal microbiome based on prior murine studies [27,28], our study found very little evidence of fungal colonization in the overall microbiome in these infants (Supp. Fig. 1). Fungal sequences made up less than 1% of our total shotgun sequences and only 3 samples out of 51 had identifiable fungal species. To further evaluate this, we did direct fungal sequencing using the ITS region of fungal ribosomal DNA. Again, we found few fungal sequences and were therefore unable to do further statistical analysis. That being said, the 3 subjects that had fungal sequences were associated with a lack of bacterial diversity (Supp. Fig. 1). Two of the samples were primarily *Enterococcus* and *Candida* species with the third being primarily *Staphylococcus* and *Aspergillus* species.

Discussion

Antibiotics have been shown to disrupt the human microbiome, but the impact they have on infants remains poorly understood. Studies have now shown that early antibiotic exposure may be associated with diseases such as asthma and obesity, but the mechanisms have not been fully elucidated [29–32]. Through this study we further characterize the effect

antibiotics have on the gut microbiome of infants in the NICU by looking at metagenomic sequencing data over time.

Even when accounting for age, gestational age, mode of delivery, and feeding, we demonstrate that antibiotics given at birth significantly affected the overall bacterial microbiome compared to those infants who did not receive antibiotics. This is corroborated by many recent studies looking at the microbiome in infants receiving early courses of antibiotics [11,16,33–35]. These studies demonstrate a relative decrease in species richness within the gut microbiome that persists even after the cessation of antibiotics [11,16,35]. The first years of life is thought to be critical in the establishment of a healthy microbiome. We show that even disruptions due to antibiotics in the first days of life can lead to recognizable dysbiosis later on. Studies have demonstrated that these early courses of antibiotics can lead to the development of populations of bacteria carrying antibiotic resistance genes which may put the infants at increased risk for more difficult to treat infections [33,34]. Further clinical studies have showed increased mortality in infants on prolonged antibiotic courses at birth who are culture negative [36].

Functional analysis of the microbiome demonstrated changes in multiple pathways after antibiotics given at birth. Those significantly affected included folate synthesis, glycerolipid metabolism, fatty acid biosynthesis, and glycolysis. These changes were driven by *Bifidobacterium breve*, *Escherichia coli, Klebsiella* species, and *Bacteroides* species, all bacteria that are thought to be key components of the developing infant microbiome. In particular, we found further evidence that a small loss of *Bifidobacterium* drives large functional changes in the microbiome. *Bifidobacterium* species are the targets of probiotic interventions in newborns [37]. Although studies have not demonstrated significant long-term changes in the microbiome due to probiotic supplementation, they have shown a difference in the metabolome reflecting the functional pathways of the bacteria themselves. Functional analysis tools such as FishTaco will continue to expand our knowledge of the functional microbiome [21].

In contrast to the changes we saw with antibiotics given at birth, we found that later antibiotic use did not appear to significantly change the microbiome in a directed fashion. Instead, we found that these courses of antibiotics led to disruptions in the gut microbiome that were more random. Instability and dysbiosis have separately been linked necrotizing enterocolitis [38,39]. It is thought that disruptions due to an antibiotic course may predispose infants to episodes of necrotizing enterocolitis and late onset sepsis. We did find a significant decrease in *Escherichia coli* populations in these later courses of antibiotics. This makes sense as the antibiotics used are frequently active against common pathogens such as *Escherichia coli.*

Beyond focusing on the bacteria, through this study we also attempted to identify the impact antibiotics have on the infant gut mycobiome. Infants in the NICU setting are at increased risk for invasive fungal disease [18]. Furthermore, they are frequently exposed to broad spectrum antibiotics which have been associated with candidiasis. Few descriptive studies of the human mycobiome exist [23] and only recently have any been published in neonates [40]. Murine and now human studies indicate that fungal communities change

significantly during development and demonstrate increases in fungal populations after courses of antibiotics [27,28,40]. Despite extensive efforts to identify the fungal signature within the stool samples from our cohort, we found few fungi. What we did find was not enough to identify any impact based on antibiotic use. That being said, anecdotally, we saw loss of bacterial diversity associated with a fungal signature. Two samples contained predominantly *Enterococcus* which has been shown in prior studies to inhibit the virulence of *Candida* species in animal models [41]. The importance of this finding is unclear and merits further investigation.

This study is limited by its small sample size. Larger studies with more subjects and more frequent stool collection are underway to further evaluate this critical period of development. Without a control group of vaginally delivered, full-term infants without antibiotic exposure makes this primarily an observational study. Much of the previous research looking at the effects of antibiotics were done in premature infants. The infants enrolled in our study were in general older by gestational age. Furthermore, these results reflect the population of a single tertiary care NICU that is not associated with a birthing center and may not be applicable to other centers. As a major surgical center, gastroschisis was somewhat overly represented in our enrolled subjects, though this was not by design and more a function of sampling at time of enrollment. As with most microbiome studies, we are further limited in how we interpret the data based on relative abundances and not absolute values.

Through this study we have further characterized the role antibiotics may have in the development of the infant microbiome. Antibiotics given at birth led to clear alterations in the gut microbiome that had significant impact on the functional pathways involved in folate synthesis, glycerolipid metabolism, fatty acid biosynthesis, and glycolysis. Later courses of antibiotics led to stochastic dysbiosis and a significant decrease in *Escherichia coli*. Further characterization of the infant mycobiome and how it is impacted by antibiotics is still needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Availability of data and materials- Sequence data has been deposited to the NCBI Sequence Read Archive under BioProject accession number PRJNA521878. All code and intermediate files to reproduce the analyses are available at https://github.com/fanli-gcb/NICU_microbiome.

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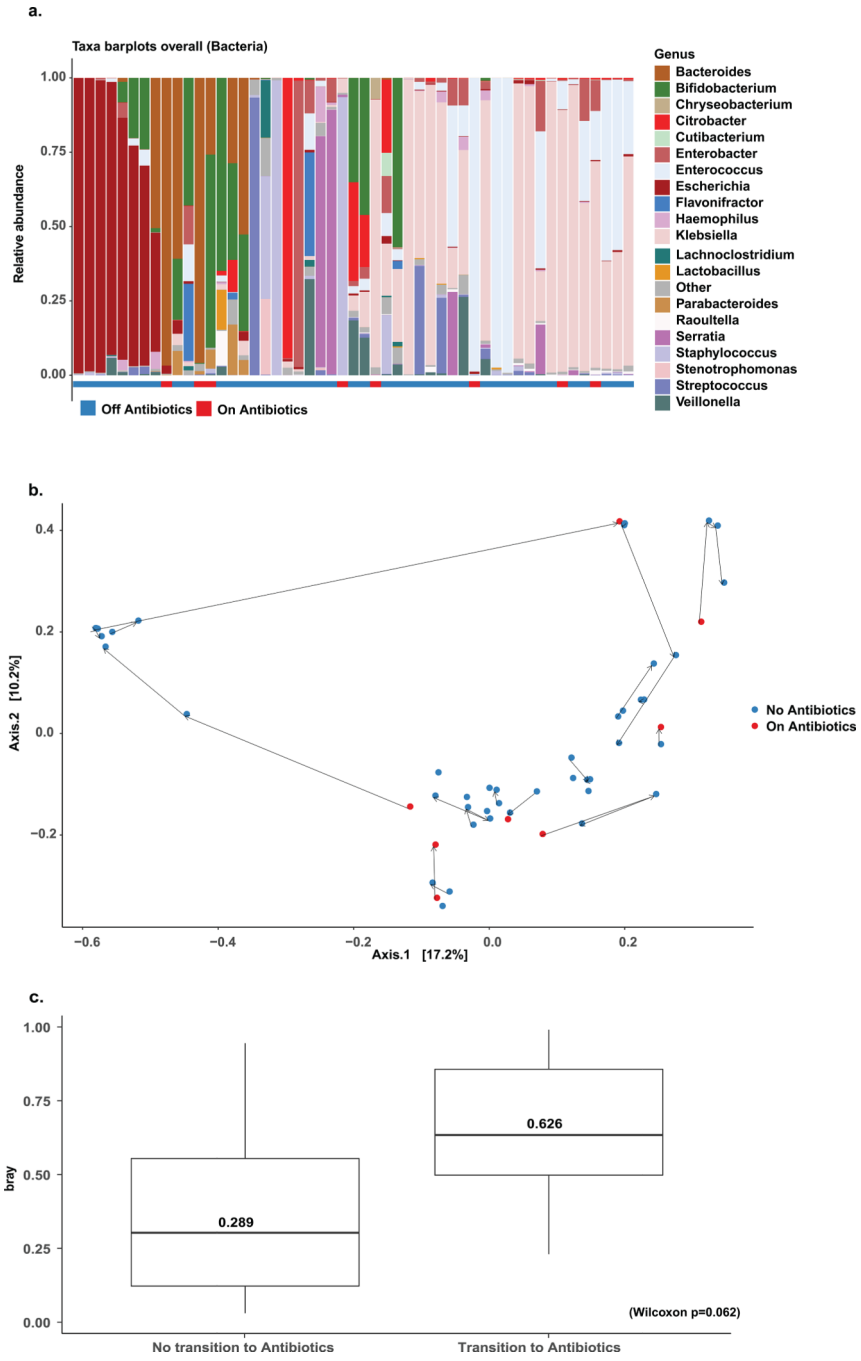

Fig. 1.

Effect of antibiotics exposure on the infant gut microbiome. (a) Genus-level composition for n=51 samples collected either during antibiotic treatment (red) or without current exposure (blue). Genera with a mean relative abundance less than 1% are grouped into ‘Other’. (b) Principal coordinates analysis (PCoA) plot using Bray-Curtis distances with lines connecting samples from the same infant. Percentages in brackets indicate the percent variation explained by each axis. (c) Boxplot of Bray-Curtis distances between samples encompassing a transition either on or off antibiotics versus those without a transition.

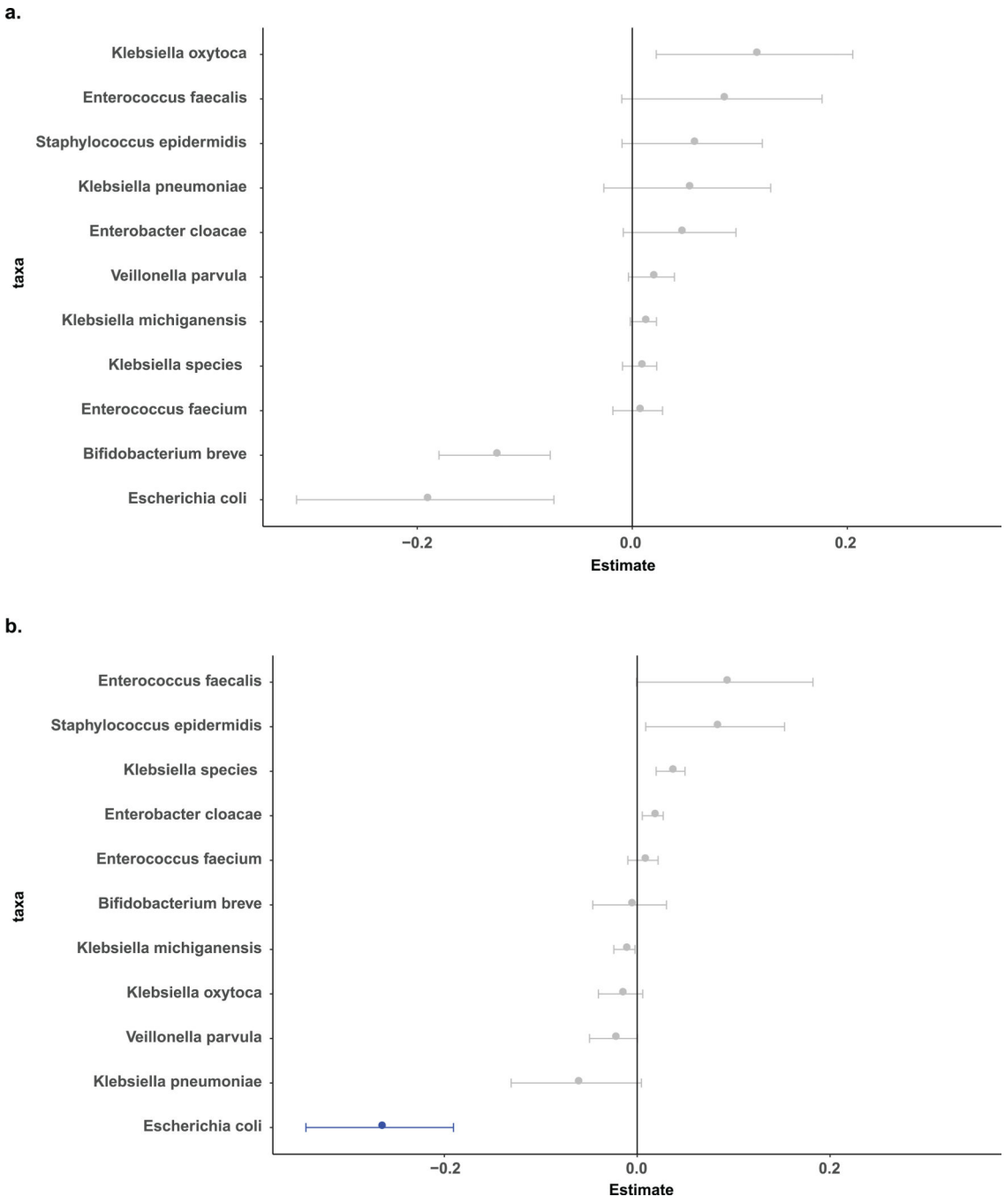

Fig. 2.

Effect of antibiotics on bacterial relative abundances. Forest plot of results from linear mixed effects modeling of species-level relative abundances as a function of antibiotics exposure at birth (a) or current antibiotics exposure (b). Statistically significant effects are shown in blue (decreased) and error bars denote 95% confidence intervals.

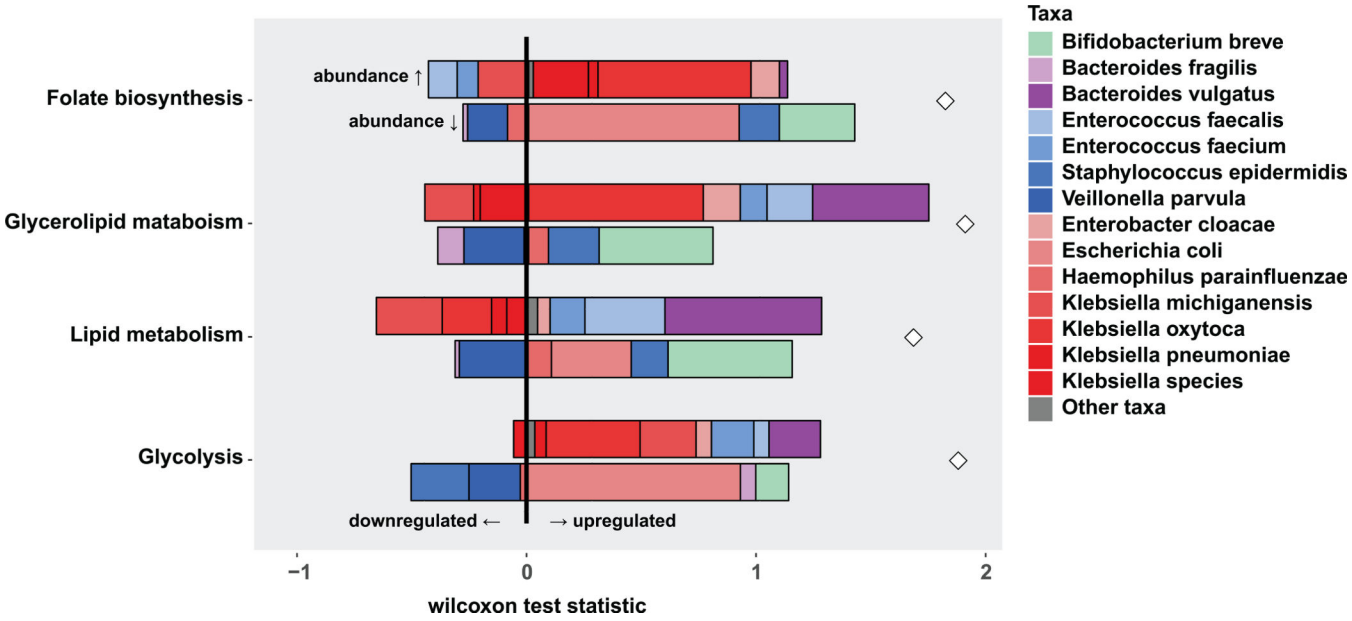

Fig. 3.

Taxonomic drivers of functional shifts in infants who were exposed to antibiotics at birth ($p<0.1$). Overall enrichment of each KEGG pathway is denoted by an open diamond (◊). Taxa attenuating each functional shift are shown to the left of the vertical line, and taxa driving each functional shift are shown to the right of the vertical line. For each KEGG pathway, taxa shown along the top are increased in infants with at-birth antibiotics exposure and taxa shown along the bottom are decreased in infants with at-birth antibiotic exposure.

Table 1.

Demographic characteristics comparing infants in a tertiary care neonatal intensive care unit who received no antibiotics during sample collection compared to 7 infants on antibiotics during sample collection.

	No antibiotics during sample collection	**On antibiotics during sample collection**	**p-value**
Number of subjects	**18**	**7**	
Number of samples	34	17	
Sex			
male	10 (55.6%)	5 (71.4%)	0.66
female	8 (44.4%)	2 (28.6%)	
Gestational age in weeks, mean [range]	37.4 [30.4–40.4]	35.6 [29.7–39.9]	0.29
Gestational age <34 weeks	3 (17%)	1 (14%)	0.88
Intrapartum antibiotics	6 (33.3%)	2 (28.6%)	1
Delivery Mode			
Vaginal	5 (27.8%)	3 (42.9%)	0.64
C-section	13 (72.2%)	4 (57.1%)	
Birth weight, mean [range]	2.81 [1.50–4.31]	2.27 [1.20–3.25]	0.30
Antibiotics given at birth	12 (66.7%)	6 (85.7%)	0.63
Gastroschisis	5 (27.8%)	2 (28.6%)	1
Infant age at sample collection in days, mean [range]	36.0 [6–122]	24.3 [6–82]	0.13