Antibiotic Exposure Prevents Acquisition of Beneficial Metabolic Functions in the Preterm Infant Gut Microbiome

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Research

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Abstract

Background: Aberrations in the preterm microbiome following antibiotic therapy have been reported in previous studies. The objective of this study was to probe potential underlying mechanisms between this observation and susceptibility to adverse prematurity-related outcomes.

Results: Metagenomic shotgun sequencing was performed on 132 fecal and 264 skin samples collected at 1 and 3 weeks of age from 68 infants born at <36 weeks postmenstrual age and birth weight <2000 g. After accounting for gestational age and maternal antibiotics, the distribution of organisms in all samples and the corresponding metabolic pathway abundance were compared between infants exposed to postnatal antibiotics and antibiotics-naïve infants.

In antibiotic-naïve infants, gestational and postnatal age imparted similar trajectories on maturation of the microbial community and associated metabolic functional capacity, with postnatal age exerting greater contribution. Antibiotic exposure was associated with reversal in maturation trajectory from the first week to the third week of age ($p < 0.001$). Butyrate producing genera, including *Clostridium* and *Blautia* were significantly more abundant in antibiotic-naïve neonates at 3 weeks postnatal age. Correspondingly, metabolic pathways required for short chain fatty acid synthesis were significantly increased in the antibiotic-naïve infants, but not in antibiotic-exposed neonates, at three weeks after birth.

Conclusions: Early brief antibiotic exposure markedly disrupts developmental trajectory of the neonatal microbiome and its corresponding functional capacity. Our findings may provide a mechanistic explanation for the known associations between antibiotic use and adverse outcomes in preterm infants.

Background

Perturbations of the evolution of early life microbiota have been implicated in short- and long-term health outcomes [1–3]. Previous work had linked antibiotic exposure with devastating prematurity-related complications, such as necrotizing enterocolitis and late-onset sepsis, secondary to altered microbiome. However, the mechanisms underlying this association have not been determined.

Shotgun metagenomic sequencing and other recent advances in next-generation DNA sequencing methodology have facilitated probing for presence and changes in microbial metabolic genes [4, 5], which may provide insights into the mechanisms of antibiotic-exposure-induced complications in preterm infants. We conducted a population-based prospective cohort study of infants born at < 36 weeks postmenstrual age and birth weight < 2000 g to, firstly, assess the taxonomic composition and potential metabolic function of the early neonatal microbiota across multiple body sites and up to 3 weeks of age, a period of peak vulnerability to prematurity-related complications. Secondly, we determined the effect of postnatal antibiotics on the preterm infants' microbiome structure and function.

Results

Study population and antibiotic exposure

Samples from the skin (axilla and groin) and stool were collected from 68 preterm infants, median (range) birthweight 1384 (648–1940) g and gestational age 30.5 (24.0–35.0) weeks (Table 1). Fifty-nine (86.8%) infants were exposed to maternal antibiotics, administered within 72 hours prior to birth. In addition, 22 (32.4%) infants received postnatal antibiotics (classified as antibiotic-exposed) while 46 (67.6%) infants did not (classified as antibiotic-naïve; Table 1). Infants in both groups were similar in demographic characteristics and sample collection times, except that antibiotic-exposed infants were modestly smaller than antibiotic-naïve infants, Table 1. This was accounted for using generalized linear mixed modeling in subsequent analysis. Most (97.1%) of the infants were fed human milk during the study period. Two neonates developed necrotizing enterocolitis (NEC) in the antibiotic-exposed group during the study period.

Table 1
Demographic characteristics

	Antibiotic-naïve group (n = 46)	Antibiotic-exposed group (n = 22)	p-values
Birth weight in g, median (range)	1478.0 (688–1940)	1173.0 (648–1924)	0.003
Gestational age in weeks, median (range)	31.5 (28–35)	28.5 (24–32)	< 0.001
Female gender, <i>n</i> (%)	25 (54.3)	12 (54.5)	0.988
Multiple gestations, <i>n</i> (%)	35 (76.1)	17 (77.3)	0.914
Race, <i>n</i> (%)			
White	23 (50.0)	11 (50.0)	1
Black or African American	18 (39.1)	10 (45.5)	0.62
Asian	2 (4.3)	0 (0.0)	0.321
Native Hawaiian or other Pacific Islander	1 (2.2)	0 (0.0)	0.486
Unknown or not reported	2 (4.3)	1 (4.5)	0.97
Hispanic or Latino, <i>n</i> (%)	2 (4.3)	1 (4.5)	0.97
Cesarean section, <i>n</i> (%)	42 (91.3)	20 (90.9)	0.957
Diet, <i>n</i> (%)			
Mostly breast milk	39 (84.8)	19 (86.4)	0.863
Any receipt of formula	4 (8.7)	1 (4.5)	0.54
Any receipt of breast milk	44 (95.7)	22 (100.0)	0.321
Perinatal maternal antibiotic exposure, <i>n</i> (%)	39 (84.8)	20 (90.9)	0.486
Specimen Collection time			
Week 1	7 (5–9)	6.5 (5–9)	0.907
Week 3	16 (15–21)	17 (15–24)	0.562

Maturation And Differentiation Of The Microbiome In Preterm Infants

To understand the ontogeny of the microbial community of the stool and skin in the preterm infant during the first three weeks of age, we focused on infants that did not receive postnatal antibiotics during this 3-

week period. Microbial DNA from a total of 90 fecal and 180 skin swabs (90 from the groin and 90 from the axillae) from 46 antibiotic-naïve infants was sequenced and data from week 1 were compared with week 3. We found, as expected, that at all three body sites examined, the number and diversity of genera increased from week 1 to week 3 (Fig. 1A). Similarly, comparison of samples at Week 1 and Week 3 postnatal ages by PCA revealed maturation and differentiation at the three body sites. While at Week 1, samples from the three body sites were more closely clustered together, by Week 3, microbial composition had become more distinct across the three sites (Fig. 1B). As might be expected, the composition of groin skin microbiome was more similar to stool microbiome than was axillary skin microbiome, especially at Week 3. The composition at each body site at week 3 was distinct from composition at week 1 (multi-response permutation procedures (MRPP, p-values < 0.001 for Week 1 versus Week 3 stool, axilla, and groin microbial composition).

We next investigated which organisms made the largest contribution to microbiome maturation with a focus on fecal microbiome in antibiotic-naïve infants. Among the genera that significantly changed from Week 1 to Week 3, *Clostridium* demonstrated the most significance in abundance in week 3 after accounting for potential confounders. Several other genera, *Klebsiella*, *Veillonella*, *Serratia*, *Escherichia*, were also significantly increased (Fig. 2A and 2B, all $p < 0.05$). Conversely, *Staphylococcus* demonstrated the greatest decrease in abundance from Week 1 to Week 3 (Fig. 2C).

Impact of gestational and postnatal ages on developmental trajectory on stool microbiome composition

We examined the contribution of gestational age to microbiome composition at Weeks 1 and Weeks 3 in antibiotic-naïve infants. We found diversity and overall composition of the microbiome were not significantly different between infant born at 28–32 weeks compared with 33–36 weeks gestational age ranges (Supplemental Fig. 1A) whereas postnatal age had a significant impact on microbiome composition at all body sites among gestational age cohorts (Supplemental Fig. 1B).

We directly compared the contribution of gestational age versus postnatal age to microbiome composition by performing unsupervised PCA, then coloring samples based on gestational age in 2-week gestational age increments, as well as postnatal age at Weeks 1 and 3. Further, we calculated the mean Bray-Curtis distance of all pairwise comparisons between the two-week gestational age cohorts. Three trends are observed in the data, although none of the differences in microbial composition between any two-week gestational age cohorts were statistically significant as assessed by MRPP (Fig. 3A). However, the trend for both developmental variables was in the same direction- in all cases in this PCA analysis (compare the orientation of directional arrows in Figs. 3A and 3B). We then compared the impact of postnatal age on microbiome composition for each gestational age (GA) cohort in antibiotic-naïve infants. Microbiome composition demonstrated progression from Week 1 to Week 3 at all gestational ages (Fig. 3B). Postnatal age-dependent differences in microbiome composition were greatest at the earliest gestational ages ($p = 0.017$ for 28-to-30-week GA infants and $p = 0.001$ for 30-to-32-week infants) and declined with advancing gestational age (Fig. 3B). Overall, the data indicate that two postnatal weeks

time period had a greater impact on microbiome composition than two gestational weeks, but there is a trend toward increased contribution of gestational age later in pregnancy.

Effect of perinatal maternal antibiotic exposure on neonatal microbiome structure

In our cohort, although most infants did not receive antibiotics postnatally, 86.8% of mothers received antimicrobials prior to delivery. We evaluated the effects of maternal antibiotics on the developing skin and gut microbiome and found no significant difference in Shannon Diversity over the sampling period in gut microbiome in infants who had been exposed to maternal antibiotics, administered within 72 hours prior to birth compared to those who were not. Maternal antibiotics have a non-significant trend toward decrease diversity at all body sites at Week 1, but this had resolved by Week 3 (Supplemental Fig. 2A). Similarly, PCA failed to reveal significant difference in the microbial composition and maturation of the microbiome at any of the three body sites (Supplemental Fig. 2B; only stool and groin samples are shown for clarity). However, we found using ZINB-GLMM, that prenatal antibiotic exposure was associated with alterations in abundance of several genera after accounting for other variables, including gestational age, breast milk receipt, and delivery mode both at week 1 and week 3 (Supplemental Fig. 2C).

Effect Of Antibiotic Treatment On Microbiome Diversification And Maturation

We next sought to understand how postnatal antibiotic exposure impacted composition and maturation of the preterm infant gut and skin microbiota. Unlike prior investigations of the preterm infant microbiome, the majority of infants in our study received no antibiotics up to 3 weeks of age. Among those that received postnatal antibiotics, the duration and intensity were generally short. Indeed, among the 22 infants that received antibiotics, 16 infants (72.7%) received < 48 hours of ampicillin and gentamicin, and predominantly during the first 3 days of age. We first compared microbiome diversity across body sites at Week 1 and Week 3 between antibiotic-exposed and antibiotic-naïve groups. Fecal samples showed a decrease in diversity during the first and third week and skin samples from the groin demonstrated decreased diversity by the third week post-antibiotic therapy ($p < 0.001$, Fig. 4A). There were no significant differences in diversity of microbiota from the axillae at Week 1 and Week 3 between antibiotic-exposed and antibiotic-naïve groups. We next examined overall microbial composition using PCA. There was decreased differentiation of the gut microbial composition in antibiotic-exposed infants compared to antibiotic-naïve infants (Fig. 4B). Overlay of the PCA plots from Week 1 and Week 3 demonstrates that antibiotic treatment tends to shift microbial composition in the opposite direction relative to changes seen with postnatal age, indicating that antibiotics therapy is associated with blunting of the maturation of the microbiome at all three body sites (Fig. 4C). In addition, microbiome maturation across body sites measured by Bray–Curtis distance shows significant decrease in differentiation ($p < 0.001$) between antibiotic-exposed and antibiotic-naïve groups from week 1 to week 3 (Fig. 4D).

To identify organisms most impacted by antibiotic exposure, we performed ZINB-GLMM with FDR correction followed by calculation of effect size using SLDA to identify genera that differed between the microbial composition of antibiotic-exposed versus antibiotic-naïve infants at Weeks 1 and 3, after accounting for gestational age, maternal antibiotics, breast milk receipt, and delivery mode. We found that *Sphingomonas*, *Acidovorax* and *Candida* were significantly enriched in the antibiotic-exposed group, whereas several genera, including *Blautia*, *Streptococcus*, *Enterococcus* and *Staphylococcus* were significantly more abundant in the antibiotic-naïve infants in the first week after birth (Fig. 5A and Supplemental Fig. 3). At week three, there were no genera found at significantly higher abundance in antibiotic-exposed infants, while several genera including *Clostridium*, *Clostridioides*, *Blautia*, as well as *Streptococcus*, and *Staphylococcus* were significantly increased in the antibiotic-naïve infants at three weeks after birth (Figs. 5B and Supplemental Fig. 3). Antibiotic exposure resulted in domination of the gut microbiome by a small number of genera, as indicated by the Berger-Parker Dominance index both at week 1 and week 3 (Figs. 6A). In antibiotic-treated infants, *E. coli* and *Klebsiella* dominate at both time points (Figs. 6B).

Effect of antibiotic exposure on maturation of metabolic functional capacity of stool microbiome

Microbes are believed to influence human health, in part, through their ability to produce metabolites with either beneficial or harmful effects on the host. Given the primacy of metabolic activity in the preterm infant gut microbiota and potential direct impacts in health outcome, we characterized the metabolic gene content in antibiotic-naïve versus antibiotic-exposed. DNA reads were assigned to metabolic genes using HUMAnN2 [7]. To assess overall metabolic pathway abundance, we used unsupervised principal component analysis (PCA) comparing samples based on the infant's postnatal age and antibiotic exposure status (Fig. 7A). There was a significant change in the distribution of metabolic pathways represented in the preterm infant gut between Week 1 and Week 3 in antibiotic-naïve infants ($p < 0.001$), indicating the metabolic capacity demonstrated significant functional maturation in these infants. However, the change from Week 1 to Week 3 in antibiotic-exposed infants was not significant ($p = 0.064$), indicating that antibiotics impaired maturation of metabolic functional capacity. PCA analysis demonstrated that antibiotic exposure was associated with a shift to a more immature metabolic pathway abundance at Week 3 compared to antibiotic-naïve infants. These two features of the PCA plot reveal that antibiotic exposure is associated with stunting of functional maturation of the preterm infant microbiome. To specifically address the role of antibiotic exposure and account for differences in gestational age, maternal antibiotic exposure between groups, we employed generalized linear mixed modeling (GLMM) with fixed and random effects. We found that genes encoding a total of 20 metabolic pathways that were significantly different from Week 1 to Week 3 in the stools of antibiotic-naïve infants. In contrast, 34 metabolic pathways were significantly different in infants that received antibiotics (Fig. 7B). Notably, there was very little overlap in the metabolic pathways enriched from Week 1 to Week 3 in antibiotic-naïve versus antibiotic-exposed infants (Supplemental Fig. 4) indicating that antibiotic exposure is associated with profoundly different trajectories in the development of metabolic functional pathways in the preterm infant gut. Among metabolic pathway genes that were significantly increased in antibiotic-naïve infants were pathways involved in synthesis of the short chain fatty acid butyrate

(PWY.5676. acetyl.CoA.fermentation.to.butanoate.II), such as butyrate and acetate pathway, Fig. 8. It is particularly notable that antibiotic exposure completely suppressed increased abundance of the butyrate and acetate synthesis pathway seen in antibiotic-naïve infants, suggesting that maturation of the fecal microbiota from Week 1 to Week 3 is associated with increased capacity to produce metabolites beneficial to the developing neonatal intestinal epithelium (Fig. 8).

Discussion

Antibiotics are the most-commonly prescribed medications in Neonatal Intensive Care Units. Previous reports have described the effects of antibiotics on the infant microbiome maturation but implication on microbial functional capacity has not been studied. In this prospective population study, in addition to altered maturation and differentiation, the functional capacity of the microbiome was deranged by short-term exposure of preterm infants to antibiotics. Postnatal age plays a greater role than gestational age in shaping the microbiota in the gut and skin. Intrauterine exposure through maternal administration just prior to delivery did not significantly impact diversity but altered abundance of gut microbes. Acquisition of putatively beneficial metabolic pathways was particularly impacted following brief antibiotic exposure, as exemplified by blunting of genes associated with butyrate and acetate synthesis at week 3 in the microbiota of antibiotic-exposed infants. Alteration in the trajectory of microbial metabolic pathways may contribute to the pathomechanism of antibiotic-associated adverse prematurity-related adverse events, such as NEC and late-onset sepsis [8]. These data add to the growing body of evidence of the potential deleterious effects of antibiotic therapy on the abundance and diversity of the developing microbiome of premature infants.

The developmental trajectory to microbiome maturation is probably innate, but it is influenced by the physiologic state of the developing intestine and the environment. Exposure to antibiotics appears to alter the developmental trajectory. In agreement with previous reports [9–13] and contrary to others [14–16], gestational age at birth appeared to exert less influence on the microbiota development than postnatal age. In our study, both gestational age and postnatal age independently exerted effects on the preterm infant microbiome. In the intestine in particular, gestational age was associated with overall similar trajectories in microbiome maturation. However, postnatal age had a greater impact on microbiome maturation than gestational age.

A major link between microbiota and host cells is the production of short-chain fatty acids through bacterial metabolism[21–23]. We demonstrated that at week three several genera that are beneficial to the host, including *Blautia* and *Clostridium*, were significantly increased in antibiotic-naïve infants. *Blautia* is a dominant genus in the human gut microbiota and belongs to the clostridial subcluster XIVa, along with *Clostridium*, is a main butyrate-producing bacterium that is associated with the host's metabolic function [24] [25]. Butyrate plays a pleiotropic role in the gut, including enhancing epithelial barrier integrity, and inhibition of inflammation. Alterations in *Blautia* and *Clostridium* abundance may also be important in the pathogenesis of Crohn's disease, which, similar to NEC, is associated with disrupted intestinal barrier function [26]. In our study, we demonstrate that butyrate and acetate synthesis genes are

enriched at the critical week 3 timepoint, while this enrichment is completely abrogated in antibiotic-exposed infants. Taken together, our findings have implications for a putative host-microbe crosstalk pathway wherein protective butyrate signaling is dampened by antibiotic therapy.

Despite the relatively large sample size of our cohort, this is a single institution study. The vast majority of our infants were exposed to perinatal maternal antibiotics administered within 72 hours of delivery and most of them were delivered by cesarean section. These may limit the generalizability of our findings. However, most VLBW infants are products of high-risk pregnancies, which are often associated with high rate of cesarean births. On the upside, most infants in our cohort were not treated with antibiotics postnatally. This enabled us to characterize the ontogeny of the microbiome in preterm infants, and to assess the impact of gestational and postnatal age on microbiome composition and metabolic functional capacity. Unlike previous studies that relied on 16S RNA sequencing, we used high resolution shotgun metagenomic sequencing and multidimensional analysis platforms to assess microbial species composition and the corresponding metabolic functional genes.

Conclusions

Our data demonstrate that brief early antibiotic therapy to preterm neonates engenders alterations in microbial composition and differentiation with resulting in delay in acquisition of beneficial microbial metabolic pathways.

Methods

Study subjects and sample collection

The study was approved by the Institutional Review Boards of Cincinnati Children's Hospital Medical Center and the University of Cincinnati (CCHMC #2018-7474). Infants born at <36 weeks postmenstrual age and birth weight <2000 g admitted to the Neonatal Intensive Care Unit at the University of Cincinnati Medical Center (UCMC) from February 2019 to October 2019, with anticipated length of stay of >21 days, were enrolled on admission. Infants were stratified into 1) antibiotic-exposed or 2) antibiotic-naïve groups, based on postnatal antibiotic therapy. Maternal antibiotics therapy during labor within 72 h prior to delivery was documented. Maternal and neonatal demographic data, labor complications and NICU courses, including postnatal antibiotic therapy were collected prospectively during the study period. Stool and skin samples were collected at ~1 and 3 weeks of postnatal age.

Illumina sequencing

Genomic DNA was isolated using Qiagen QIAamp® PowerFecal® Kit and Genomic DNA Mini Kit (Invitrogen), respectively [27]. Amplified library generation was performed with Nextera XT adapters, and sequencing performed to obtain 150-bp DNA paired end reads to a depth of approximately 2 million reads per sample using Illumina NextSeq 500 in the Microbial Genomics and Metagenomics Core at Cincinnati Children's Hospital.

Data Analysis

To establish differences in fecal and skin microbiota between antibiotic-exposed versus antibiotic-naïve groups and between time points, shotgun metagenome sequence data were analyzed using MRPP. Visualization of overall microbiome composition among groups was performed using PCA. Examination of clinical covariates were performed using zero inflated negative binomial generalized linear mixed models (ZINB-GLMM). Generalized linear mixed modeling was performed using the “glmer” command from the lme4 package in R. To identify individual genera that differ between groups, we performed non-parametric tests (Mann-Whitney *U* test or Kruskal-Wallis test), followed by Bonferroni correction for multiple testing. Effect size of the statistically significantly different genera were determined using shrinkage linear discriminant analysis (SLDA). We performed generalized linear mixed modeling with parameterization for negative binomial distribution and zero inflation (NBZI-GLMM), then performed false discovery rate (FDR) correction followed by calculation of effect size using SLDA [6]. All data analyses were carried out using the R programming language or SPSS software for Windows (version 20.0; SPSS, Inc., Chicago, IL, USA).

Declarations

•Ethics approval and consent to participate

This study was approved by the Institutional Review Boards of Cincinnati Children’s Hospital Medical Center and the University of Cincinnati (CCHMC #2018-7474), and all participants provided written informed consent.

•Consent for publication

Not applicable

•Availability of data and material

Sequence data is available at the Short Read Archive with project number (*pending*). Linux shell and R programming language scripts used to analyze the data and generate the manuscript figures are available upon request.

•Competing interests

The authors declare no conflicts of interest.

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•Authors' contributions

David Haslam and Henry Akinbi conceptualized and designed the study, assisted with samples collection, performed data interpretation and edited the manuscript. David Haslam is the Director of the Microbial Genomics and Metagenomic Core at Cincinnati Children's Hospital Medical Center. He directed critical experimental techniques. Yanping Xu collected inform consent, collected samples, performed experiments, data acquisition and wrote the initial draft of the manuscript. Olivia Milburn obtained inform consents, collected samples, performed experiments and maintained our database. Traci Beiersdorfer enrolled patients and obtained inform consents. Lizhong Du participated in conceptualizing the study and revised drafts of the manuscript.

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References

1. Morais J, Marques C, Teixeira D, et al. Extremely preterm neonates have more *Lactobacillus* in meconium than very preterm neonates - the in utero microbial colonization hypothesis. *Gut Microbes*. 2020;12(1):1785804.
2. Bokulich NA, Blaser MJ. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Science*. 2016;343:82.
3. Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. *Nat Med*. 2016;22(3):250–3.
4. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486(7402):207–14.
5. Arboleya S, Sanchez B, Solis G, et al. Impact of Prematurity and Perinatal Antibiotics on the Developing Intestinal Microbiota: A Functional Inference Study. *Int J Mol Sci* 2016; 17(5).
6. Hellmann J, Andersen H, Fei L, et al. Microbial Shifts and Shorter Time to Bowel Resection Surgery Associated with *C. difficile* in Pediatric Crohn's Disease. *Inflamm Bowel Dis*. 2020;26(8):1212–21.
7. Franzosa EA, McIver LJ, Rahnavard G, et al. Species-level functional profiling of metagenomes and metatranscriptomes. *Nat Methods*. 2018;15(11):962–8.
8. Masi AC, Stewart CJ. The role of the preterm intestinal microbiome in sepsis and necrotising enterocolitis. *Early Hum Dev*. 2019;138:104854.

9. Brooks B, Firek BA, Miller CS, et al. Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. *Microbiome*. 2014;2(1):1.
10. Korpela K, Blakstad EW, Moltu SJ, et al. Intestinal microbiota development and gestational age in preterm neonates. *Sci Rep*. 2018;8(1):2453.
11. Greenwood C, Morrow AL, Lagomarcino AJ, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of *Enterobacter*. *J Pediatr*. 2014;165(1):23–9.
12. Fjalstad JW, Esaiassen E, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: a systematic review. *J Antimicrob Chemother*. 2018;73(3):569–80.
13. Rooney AM, Timberlake K, Brown KA, et al. Each Additional Day of Antibiotics Is Associated With Lower Gut Anaerobes in Neonatal Intensive Care Unit Patients. *Clin Infect Dis*. 2020;70(12):2553–60.
14. Fouhy F, Watkins C, Hill CJ, et al. Perinatal factors affect the gut microbiota up to four years after birth. *Nat Commun*. 2019;10(1):1517.
15. Yassour M, Vatanen T, Siljander H, et al. Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci Transl Med*. 2016;8(343):343ra81.
16. Yatsunencko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature*. 2012;486(7402):222–7.
17. Pammi M, Cope J, Tarr PI, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome*. 2017;5(1):31.
18. Deshmukh HS, Liu Y, Menkiti OR, et al. The microbiota regulates neutrophil homeostasis and host resistance to *Escherichia coli* K1 sepsis in neonatal mice. *Nat Med*. 2014;20(5):524–30.
19. Gibson MK, Wang B, Ahmadi S, et al. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat Microbiol*. 2016;1:16024.
20. Tanaka S, Kobayashi T, Songjinda P, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol*. 2009;56(1):80–7.
21. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota-gut-brain communication. *Nat Rev Gastroenterol Hepatol*. 2019;16(8):461–78.
22. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes*. 2016;7(3):189–200.
23. Watson H, Mitra S, Croden FC, et al. A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. *Gut*. 2018;67(11):1974–83.
24. Jia L, Li D, Feng N, et al. Anti-diabetic Effects of *Clostridium butyricum* CGMCC0313.1 through Promoting the Growth of Gut Butyrate-producing Bacteria in Type 2 Diabetic Mice. *Sci Rep*. 2017;7(1):7046.
25. Kim YG, Sakamoto K, Nagler CR, Núñez G. Neonatal acquisition of *Clostridia* species protects against colonization by bacterial pathogens. *Science*. 2017;356:315–9.

26. Geirnaert A, Calatayud M, Grootaert C, et al. Butyrate-producing bacteria supplemented in vitro to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Sci Rep.* 2017;7(1):11450.
27. McGaughey KD, Yilmaz-Swenson T, Elsayed NM, et al. Comparative evaluation of a new magnetic bead-based DNA extraction method from fecal samples for downstream next-generation 16S rRNA gene sequencing. *PLoS One.* 2018;13(8):e0202858.

Figures

Figure 1A

Figure 1B

Figure 1

Maturation of the microbial community during the first three weeks of age. Ontogeny of overall microbiome composition was assessed in antibiotic-naïve preterm infants (n=46) by analyzing sequencing data for microbial diversity. A. Box and whisker plot of microbiome diversity by Shannon Index using the median and interquartile ranges for each cohort. There was significantly increased microbial diversity in the week 3 compared to that in week 1; $p = 0.008$, <0.001 , $=0.002$ in axilla, groin and

stool samples respectively in antibiotic-naïve infants. B. Principal component analysis of the microbiome. Genera abundance data was subjected to generalized log2 transformation followed by principal component analysis. Samples were then colored by group membership and an ellipse was drawn at the 95% confidence interval around the group centroid. Arrows were drawn between the centroids to indicate the magnitude and direction of microbiome change in the first two dimensions. MRPP was used to assess the significance of difference in microbiome composition between groups. Genera composition was statistically distinct from weeks 1 to 3 between all three cohorts by MRPP ($P < 0.001$).

Figure 2A

Figure 2B

Figure 2C

Figure 2

Identification of genera undergoing greatest change during postnatal maturation in antibiotic-naïve infants from week 1 to week 3. Genera that significantly differed from week 1 to week 3 in antibiotic-naïve infants was determined by pairwise Wilcoxon rank sum test followed by FDR correction for multiple testing. Among those organisms, shrinkage linear discriminant analysis was used to identify those with greatest effect size. A) Genera that were significantly changed from week 1 to week 3 (uncorrected p -value < 0.05 , $FDR < 0.1$, and effect size > 0.3) are shown for organisms more abundant at week 1 than week 3 (red) or the converse (blue). Demonstrative genera include B. *Clostridium* and C. *Staphylococcus*.

Figure 3A

Figure 3B

Figure 3

Impact of gestation and postnatal ages on developmental trajectories in the antibiotic-naïve preterm infant gut microbiome. Infants were assigned to gestational age cohorts in two-week intervals. PCA was applied to generalized log2 transformed microbiome composition data from antibiotic-naïve infants at week 1 and week 3. Samples were then colored by group membership and an ellipse was drawn at the 95% confidence interval around the group centroid. Arrows were drawn between the centroids to indicate

the magnitude and direction of microbiome change in the first two dimensions. PCA calculation and graphing on the first two dimensions is the same for Figure 3A and 3B. A MRPP was used to assess the significance of difference in microbiome composition between groups. Mean Bray-Curtis distance was computed for all pairwise comparisons between group members. At both Week 1 and Week 3 there was stepwise progression in microbiome composition across the 4 gestation age cohorts, however none of the differences in composition between gestation cohorts were statistically significant. Mean Bray-Curtis distances are indicated (BC). B. Microbiome composition demonstrated progression from Week 1 to Week 3 in a similar trajectory in the first two dimensions at all gestational ages. Differences in composition were statistically significant at the earliest two gestational ages. Mean Bray-Curtis distances between the two groups are indicated (BC).

Figure 4

Impact of postnatal antibiotic exposure on skin and stool microbiome diversity and maturation. A. Box and whisker plot of microbiome diversity by Shannon Index using the median and interquartile ranges for each cohort. Fecal samples showed a decrease in diversity during the first and third weeks after antibiotic therapy, while skin samples from the groin demonstrated decreased diversity by the third week post-antibiotic use. ($p<0.001$). B. PCA was applied to generalized log2 transformed microbiome composition data from antibiotic-naïve and exposed infants at week 1 and week 3. Samples were then colored by group membership and an ellipse was drawn at the 95% confidence interval around the group centroid. PCA calculation and graphing in the first two dimensions is the same for Figures 4A and 4B. Dotted

arrows were drawn between the centroids to indicate the magnitude and direction of microbiome difference in the first two dimensions between antibiotic exposed and naïve infants. MRPP was used to assess the significance of difference in microbiome composition between groups. A. At Week 1, the composition of stool microbiome was significantly different among antibiotic-exposed infants when compared to antibiotic-naïve infants. At Week 3 the composition of groin and stool microbiome was significantly different in antibiotic-exposed and -naïve infants. C. Overlay of Week 1 and Week 3 data reveals that antibiotic exposure (dotted arrows) tends to drive microbiome composition in a direction opposite or perpendicular to postnatal age (solid arrows), suggesting that antibiotics blunt the impact of postnatal age on microbiome composition. D. Mean Bray-Curtis distance for all pairwise comparisons from Week 1 to Week 3 were determined in samples from the axilla, groin, and stool in antibiotic-naïve and -exposed infants. The mean Bray-Curtis distance is indicated as a solid line. There was a statistically significant decrease in mean Bray-Curtis distance at each body site in antibiotic-exposed infants compared to antibiotic-naïve infants (unpaired t-test $p < 0.05$) indicating that antibiotic exposure blunts microbiome differentiation from week 1 to week 3 at all three body sites.

Figure 5

Figure 5

Identification of genera most impacted by antibiotic exposure in the preterm infant gut microbiome at Week 1 and Week 3. Genera that significantly differed in antibiotic-naïve and -exposed infants at week 1 to week 3 was determined by pairwise Wilcox rank sum test followed by FDR correction for multiple testing. Among those organisms, shrinkage linear discriminant analysis was used to identify those with greatest effect size. Genera that were significantly differed in antibiotic-naïve and -exposed infants

(uncorrected p-value < 0.05, FDR < 0.1, and effect size > 0.3) are shown for organisms more abundant in antibiotic-exposed infants 3 (blue) or antibiotic-naïve infants (gold).

Figure 6A

Figure 6B

Figure 6

Association of antibiotic exposure with intestinal dominance by *E. coli* and *Klebsiella*. A. Berger-Parker Dominance index between the antibiotic-naïve and -exposed infants both at week 1 and week 3 (p-

value=0.02 and 0.004). B. Bubble chart demonstrating the composition of preterm infant gut microbiome at weeks 1 and 3 in antibiotic-naïve and -exposed infants.

Figure 7A

Figure 7B

Figure 7

Maturation of metabolic functional capacity of stool microbiome as function of postnatal age and antibiotic exposure. A. Principal component analysis of metabolic pathway composition. Overall composition of metabolic pathway genes and their maturation over three weeks was assessed in stools

from antibiotic-naïve preterm infants and compared to antibiotic-exposed assessed by unsupervised PCA. Normalized pathway abundance data was subjected to generalized log2 transformation then PCA. Samples were then colored by group membership and an ellipse was drawn at the 95% confidence interval around the group centroid. Arrows were drawn between the centroids to indicate the magnitude and direction of microbiome change in the first two dimensions. MRPP was used to determine significance of the difference in pathway abundance between groups. A. There was a statistically significant difference in metabolic functional capacity at Week 1 compared to Week 3 in antibiotic-naïve infants ($p < 0.001$). In contrast, there was a smaller and non-significant change in metabolic pathway composition in antibiotic-exposed infants from Week 1 to Week 3 ($p = 0.064$). B. Heatmap demonstrating the fold change of metabolic pathways that differ from week 1 to week 3 in antibiotic-naïve and exposed infants. Generalized linear mixed modeling with fixed and random effects (GLMM) was used to identify metabolic pathways that significantly differed from Week 1 to Week 3 in antibiotic-naïve and exposed infants. After accounting for maternal antibiotics, gestational age, delivery mode, and receipt of breast milk, genes encoding a total of 20 metabolic pathways were significantly different and had fold-change > 2 from Week 1 to Week 3 in antibiotic-naïve infants. In contrast, 34 metabolic pathways genes were significantly different in infants that received antibiotics.

Figure 8

Figure 8

Acquisition of a major butyrate and acetate synthetic pathway in the preterm infant gut microbiome was markedly impaired in antibiotic-exposed infants. The relative abundance of metabolic pathway genes was compared from Week 1 to Week 3 in antibiotic-exposed and naïve infants using GLMM. The Acetyl-

CoA to butyrate and acetate synthesis pathway, a contributor to butyrate and acetate synthesis in the infant gut, was significantly increased in abundance from Week 1 to Week 3 in antibiotic-naïve infants. Acquisition of the same pathway was markedly blunted in antibiotic-exposed infants.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalData20210711.pdf](#)