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Clinical Sequelae of Gut Microbiota Development and Disruption in Hospitalized Preterm Infants

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Supplementary Data

Supplementary Figures. PDF containing Figures S1–S9

Declaration of Interests

P.I.T. is a holder of equity in, a consultant to, and a member of the Scientific Advisory Board of MediBeacon Inc., which is developing a technology to noninvasively measure intestinal permeability in humans. P.I.T. is a coinventor on patents assigned to MediBeacon (U.S. patents 11,285,223 and 11,285,224 titled "Compositions and methods for assessing gut function" and U.S. patent application 2022-0326255, "Methods of monitoring mucosal healing"), which might earn royalties if the technology is commercialized. P.I.T. receives compensation for his roles as Chair, Scientific Advisory Board of the AGA Center for Microbiome Research and Education, and consultant to Temple University on waterborne enteric infections. He is a member of the Data Safety Monitoring Board of Inmunova, which is developing an immune biologic targeting Shiga toxin-producing E. coli infections, for which he receives no compensation, except for reimbursement of expenses. P.I.T. receives royalties from UpToDate from two sections on intestinal E. coli infections. G.D. is a cofounder, holder of equity in, a consultant to, and a member of the Scientific Advisory Board of Viosera Therapeutics, which is developing combination antimicrobial therapy against bacterial pathogens. G.D. is a coinventor on a patent assigned to Viosera Therapeutics ("Compositions and methods of use of antibacterial drug combinations," U.S. Patent Application No. 62/190,588), which might earn royalties if the technology is commercialized. G.D. is a consultant to and a member of the Scientific Advisory Board of Pluton Biosciences, which is developing methods for discovering environmental microbes for commercial applications. G.D. has consulted for SNIPR Technologies Ltd. in the last 5 years, but not presently. The authors declare no other competing interests.

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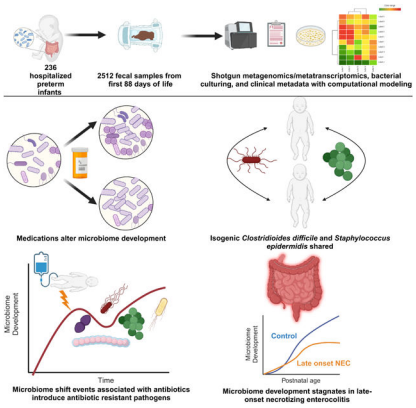
Summary

Aberrant preterm infant gut microbiota assembly predisposes to early life disorders and persistent health problems. Here, we characterize gut microbiome dynamics over the first three months of life in 236 preterm infants hospitalized in three neonatal intensive care units using shotgun metagenomics of 2,512 stools and metatranscriptomics of 1,381 stools. Strain tracking, taxonomic and functional profiling, and comprehensive clinical metadata identify *Enterobacteriaceae*, Enterococci, and Staphylococci, as primarily exploiting available niches to populate the gut microbiome. *Clostridioides difficile* lineages persist between individuals in single centers, and *Staphylococcus epidermidis* lineages persist within and, unexpectedly, between centers. Collectively, antibiotic and non-antibiotic medications influence gut microbiome composition to greater extents than maternal or baseline variables. Finally, we identify a persistent low diversity gut microbiome in neonates who develop necrotizing enterocolitis after day of life 40. Overall, we comprehensively describe gut microbiome dynamics in response to medical interventions in preterm, hospitalized neonates.

Graphical Abstract



eTOC

Preterm neonates undergo numerous interventions. Thänert, Schwartz, and Keen et al. use multi-omics to show that antibiotics and other drugs alter gut microbiome development, more so than prenatal factors. Microbiome development stagnates before necrotizing enterocolitis ensues, but only among infants who experience this devastating complication after 40 days of life.

Introduction

Newborns acquire microbes during and after delivery in all body sites from their mothers and the environment[1]. Neonates continue to accrue microbes in their gut microbiome, an organ essential for immune and nutritional development[2]. Microbiome development is shaped by diverse factors, including environment, mode of delivery, antibiotic exposures, diet, and gestational age[3–8]. Worldwide, 10.6% of all infants are born prematurely[9] (defined as birth before the 37th week of gestation) and many require months-long initial hospital care in neonatal intensive care units (NICU)[10]. NICU hospitalization and accompanying necessary interventions, including antibiotics and altered feeding interventions that differ from the home environment, can dramatically influence the developing gut microbiome[4,11,12]. In contrast to the rapid acquisition of commensal anaerobes observed in their term counterparts, the preterm infant gut is initially seeded by bacteria that include nosocomial pathobionts of hospital origin including Staphylococci, Enterococci, and *Enterobacteriaceae*[4,13,14]. These nosocomial pathobionts are abundant in the stool of infants born preterm, frequently dominate their host's gut microbiome (to >50% of the overall community), and are transmitted between infants in the NICU environment[11,13–17]. The specific clinical variables and exposures governing pathobiont and commensal entrance and exclusion into the preterm microbiome remain incompletely understood.

The hospitalized preterm neonatal gut microbiome undergoes dynamic, choreographed development with species acquisition, *in vivo* evolution of individual microbes and the community collectively, and species loss, which are each governed by microbe-microbe, microbe-host, and exogenous factors[2,4,11,18–20]. Frequent antibiotic administration exacerbates this dynamicity by rapidly reshaping the content of the developing gut microbiome[11,15]. These rapid microbiome shifts have been well characterized in other dynamic populations such as adults with inflammatory bowel disease and traveler's diarrhea[21,22], but the relative contributions of each factor that dictate how the microbiome changes in hospitalized preterm neonates are largely undetermined. Much is known about the impact of antibiotics on the preterm gut microbiome[11,12,19,20]. However, we understand little about the early-life impact of other factors, together referred to as the exposome[23], including comorbidities and non-antibiotic medications, many of which are known to have *in vitro* effects on human gut bacteria[24]. This is an important knowledge gap, because extremely low birthweight infants are routinely exposed to more medications, and for longer durations, than their higher birthweight counterparts in the same neonatal units[25]. The exposome varies in isolation and combination across intervals in neonatal care[25], and the effect on microbiome composition and function is poorly understood. Importantly, alterations in specific functional units (*e.g.*, genes, pathways) encoded in bacterial genomes, including those affecting immune homeostasis, can explain microbiome responses to perturbations not captured by taxonomic composition alone[26–28]. Thus, it is critical to study the presence or absence of both specific taxa and encoded microbiome genetic content to determine an infant's susceptibility to infections and pathologies, and, more importantly, identify the precipitants of changes in community composition[26,29].

In addition to unique medications and exposures, preterm neonates are at immense risk for devastating pathologies influenced by the gut microbiome, most notably necrotizing enterocolitis (NEC)[30–34] and bloodstream infections caused by gut-resident pathobionts[15,35,36]. NEC is a necroinflammatory gastrointestinal disorder affecting ~5–10% of all very low birthweight infants in the US and remains fatal in 15–40% of cases[37]. Prediction of NEC prior to onset has been elusive, perhaps because a wide spectrum of disease presentation exists, and variable times to onset in the first several months of life[33,38]. Failure of the immature preterm neonatal immune system to control pro-inflammatory responses to an inciting gut microbiome is thought to be at the heart of NEC etiology[33,39]. Studies have variably reported reduced microbiota diversity, increased abundance of *Enterobacteriacae*, and under-representation of obligate anaerobes in the prelude to disease onset[30,32,34,39]. However, consistent compositional and functional signatures of microbiome communities preceding NEC, the identification of which would be essential to establish reliable biomarkers, remain elusive[30–33,38–40].

Here, we sequence 1479 stools from 96 very low birthweight preterm infants, and analyze them with 409 previously sequenced stools from 92 additional preterm infants from the same cohort[11,12,34,40] to comprehensively and precisely define gut microbiota assembly in preterm infants hospitalized in three NICUs in the central United States. We interrogate independent variables that are components of the NICU exposome, including antibiotic and non-antibiotic treatments, along with dependent variables of microbiome taxonomic and functional development during hospitalization. We use strain-resolved metagenomic assembled genomes (MAGs) to identify microbial dynamics within and between individuals in response to maternal factors and NICU interventions over time. Finally, we compare 96 infants without NEC using a 2:1 match to 48 infants with NEC, interrogating an additional 624 stools using shotgun metagenomics and metatranscriptomics to investigate taxonomic and functional microbiome signatures which may precede NEC onset.

Results

Gut microbiota assembly in hospitalized preterm infants

To characterize bacterial succession in the preterm gut, we performed fecal shotgun-metagenomic sequencing on 1479 stool samples (Data S1) collected at near-daily frequency from 96 preterm infants without NEC hospitalized in NICUs in St. Louis, Missouri (54/96, 56%), Oklahoma City, Oklahoma (32/96, 33%), and Louisville, Kentucky (10/96, 10%). These infants were part of a prospective cohort investigating the bacterial etiology of NEC among preterm infants but who did not develop NEC during hospitalization (Figure 1A, Data S1–S2)[34,40]. They were born at a mean gestational age (standard deviation) (GA) of 26.4 (2.5) weeks, with a mean birthweight of 938 g (273); 48/96 (50%) were female (Data S2).

Gut microbiota development of hospitalized preterm infants has previously been described as following a common pattern, proceeding from initial dominance of *Staphylococcus* and other Bacilli in virtually all infants, to communities defined by pathobionts[4,40]. Our shotgun metagenomic data support these choreographed trajectories of the earliest microbiota assembly in preterm infants. The gut microbiota of preterm infants in our cohort

diversified rapidly over the first month of life (Figure 1B, Figure S1), accruing taxa (Figure 1C, S1), and transitions from *Staphylococcus epidermidis* dominance to communities defined by potential pathobionts, specifically *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Escherichia coli* (Figure 1C, S1A). We recapitulate prior data[4,11] demonstrating that pathobionts such as *Enterobacteriaceae*, *E. faecalis*, and *S. epidermidis* are highly prevalent and often dominate the microbiota of individual infants with >50% abundance (Figure S1B–C). Notably, we also find putatively beneficial early life colonizer microbes[2,41], including *Bifidobacterium* spp. and *Veillonella* spp. (Figure S1A, S1C), demonstrating that important commensals are acquired even during hospitalization in the NICU.

Staphylococcus epidermidis and *Clostridioides difficile* strains are shared among infants

The earliest preterm gut microbiota is influenced by microbes acquired from the NICU environment[4,13,14,17]. While our study did not include environmental samples, strain-sharing between unrelated individuals is indirect evidence for environmental acquisition. Thus, to characterize species-specific microbe acquisition from environmental sources systematically, we co-assembled, binned, and taxonomically annotated infant-specific metagenome-assembled genomes (MAGs). We de-replicated a total of 1176 high- (n=864 MAGs, ≥90% completeness, ≤5% contamination) and medium- (n=312 MAGs, ≥50% completeness, ≤10% contamination) quality MAGs (Data S3), generating a set of 197 species-level representative genomes. Using this MAG database, we profiled species-specific pairwise average nucleotide identity (ANI) values for all metagenomic sample pairs using inStrain (see Methods)[42] to identify instances of strain-sharing between unrelated infants.

We identified a total of 28,251 instances in which the same strain (≥99.999% popANI) was detected in multiple specimens across all species and sample pairs, of which 26,378 (93.4%) were from samples collected from the same infant over time. Eighty-two (85.4%) of the 96 infants studied shared at least one strain of at least one bacterial species with another infant. We specifically counted strain-sharing events only when ≥2 unique samples from the same infant pair shared a strain, to limit false positive associations. Strains of *S. epidermidis* and *C. difficile* were the most frequent species identified in stool samples of multiple, unrelated infants (Figure 1D, E). We did not observe a correlation between strain-sharing events and prevalence (p=0.53, Spearman). Strain-sharing events for *S. epidermidis* were more frequently observed than for other species with comparable cross-cohort prevalence (Figure 1D, Chi Square, p<0.001), indicating that environmental vectors may disproportionally contribute to their spread in the NICU or that these species are well-adapted to persist in the hospital environment. Strain sharing events for *C. difficile* were also more likely for its prevalence than other species (Figure 1D, Chi Square, p<0.05). *S. epidermidis* prevalence decreased with increasing day of life and was positively associated with carbapenems and glycopeptide exposure (Figure 1F). Conversely, *C. difficile* prevalence increased with day of life in the NICU and was negatively associated with administration of aminoglycosides, 4th generation cephalosporins, and carbapenems (Figure 1F). These observations suggest organism-specific dynamics of acquisition and spread, potentially associated with distinct environmental influences.

Surprisingly, we inferred 359 *S. epidermidis* strain sharing events across hospital systems (Figure 1E). To exclude the possibility that laboratory contamination explained this commonality, we randomly selected never-thawed stools from infant pairs hospitalized in three different centers (St. Louis, Louisville, and Oklahoma City) predicted to share *S. epidermidis* (n=21 pairs) and *C. difficile* (n=10 pairs) strains by sequence analysis. We then isolated single colonies from these stools and performed metagenomic sequencing, which corroborated our direct from stool sequencing that demonstrated within-hospital sharing of *C. difficile* and cross-hospital strain-sharing of *S. epidermidis* (Figure S2). Specifically, 14/21 (67%) and 7/10 (70%) of inStrain-predicted sharing events of *S. epidermidis* (Figure S2A–B) and *C. difficile* (Figure S2C–D), respectively, were validated with culturing using a relatedness cutoff of 99.999% popANI (Figure S2B,D, solid line). Strain sharing of *Enterobacteriaceae*, *E. faecalis*, and administered *Bifidobacteria* probiotics[15,29] has been demonstrated within NICUs, and *Enterobacteriaceae*, *Enterococcus faecium, Acinetobacter baumannii*, and commercial probiotics[43,44] in adults in ICUs. The identified network of *S. epidermidis* strains shared across large spatiotemporal distances suggests that specific strains are uniquely adapted to persist in the NICU environment.

Microbiome shifts introduce pathobionts and antibiotic resistance into the preterm gut microbiome

Changes in gut microbiota composition can rapidly alter microbial cues to the host's immune system[21]. Such 'microbiome shifts' are thought to contribute to the pathophysiology of gastrointestinal disorders and infections in adult populations, such as inflammatory bowel disease and traveler's diarrhea[21,22]. These events are characterized by interval community alterations between samplings that exceed the boundaries set by regularly observed intra-patient variation and are indistinguishable from inter-patient variation[21,22].

To characterize the frequency, cause, and consequences of taxonomic microbiota shifts, we profiled Bray-Curtis dissimilarity in samples consecutively collected from the same participants. The composition of consecutive samples within a 20-day period were more similar to each other than to those from unrelated individuals (Figure S3A–B), allowing us to set a threshold for detecting microbiome shift events. We identified 131 microbiota shift events (Figure S3, 1.36/patient), defined as within-individual between-sample Bray-Curtis dissimilarity greater than the average dissimilarity of between-individual comparisons[21,22] (Figure S3B). Shift events occurred significantly earlier than the average postnatal day of sample collection (Figure S3C, P=1.34e^{-07}, two sample t-test), indicating greater instability closer to birth, and were associated with taxa appearances as well as disappearances (Data S19). Overall, shifts indicated punctuations of microbiome destabilization with dramatic changes in pathobiont abundance (Figure 2A, left) as well as discrete introductions (Figure 2A, right). Shifts were significantly more likely to introduce pathobionts (*i.e.*, *E. cloacae*, *E. coli*, *E. faecalis*, and *K. pneumoniae*), into the preterm gut compared to non-shift sample pairs (Figure 2A, P≤0.01, permutation test). Shifts also depleted putatively beneficial taxa, including *Bifidobacterium longum* and *Veillonella* species as well as the same pathobionts introduced above (Figure S3). Common pathobionts introduced in shifts usually persisted

over the subsequent 10 days (Figure 2B), suggesting that these community alterations have lasting influence on the developing preterm gut microbiome.

We also found that exposure to antibiotics in the prior 14 days, especially to broad-spectrum agents like 3rd and 4th generation cephalosporins, carbapenems, glycopeptides, and lincosamides, was associated with microbiome shifts (Figure S4A). Alternatively, it is also possible that the microbiome changes prompted clinical changes leading to antibiotic administration. Notably, antibiotic resistance genes not previously identified in the same infant's gut microbiome were introduced during microbiota shifts compared to non-shift timepoints (Figure 2C, T-test $P<0.001$). These genes encode diverse mechanisms of antibiotic resistance (Figure 2D), including broad spectrum ß-lactamases (Figure S4B), suggesting that pathobionts introduced around shifts may proliferate because of their antimicrobial resistance gene repertoire. Consequently, our data suggest that microbiome shifts may act as a prime moment of vulnerability for clinical changes necessitating the introduction of antibiotics or antimicrobial-resistant pathobionts into the gut of hospitalized preterm infants, which further disrupt microbiome development.

The NICU exposome shapes microbiota development during hospitalization

In addition to antibiotics, hospitalized preterm infants are frequently exposed to a spectrum of host-directed medications and interventions[45,46]. These and other variables, such as mode and hospital of birth, maternal prenatal exposures, and infant diet, are collectively known as the 'exposome' and shape preterm infant gut microbiome trajectories[5,11,12,47,48]. To comprehensively characterize how the NICU exposome affects the preterm infant gut microbiota assembly and functional maturation during hospitalization, we integrated the 1479 metagenomic profiles of 96 infants generated above with shotgun metagenomic data from 409 stools from 92 different preterm infants from the same study population, described in previous work[11,12]. From this set, we synthesized 1,888 taxonomic profiles (Data S4) from 188 preterm infants collected over the first 88 days of life with extensive clinical metadata, including baseline variables, maternal information, antibiotic therapies, dietary exposures, and non-antibiotic medications (Data S5). Because of the rapid change of microbiota composition over the first weeks of life and the microbe-specific effects of some drugs[4,11,24,49], we hypothesized that postnatal exposures would affect microbiome composition more individually and collectively than prenatal exposures. To address this hypothesis, we profiled the impact of metadata and the NICU exposome on Bray-Curtis dissimilarity of the gut microbiome (Figure 3A) using repeat-measures permutational analysis of variance (repeat PERMANOVA). As expected, given the longitudinal nature of our study, sample ID explained 60% of taxonomic and 59% of functional variance of the gut microbiome (Data S19, p=0.003). We divided variables into three categories: baseline/maternal/diet, antibiotic, and non-antibiotic medications, and found that only 3/27 (11%) of baseline/maternal/dietary variables were significantly ($p<0.1$) associated with taxonomic variance (Fig. 3B, Data S19). Postnatal age was the most explanatory feature (p=0.003), explaining 4% of taxonomic (Fig. 3A) and 6% of functional variance (Data S19). Cumulative formula and breastmilk exposure collectively contributed 1% to taxonomic variance. No other tested baseline or maternal variables, including hospital site, delivery mode, and gestational age were significantly associated with microbiome taxonomy or

functional potential in the first 3 months of life (p>0.1, Data S19). In subsequent analyses, we focused on postnatal age instead of postconceptional age as we have previously done[40] because gestational age was not significantly associated with metagenomic variation (p>0.05, Figure 3, Data S19). To further define the impact of postnatal age on microbiome variation, we divided our cohort into 10 DOL windows with samples from infants DOL > 50 lumped together. We found that postnatal age was significantly associated (p<0.005) with microbiome development in all DOL windows with the largest effect of 1.5% variance explained from samples at the highest DOL in our cohort (DOL 51–88, Data S19). As maternal and baseline variables have been associated with microbiome differences in term infants[5,19,50], these data lead us to hypothesize that in preterm infants hospitalized in the NICU, early-life exposures contribute more to microbiome development than do prenatal exposures.

Collectively, 6/11 (55%) of antibiotic class exposures in the prior 14 days were significantly associated with microbiome taxonomic composition, and contributed 6% variance (Fig. 3B, Data S19). Surprisingly, 17/25 (68%) of non-antibiotic medication exposures in the 7 days prior to sample collection were associated with taxonomic composition, collectively contributing 10% variance (p<0.1, Fig. 3A–B, Data S19). Because medications in the NICU are often used concurrently, we determined how frequently medications were co-administered to understand the influence of non-antibiotic medications. We found that apart from the co-administration of ampicillin and gentamicin, vancomycin and gentamicin, and caffeine and gentamicin, most medication exposures did not overlap (Figure S5). Taken together, these findings suggest that the dynamics of microbiota assembly in the NICU during the first weeks of life are ordained by postnatal environmental exposures to a greater extent than by prenatal biology.

Antibiotic and non-antibiotic treatments affect early-life microbiome composition

To identify how the NICU exposome shapes taxa abundance trajectories, we implemented per-feature general linear mixed models (GLMMs) using MaAsLin2[51]. We included participant, hospital site, and study (this study, Gibson et al[11], or Gasparrini et al[12]) as random effects and evaluated all 62 baseline/maternal/diet, antibiotic, and non-antibiotic medication variables (Figure 3B, Data S5). This method corrects for known confounders and deconvolutes the effects of concurrent exposures but does not address the interactions between variables[51]. Although prenatal and maternal variables did not explain overall taxonomic variance (Figure 3B, Data S19), pre-birth maternal outpatient antimicrobials and increased gestational age were each associated with increases in single species (Figure 3C, q≤0.05). We hypothesized that prenatal and baseline variables might be more important very early in life because we did not observe that they drove microbiome composition overall (Figure 3B). We then reduced our dataset to 190 samples from 73 participants from the first 10 DOL and evaluated only the 27 baseline/maternal/diet variables and similarly found no maternal or baseline variables impacting taxonomy (q>0.05, Data S19). We conclude that maternal and baseline variables have minimal impact on early-life microbiome composition for preterm hospitalized infants.

Increasing postnatal age was associated with decreases in *S. epidermidis*, *S. warneri*, and *C. avidum* (Figure 3C) consistent with what we observed on the smaller cohort above (Figure S1C). DOL was also associated with increases in anaerobes such as 2 *Veillonella* spp., *C. difficile*, *Finegoldia magna*, and 2 *Clostridium* spp. as well as potentially pathogenic *Enterobacteriaceae* such as 5 *Klebsiella* spp. and *E. coli* (Figure 3C, Data S19). Cumulative dietary exposures, coded as fraction of days exposed to formula or breastmilk prior to a sample, were associated with alterations in key potential pathogens such as *Klebsiella*, *E. coli*, and *Staphylococcus* spp. (Figure 3C). Collectively, antibiotic administration within the 14 days prior to a sample was associated with abundance decreases of 24 species and increases of 5 species ($q \leq 0.05$, Figure 3D, Data S19). Aminoglycoside exposure within the prior 14 days, the most common exposure in our cohort affecting 946/1888 (50%) of samples, was associated with decreased abundance of *F. magna*, *S. aureus*, and *C. difficile* with increased abundance of *S. epidermidis* (Figure 3D). β-lactam antibiotics were associated with decreases of *Enterobacteriaceae* with the strongest effect of fourth generation cephalosporins in decreasing *E. coli* abundance (coefficient = −3.1, q=0.007). Penicillins (ampicillin, ticarcillin-clavulanate, ampicillin-sulbactam) and third generation cephalosporins were associated with decreased abundance of numerous *Klebsiella* species (Figure 3C). Recent third generation cephalosporins were also associated with increased *E. faecalis* abundance, which is likely due to their intrinsic cephalosporin resistance[52]. Vancomycin was associated with decreased abundance of *E. faecalis* consistent with known susceptibility[52] and *Veillonella dispar*, which may reflect susceptibility given strain-level variation of *Veillonella* spp.[53]. *Veillonella* spp. were also depleted by recent exposure to first generation cephalosporins (coefficient = −1.3, q=0.03) and lincosamides (coefficient = −3.7, q=0.001). Thus, we demonstrate abundance decreases of many potential pathogens (*Enterobacteriaceae*, *Staphylococcus* spp., *E. faecalis*) as well as putatively beneficial taxa including *Veillonella*. While we posit that the observed changes are likely due to direct killing of susceptible bacteria from the administered antibiotic, we cannot rule out that abundance changes may be secondary to the time gap between the last dose of antibiotics and sample production with concomitant increases in other microbes or the co-administration of ampicillin or vancomycin with gentamicin (Figure S5). Indeed, we have demonstrated co-exposure of these antibiotics can alter gut microbiome abundance of *Enterococcus* and *Enterobacteriaceae* distinctly than when they are administered separately[15].

We next investigated specific associations between non-antibiotic medications and gut microbiome taxonomic content. Unlike antibiotics, which were largely associated with taxonomic decreases, non-antibiotic medication exposures were associated with bi-directional changes, with 41 increases and 38 decreases of key taxa with overall prevalence greater than 10% ($q \leq 0.05$, Figure 3E, Data S19). Interestingly, the coefficient of change was often greater for non-antibiotic than antibiotic exposures (Figure 3C–E), effects that have been observed in adults with cardiometabolic disease[54]. 85 individual species were significantly associated ($q \leq 0.05$) with non-antibiotic exposures. *Klebsiella* spp. were the most frequently impacted genus accounting for 26/79 (32%) of associations with medications affecting greater than 1% of samples. Oral multivitamins and famotidine were associated with increases of 6 *Klebsiella* spp. and fentanyl, midazolam, and calcium

gluconate were associated with decreases of 8 *Klebsiella* spp. (Figure 3E, Data S19). Caffeine, the most common medication overall affecting 1668/1888 (88%) of samples, was associated with decreased *C. difficile*, increases in two *Klebsiella* spp., and increased *E. faecalis* (Figure 3E). Oral iron, affecting nearly 1/3 of samples, was associated with decreases in *S. epidermidis* and *K. michiganensis* and increases in *V. dispar* and *F. magna*. Though some of these changes may result from the increased age of iron supplementation (43 days in exposed versus 22 days in unexposed infants, Data S5), these data also suggest that iron supplementation may lead to increases in beneficial *Veillonella* and *Finegoldia*, reflecting a more mature microbiome composition[55]. Fentanyl exposure in 467/1888 (25%) of samples was associated with decreased relative abundance of *E. faecalis*, *Cutibacterium avidum*, *F. magna*, 3 *Klebsiella* spp., and *V. dispar*. These decreases might be related to opioid-induced changes in motility or secondary to other microbial changes leading to differences in the intestinal micro-environment[56]. *E. faecalis* abundance increases were commonly associated with non-antibiotic medications including steroids (dexamethasone and hydrocortisone), iron, multivitamins with iron, and cholecalciferol. Interestingly, enterococci must acquire iron for virulence and anaerobic growth in the intestine[57,58]. Collectively, these results demonstrate taxonomic abundance changes associated with antibiotic and non-antibiotic exposures or the medical conditions for which they were administered for both pathobionts and key gut commensals in the preterm infant gut during NICU hospitalization.

Developmental trajectory of functions encoded by the preterm gut microbiome

Functions encoded by gut microbes are critical for infant development, because they provide vital nutrients, educate intestinal immunity, and mediate gut barrier function, important for the defense against bacterial infections of gut origin[5,19,26,27]. Despite its central role for infant health, our understanding of the functions encoded by the NICU-preterm gut microbiome remains rudimentary.

To better understand the kinetics of gut microbiome development, we established detailed trajectories of functional assembly of preterm gut microbiota. To do this, we mapped our shotgun metagenomic microbiome functional profiles (Data S7) to 1381 metatranscriptomes from the same stools (Data S8–S9), to capture the transcription of the encoded metabolic potential. Generally, taxonomic potential and expression corresponded to the taxonomic composition of the preterm gut microbiome, with pathway richness and transcription increasing during postnatal development (Figure S6A–E). However, we found considerable variation in transcription by functional group. Expression of core functional groups, including glycolysis and nucleoside/nucleotide biosynthesis found in virtually all microbiomes, changed less over postnatal development than did expression of genes encoding amino acid biosynthesis, carbohydrate degradation, or fatty acid and lipid biosynthesis (Figure 4A). Critically, amino acid and fatty acid biosynthesis by the microbiome are considered important for host immune modulation and development[3,59,60], highlighting that the transcriptional trajectories of these functional groups during postnatal maturation may directly affect infant health. *Enterobacteriaceae* dominated the expression of pathways changing during postnatal development, with genomes of *Escherichia* spp., *Klebsiella* spp., and *Enterobacter* spp. responsible for their transcription (Figure 4B,

C). Conversely, the transcriptional contribution of key commensals, including *Veillonella* and *Clostridium* species (Figure S6G), was less pronounced than the dominant effect of pathobionts on the transcriptional landscape during the first weeks of life.

Because we found evidence that the NICU exposome shapes the taxonomic composition of the preterm gut microbiota (Figure 3), we hypothesized that the environment also influences the transcription of genes encoding functional trajectories. Thus, we implemented GLMMs as described above and comprehensively characterized the impact of the NICU exposome on the expression of metabolic pathways. We identified a substantial impact of dietary, antibiotic, and non-antibiotic medications on the transcription of key metabolic units, including secondary metabolite, amino acid, vitamin, and fatty acid biosynthesis (Figure 4D). While antibiotics and most non-antibiotic medications were largely associated with reduced expression of metabolic potential, chlorothiazide and dexamethasone were associated with increased expression of multiple functional pathways. Importantly, transcriptional microbiome restructuring was not restricted to single pathways but frequently affected multiple functionalities (Figure 4D), highlighting the vast and overlooked impact of the NICU exposome on the preterm gut metatranscriptome.

We next asked if the observed transcriptional response was an indirect consequence of taxa abundance alterations or was driven by transcriptional regulation. To answer this question, we profiled concordance between taxonomic responses to NICU exposures on the DNA and RNA content using GLMMs. We found that while negative transcriptional responses to antibiotics were largely explained by taxa depletion rather than regulation, pathways were often downregulated in response to non-antibiotic medications and diet exposures (Figure 4E). Specifically, transcriptional responses to milk formula and Vitamin D_3 were mostly unexplained by taxa depletion, suggesting active transcriptional regulation by the intestinal microbiome in response to early-life exposures.

Multi-omics analysis supports a variant microbial population as a risk factor for a subset of NEC

Previous work suggested that aberrant developmental progression of the preterm gut microbiome plays a fundamental role in the development of NEC[30,32,34]. Here, we used multi-omic characterization of the intestinal microbiota in hospitalized, non-NEC preterm infants to systematically query gut microbiome developmental trajectories of infants that developed NEC at any point during their NICU stay for differences in bacterial composition or function. We generated 624 longitudinal shotgun metagenomic and transcriptional profiles of the pre-NEC gut microbiome in 48 infants (see Methods, Data S1, S10–13). Comparisons were performed using a 1:2 matched case-control study design (Figure 1A), where controls were matched to cases based on NICU location (St. Louis, Louisville, and Oklahoma City), gestational age (within one week), and birthweight (within 100g). Using repeat measures PERMANOVA, and the entire NEC cohort specimens, neither the taxonomic composition of the pre-onset NEC microbiome or metatranscriptome, nor its functional profiles, varied significantly by NEC status (Figure 5A). We also found no significant difference in the bacterial replication rate of any taxonomic group prior to NEC onset (Figure 5B, Data S14), as has been previously reported[32]. Similarly, we found no

difference in the community level virulence factor repertoire of cases and controls (Figure S7A–B, Data S15–17). Further, microbiota shifts were neither more frequent (Chi-square test P=0.876) nor occurred at different times relative to disease onset in the NEC case group (Figure 5C, Wilcoxon rank sum test P=0.664).

As signatures of microbiome alterations preceding NEC might be more subtle, i.e., with effects limited to individual taxa or pathways, we next implemented GLMMs. Adjusting for all confounders that were associated with preterm gut microbiota development independent of disease status (i.e., in the control-only analysis), we found no association of any species, metabolic pathway, or transcriptional signature with case or control status (MaAsLin2 all q≥0.25). Further, investigating high-quality MAGs of the species *K. pneumoniae*, a genomically diverse species that has previously been implicated in NEC development[32], we found no virulence repertoire differences between cases and controls (Figure S7C).

To account for the possibility that multi-level associations may explain individualized risk for NEC, we combined all multi-omic data (metagenomic profiles, metatranscriptomes, bacterial replication rate, community- and MAG-associated virulence profiles) using three distinct statistical modeling approaches (logistic regression, elastic net, random forest). Critically, we accounted for repeat-measures by blocking model cross-validation by individual and nesting three iterations of feature selection (25, 50, 100 features) within the cross-validation procedure, performing feature selection for each training-fold separately. Moreover, we trained models using either all pre-NEC data, a subset of data collected during the 7 or 3 days prior to onset, or on the day of clinical diagnosis. Model performance did not differ between algorithms or by number of features included in the final model (Figure 5D, Figure S8). We observed an increase in model performance peaking on the day of diagnosis, indicating that microbiome signatures are more distinguishable between cases and controls when the clinical diagnosis is made. Generally, models did not perform better than random chance, highlighting that the population-level and genome-resolved microbiome features collected in this study do not support a common microbiome imbalance that is uniformly detectable prior to the development of NEC.

While not reliably predicting NEC in all infants, we did observe a significant difference of microbiota diversity when examined by postnatal age of NEC occurrence (Figure 5E, GLMM P<0.05). Specifically, the microbiota diversity of cases and controls separated beyond postnatal day 40, when NEC case gut microbiota failed to increase in diversity in contrast to the gut microbiota in the matched case group. Twelve infants (25% of all cases) developed NEC beyond postnatal day 40, hereafter referred to as ‘late onset’ NEC. When compared to infants with onset ≤postnatal day 40 (‘early onset’), late onset cases were born at significantly younger gestational ages (24.0 (IQR 23.0–25) vs. 27.0 (IQR 25.0–28.8) weeks, Mann-Whitney p=0.0002) and lower birthweights (695g (IQR 561–798) vs 907g (IQR 773–1175) g, Mann-Whitney p=0.0006). This is consistent with reports that infants of shorter gestational age at birth develop NEC later compared to infants born following longer gestation[33], and also inferred in the predecessor study[34].

While no microbiome feature was significantly associated with NEC status when analyzing early onset cases and matched controls separately (MaAsLin2 all q≥0.25), several taxa,

pathways, and transcriptional signatures separated the microbiomes of late onset cases from matched controls. For late onset NEC, Negativicutes, and in particular *Veillonella parvula*, and Clostridia, were significantly associated with control status, while *Klebsiella* abundance was significantly increased before the event (Figure 5F, G). When applying Dirichlet multinomial modeling to the taxonomic gut microbiota composition of cases and controls we similarly found that early onset cases and controls were indistinguishable, while the developmental enterotype trajectories of late cases and controls diverged (Figure S9). Specifically, we found that enterotypes 2–5 were over-represented among late-onset NEC cases relative to controls. Conversely, the stools of none of the late onset cases had gut microbiome compositions consistent with enterotypes 6–8 (Figure S9). Further, late-onset NEC was preceded by significant alterations in the functional maturation of the gut microbiome compared to control infants (Figure 5H). While these signatures were apparent at the level of the metagenome, they were even more pronounced, and often only detectable, in analysis of bacterial transcripts. Thus, several metabolic groups, including cofactor/prosthetic group/electron carrier/vitamin biosynthesis, TCA cycle, secondary metabolite degradation, and amino acid degradation exhibited altered transcription (vs. controls) without underlying metagenomic differences between cases and controls for several pathways (Figure 5H). Collectively, these data show that while the microbiome may not hold the key for individualized risk prediction for all infants born preterm, it may forecast disease onset after postnatal day 40, and that transcriptional analysis offers greater discriminatory information than metagenomic profiling alone.

Discussion

Intestinal host-microbe interactions educate a newborn's immune system and affect health trajectories into adulthood[50,61]. Preterm infants often require months-long hospitalization in the NICU, an environment depleted of reservoirs of normal microbiota and enriched in microbiota-altering medical therapies and dietary regimens[14,19]. It has been proposed that preterm birth and the NICU exposome interact to individualize neonatal microbiota trajectories that affect early-life health outcomes[11–13,15,19]. Here, we characterized functional and taxonomic trajectories of the neonatal microbiome of 188 hospitalized preterm infants without NEC in the context of their baseline characteristics and NICU exposures, and found that the hospital environment is a major factor in seeding preterm infant bacterial gut microbiomes. Most noticeably, isogenic *S. epidermidis* are present in sites of care in three different states within the USA. This suggests that NICU-adapted lineages of this species could be universal. Given that *S. epidermidis* can be pathogenic in the NICU, including as a cause of late onset sepsis[62,63], further work is warranted to validate and extend this unexpected finding. Sharing of other species previously reported to be common between infant guts and NICU surfaces[13–15,17], specifically *Enterobacteriaceae*, *Enterococcaceae*, and *Pseudomonas*, was less frequently observed across infants and between centers in this study.

Consistent with previous reports[11,12,15], we also find that antibiotic exposure is associated with profound and consequential microbiome shifts throughout the first 88 days of life. These events are not benign: they allow pathobionts to colonize hospitalized preterm infants and enable bacteria with genes encoding antibiotic resistance to enter the gut

habitat. Alternatively, it is possible that gut entry of the organism leads to a clinical change prompting antibiotic administration. Teasing apart the temporal nature of these events will be important to minimize negative consequences of antibiotic prescribing[64,65] while ensuring rapid treatment of potential sepsis[66]. Hence, our data reinforce concern that antibiotics alter bacterial community shifts that spread clinically-relevant resistance elements into and within newborn gut microbiomes, and that these organisms persist as colonizers[4,11,12,36,67]. Drug-resistant microbes and resistance genes enriched in preterm infant gut microbiomes during early-life hospitalization can be detected in stool long after infants have been discharged and can be sources of bloodstream infection in the NICU[12,67], highlighting the durability and clinical relevance of early-life microbiome disruptions.

We also demonstrate significant microbiota alterations associated with non-antibiotic medications in preterm infants. While some effects might be explained by co-administration with antibiotics, we found that most medications that affected the microbiome were prescribed in isolation (Figure S5). Importantly, we cannot exclude that observed effects on microbial communities are caused by the pathology that obligates the medications rather than the medications per se. Our results are further supported by *in vitro* work that showed broad effects of non-antibiotic medications on gut microbiota using drug-by-microbe growth assays[24]. Intriguingly, microbial resistance mechanisms can be shared between some human-targeted drugs and antibiotics[24]. Therefore, our findings suggest that non-antibiotic medications may contribute to the high prevalence of drug resistance in the NICU microbiota. Further, we find that postnatal exposures explained more microbiome variation and were significantly associated with greater taxonomic changes than prenatal and baseline variables. These observations highlight the importance of high-frequency temporal sampling and integration of clinical variables and environmental exposures when profiling microbiome-associated health risks in preterm infants.

Our research adds to the literature on the role of the preterm infant gut microbiome in the multifactorial pathophysiology of NEC[30,33]. A current hypothesis of NEC development proposes that aberrant microbial colonization interacts with immature intestinal immunity to generate an uncontrolled inflammatory response, causing loss of barrier integrity and tissue necrosis[33]. At the aggregate cohort level, our results do not support this variant microbiome hypothesis, as pre-NEC multi-omic trajectories do not vary between cases and matched controls. Also, we did not identify correlates of disease onset and multi-omic integration via statistical modeling as predictors of NEC risk better than random chance before the day of onset. Nonetheless, we identified a subset of infants born after significantly shorter gestations and who developed NEC after postnatal day 40, whose microbiome trajectories differed compared to their matched controls. Despite some differences in the cases and controls studied, this finding resembles trends reported from these cohorts using 16S rRNA gene sequencing[34]. These infants' microbiomes are characterized by reduced microbial diversity, increased abundance of *Klebsiella*, and decreased abundance of specific commensals, predominantly *Veillonella*. Signatures of functional microbiome disruption were most apparent in the metatranscriptome, which showed extensive functional alterations compared to matched control infants. Importantly, however, the microbiome profiles of early- and late-onset NEC cases do not differ, suggesting that NEC in infants who are born most preterm could be caused by failure of microbiome maturation, with the consequence

that pathobionts persist in a more susceptible intestinal tract, i.e., a two-hit predilection. While this refined hypothesis de-emphasizes a microbiome-exclusive role in NEC causality, it extends prior work in this field that suggests fundamental biologic differences in NEC by postnatal age of onset[33]. Even though histologically and clinically early- and late-onset NEC are quite similar (though the latter has greater mortality[37]), the actual occurrence of late onset NEC (*i.e.*, in this study after day of life 40) could relate to yet-to-be identified host responses to specific gut bacteria. It remains to be established if pre-NEC microbiome differences reflect a precipitating effect (*e.g.*, from *Enterobacteriaceae*[39]), a lack of a protective effect (*e.g.*, from *Veillonella*), or if the at-risk mucosa is more hospitable for an aberrant microbiome. Nevertheless, our data suggest that strategies to promote maturation of gut bacterial communities such as limiting unnecessary antimicrobials[20,68] and possibly non-antibacterial agents might protect children born most prematurely from NEC[69].

Our study has several important limitations. First, at the time of fecal sample collection, RNA transcriptomics was not planned, so preservatives were not included in storage. All samples were handled and stored for similar intervals, but we cannot exclude transcript degradation or other changes before RNA extraction. Our metagenomic associations and strain tracking are conducted on an average of 5 million reads per sample. We have previously shown this to be adequate for tracking strains between the bloodstream and gut for organisms with detectable metagenomic depth at this sequencing threshold[15]. Although the gut microbiome of the preterm infants studied here has low diversity, it is possible we cannot adequately track low abundance strains with inStrain or iRep[42]. Given these limitations, we suggest that future studies utilize appropriate preservatives and incorporate higher sequencing depth and measurements of absolute abundance to validate and extend the findings presented here. Further, our associations, like many in the microbiome field, are determined using relative abundance, which can obscure changes in absolute microbial abundance[4]. We would also note that breastmilk from each mother differs in composition and antibody content with similarly varied potential effects on the infant gut microbiome[55,70]. Additionally, the daily fraction of nutrition derived from formula versus breastmilk impacts microbiome maturation as has been previously demonstrated[71,72]. These personalized differences would not be addressed by our study design but are likely plausible additional drivers of individual microbiome trajectories. Our study design also prevented control over indication for use, timing, or route of antibiotic and non-antibiotic medication exposures. Thus, we cannot differentiate cause and effect with the administration of these medications versus the disease process necessitating them. Variations in timing of stool intervals could result in unequal timing of exposures, including human milk. Finally, human microbiome studies alone cannot separate cause and effect, which will require complementary systems-based mechanistic investigations, including animal models.

In summary, our integration of a vast clinical metadata database with multi-omic microbiome trajectories provides a framework for studying how gut bacterial communities ordain specific outcomes in a range of medical disorders, especially those in which there is a time series component to the variables studied. Specifically, our findings endorse identifying and systematically correcting for variables that affect the gut microbiome development in attempts to thoroughly assess microbial community-driven outcome risks.

STAR Methods

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources should be directed to and reasonable requests will be fulfilled by the Lead Contact, Gautam Dantas (dantas@wustl.edu).

Materials availability—This study did not generate new unique reagents.

Data and code availability—Raw sequencing data (metagenomes, metatranscriptomes, and metagenomic-assembled genomes) generated for this study was uploaded to the SRA database under BioProject PRJNA799247. Accession numbers are listed in the key resources table. Relevant raw data and metadata can be found as extended data spreadsheets. We use well-established computational and statistical analysis software and packages. Custom code for analysis and figure generation is available at https://github.com/dantaslab/NICUExposome(10.5281/zenodo.12737979) and is publicly available as of the date of publication. DOIs are listed in the key resources table. There are restrictions to the availability of infant stool raw materials due to extreme resource limitations.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Participants for this prospective, multi-center cohort study[34,40] were recruited from among all infants born prematurely (≤37 weeks of gestation) at or below 1500 g of weight and expected to survive more than a week at St Louis Children's Hospital (St Louis, MO, USA), Children's Hospital at Oklahoma University Medical Center (Oklahoma City, OK, USA), and Kosair Children's Hospital (now Norton Children's Hospital) (Louisville, KY, USA). All infants hospitalized in the NICU were considered eligible for enrolment. Infants born with congenital noncardiac disorders were excluded. To study the impact of the gut microbiome on NEC development, we selected infants for a 1:2 case-control study from the total eligible study population. Cases were defined as infants whose clinical symptoms were consistent with NEC and whose radiographs fulfilled all criteria for Bell's stage ≥2 NEC. Two non-NEC control infants (n=96) were matched to each NEC case (n=48), based on hospital site, gestational age at birth (±7 days) and birthweight (±100g). Demographics are provided in Data S1–S2. Metadata and samples were approved under Washington University HRPO #201105492, University of Louisville Institutional Review Board (IRB) HSPO #11.0136, and University of Oklahoma Health Sciences Center IRB 2472. The IRB at each site approved the original study, and perants were provided informed consent. Secondary analyses in the current manuscript were approved by Washington University School of Medicine HRPO #201205152.

All stools produced by infants were collected, refrigerated at 4°C, and then frozen (−80°C) daily without additives until chipped for these analyses. Samples were collected prospectively from 2009 to 2013 without prior knowledge of later NEC diagnosis. All stool samples collected prior to the day of life of clinical NEC diagnosis (day of onset, DOO) were included in the analysis for this study if quantities were sufficient for analysis. We analyzed stools from 48 infants who developed NEC, including 42 of the 46 cases of NEC

in our prior publication[34], 4 infants who also had congenital heart disease, 1 infant who developed NEC late in life (day of life 121, but from whom we had stool up to day of life 81), and 1 infant for whom we lowered the threshold for residual stool to include in this analysis. We used control specimens from 26 of the 94 participants without NEC from Warner et al.[34], and 70 additional infants without NEC. For non-NEC controls all stool samples collected prior to DOO of their matched case were included. To robustly define the impact of the NICU exposome on gut microbiome trajectories in the NICU previously published shotgun-metagenomic data from stool specimens of an additional set of 92 non-NEC infants from the same cohort were included in the non-case-control analysis of this study[11,12].

METHODS DETAILS

Metagenomic/metatranscriptomic sequencing—Metagenomic DNA was extracted from ~25mg of stool, chipped on dry ice into sterile tubes without thawing. Metagenomic DNA was extracted from stool using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's protocol, except that samples were mechanically lysed for two rounds of two minutes each using a Mini-Beadbeater-24 (Biospec Products) at 2,500 oscillations per minute. Metagenomic DNA was quantified using the PicoGreen quantitation assay (Thermo Fisher Scientific) and stored at −20°C. Genomic DNA (0.5 ng) was used as input in preparation of sequencing libraries with the Nextera XT kit (Illumina) as previously described[73]. Libraries were pooled and sequenced to a depth of ~2.5 million paired-end reads (2×150 bp) on a NextSeq500 High Output platform (Illumina).

Total RNA extraction for metatranscriptomic analysis was performed from ~100mg of stool, chipped on dry ice into sterile tubes without thawing using the NucliSENS easyMAG system (bioMerieux). All stool samples with ≥200 mg of remaining stool material from the NEC case-control part of this study were included in this analysis. Frozen stool was homogenized in 1 ml of easyMAG lysis buffer in a bead beating tube containing 8–10 disruption beads zirconium/silica 23mm (Research Products International Corp.). Samples were disrupted using the MP FastPrep-24 tissue homogenizer (MP Biomedicals) at 6.5 m/s for 60 sec. The lysate was centrifuged at 12,000xg for 10 minutes and the clarified supernatant was loaded into wells of the easyMAG cartridges, avoiding visual particulates. Total lysate volume of each sample was then adjusted to 2.2 ml with EasyMAG lysis buffer followed by addition of 50 μL of NucliSENS easyMAG magnetic silica beads. Samples were then loaded onto NucliSENS easyMAG system for automated nucleic acid extraction following the manufacturer's instructions with onboard lysis "Specific A" protocol and 110 μL elution volume. Contaminating DNA was removed using the Turbo DNase (Thermo Fisher) kit and ribosomal RNA was depleted using the QIAseq FastSelect – 5S/16S/23S kit (Qiagen). Sequencing libraries with unique dual indexes were prepared using the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (New England BioLabs). Libraries were sequenced to a depth of ~13 million 2×150 bp paired-end reads on the NovaSeq S4 platform (Illumina).

Sequencing data pre-processing—Sequencing adapters were removed from demultiplexed reads with Trimmomatic 0.366 (leading = 10, trailing = 10, sliding window

= 4:15; minimum length = 60)[74]. Human reads were removed using Deconseq 4.37 with default parameters[75].

QUANTIFICATION AND STATISTICAL ANALYSIS

Metagenomic/metatranscriptomic profiling of the gut microbiome—Quality-filtered short reads were profiled using MetaPhlAn3 v3.0 and HUMAnN3 v3.0 with default parameters to quantify relative abundances of bacterial species and encoded pathways, respectively[76]. Antibiotic resistance genes (ARGs) were quantified using ShortBRED and a marker database containing all resistance gene sequences available in the CARD database as well as additional genes confirmed to mediate resistance using functional metagenomic analysis in a previous study of this same cohort[11,12,77]. All marker sequences with >1 hit and >0 RPKM per sample were retained. Similarly, virulence factor abundance in both metagenomic and metatranscriptomic datasets were profiled using ShortBRED[77] and a database of marker sequences built on all sequences downloaded from the Virulence Factor Database (VFDB) and Victors (September 2021)[78,79]. Virulence factors identified to be present in the preterm microbiome (>1 hit and >0 RPKM) were manually curated into 19 virulence categories based on literature research (*e.g.,* Adhesion/Invasion, Capsule, Motility – see Data S15–17), allowing factors to match into multiple categories. Metatranscriptomic datasets were profiled using the MetaCYC pathway database with HUMAnN3 v3.0 with default parameters.

Metagenome-assembled genomes (MAGs) were generated for each infant using a co-assembly approach to generate data of sufficient sequencing depth for assembly. Genomes were assembled using MetaSPAdes[80] using default parameters. All reads from all patient-specific samples were concatenated and aligned to indexed metagenome-assembled scaffolds using Bowtie2 (parameter: --no-mixed --very-sensitive --n-ceil 0,0.01)[81], sorted using samtools (default parameter)[82], and binned using MetaBat2 (parameter: --minContig 2000)[83] to generate MAGs. MAG assembly quality was assessed using checkM and Quast[84,85]. MAGs were categorized as high-quality (completeness ≥90%, contamination ≤5%), medium-quality (completeness 50–90%, contamination 5–10%), or low-quality (completeness ≤50%, contamination ≥10%). Both high and medium quality MAGs were used in downstream analysis as supported by prior work[86]. These were used to generate a study-database of species-level representative genomes de-replicated across all individuals included in the case-control part of the study using dRep (gANI routine, 95% ANI)[87]. To taxonomically annotate all MAGs, each MAG was screened against the RefSeq genome database (downloaded July 2019) using mash-screen (default parameter)[88]. Subsequently, each MAG's pairwise average nucleotide identity was profiled against the top three mash-screen hits using pyani (method: ANIm)[89]. Species-level taxonomical annotation was assigned to MAGs with pairwise ANI ≥94% at ≥50% reference coverage on a best-hit basis.

Bacterial replication rates were estimated based on metagenomic sequencing data using iRep as previously described[32]. Briefly, quality-filtered DNA short reads from each sample were mapped to all available dereplicated genomes from the corresponding patient with Bowtie2 (--very-sensitive mode). The resulting SAM files and dereplicated genomes were used as inputs for iRep with default parameters. Only iRep values passing all genome and mapping

quality thresholds (min cov. = 5, min wins. = 0.98, min r^2 = 0.9, max fragments/Mbp = 175, GC correction min r^2 = 0.0) were retained for further analysis.

***In silico* strain-sharing prediction and validation—**Metagenomic short-reads from stool samples of 96 non-NEC controls sequenced for this study were aligned to these de-replicated MAGs using inStrain[42]. Population ANI (popANI) values of ≥99.999% with breadth ≥0.5 at ≥25% of the reference genome being used in the comparison of two samples were considered evidence for strain-sharing. This amounts to a maximum of 50 single nucleotide polymorphisms (SNPs) for a genome with the size of 5,000,000bp. To guard against false positives resulting from contamination occurring during sample extraction or library preparation, we included infant pairs only if ≥2 unique samples from the same pair of infants indicated a strain to be shared. Strain-sharing events between unique infant-pairs were visualized as network graphs using Cytoscape[90]. Significance of strain-sharing by species was determined using a chi-square test followed by a pairwise nominal independence test with Benjamini-Hochberg correction using the r package rcompanion v2.4.35.

To confirm *in silico* predictions derived from stool metagenomes, *S. epidermidis* and *C. difficile* were selectively grown from samples of randomly selected infant pairs predicted to share the same strain. These outgrowth experiments were performed by personnel not involved in sample processing for stool-metagenomic sequencing. Approximately 10mg of fecal material was resuscitated in 0.15 ml of thioglycolate broth (Sigma). *S. epidermidis* was selectively cultured by inoculating and incubating TSB-CNA broth [TSB (BD) with colistin (20μg/ml), nalidixic acid (20μg/ml) and aztreonam (8μg/ml)] overnight at 37°C followed by inoculation of TSB-CNA broth with 6.5% NaCl and overnight incubation at 37°C. *C. difficile* was grown from stool anaerobically in reduced thioglycolate broth (Sigma) (37°C, overnight), followed by inoculation of CCMB-Tal medium (Anaerobe Systems) and incubation at 37°C overnight anaerobically. For *S. epidermidis*, cultures were streaked on blood agar plates (BD) and catalase tests (3% H2O2 in water) were performed following overnight incubation at 37°C. For *C. difficile*, cultures were streaked on selective ChromID *C. difficile* agar (BioMerieux Inc.) and incubated anaerobically overnight at 37°C for the appearance of black colonies typical for *C. difficile*. Metagenomic DNA was extracted from cultures of a single colony using the Qiagen PowerSoil Pro kit (Qiagen), libraries were prepared using a modified version of the Nextera kit (Illumina)[73], and sequencing was performed on the Illumina NextSeq platform.

Metagenomic reads were assembled using MetaSPAdes[80], binned using MetaBat2[83], annotated using mash and pyani[88,89], and de-replicated using dRep[87] as described above. Strain-sharing across sample pairs was investigated and confirmed using inStrain[42], using the same ≥99.999% popANI and ≥25% genome-compared threshold described above.

General microbiome analysis—Statistical analyses and visualizations were conducted in R v.4.0.4, v.4.2.1, and v4.4.0 using the ggplot2, labdsv, scales, vegan, ape, ggridges, reshape2, lme4, nlme, multcomp, MuMIn, MaAsLin2, DirichletMultinomial, optparse, ggpubr, rsample, purrr, tidyr, tidyverse, rowr, metR, purrr, rsample, dplyr, permute,

BiodiversityR, SIAMCAT, curatedMetagenomicData, RColorBrewer, compositions, scico, viridis, grid, glmmADMB, and rcompanion packages in Rstudio v2022-v2024.

Principal component analysis (PCA) was conducted on centered log ratio transformed relative abundance data (taxa or pathway abundances). Smoothed Shannon diversity readouts were plotted as background into the PCA space using the ordisurf function of the vegan package. For further visualizations, α-diversity was calculated with the vegan package (index = Shannon) on untransformed taxa or pathway data. Between-sample β-diversity was calculated using the vegdist function (method = Bray-Curtis). Procrustes analysis of taxonomic, functional, and transcriptional relative abundance data was performed using the procrustes function in the R vegan package.

Microbiome shift analysis—Microbiome shifts were defined as two consecutive samples collected from the same individual displaying Bray-Curtis dissimilarities equal or greater than those observed between the average of samples collected from different individuals. Bray-Curtis dissimilarity distributions were determined for consecutively collected inter- and all intra-patient sample pairings (non-NEC controls only) based on untransformed MetaPhlAn3 relative abundance data. The point at which the density distribution of interpatient dissimilarity intersects with that of intra-patient dissimilarity (Fig S3B) was set as the threshold defining a shift event. As Bray-Curtis dissimilarity between two samples increases over time (Fig S3A), shift thresholds were calculated independently for the observed range of DOL deltas between consecutively collected samples (Fig S3B).

To identify species that were significantly depleted or introduced at shift events, pre- and post-shift taxa presence was determined for all sample pairs flanking shift events (relative taxa abundance > 0%). Taxa were defined to be introduced if they were absent pre-shift but present post-shift. Conversely, depletion events were defined as taxa presence pre-shift and absence post-shift. To determine significant associations of introduction/depletion and shift events taxa-specific permutation tests were implemented. Therefore, the taxa-specific number of introduction/depletion events at shifts (n=131) was compared to the distribution of introduction/depletions observed across 1000 random permutations of an equal number of non-shift sample pairs (n=131). As shifts occurred earlier in life compared to the median sample DOL of non-shift sample pairs (Fig S3C), random permutations were restricted to generate a non-shift sample pair distribution following a similar DOL distribution as observed in the shift sample pairs using a simple bin-based density estimator in R. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg method (false discovery rate) in R.

Similar to introductions/depletions, permutation tests were implemented to identify clinical exposures associated with microbiome shifts. Antibiotics were aggregated at the class level. Binary recent exposure values (yes/no in the fourteen days prior to a shift) were counted for all shift events and compared to the distribution observed in 1000 permutations of non-shift sample pairs generated as described above. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg method (false discovery rate) in R. Significant introduction of ARGs at shifts was determined via permutation test as described above. Introduced ARGs were defined as all ARGs found directly following the shift and never

seen in an individual's gut microbiome before. ARGs were summarized by resistance mechanism for visualization purposes.

Enterotyping—Dirichlet multinomial mixture (DMM) models were implemented to generate clusters of samples of similar microbial composition ('enterotypes') based on relative abundances of microbial taxa transformed by 5000-fold multiplication to eliminate values below 1 for all samples in the case-control study using the R package DirichletMultinomial (k=40, iterations=1000)[91]. The optimal model was selected based on minimization of the model Laplace approximation. Samples were assigned cluster identity based on their highest cluster-identity probability value.

Defining the impact of the NICU exposome and baseline characteristics on gut microbiome trajectories during hospitalization—We comprehensively characterized the impact of patient baseline variables (Data S5: gestational age, birthweight, sex, delivery mode,), dietary exposures, antibiotics (aggregated on the class level), non-antibiotic medications (by route), and maternal variables (Data S5: maternal age, race, body mass index, diabetes, pre-eclampsia, chorioamnionitis, steroids, outpatient and inpatient antimicrobials, and multiple gestations) on developmental microbiota trajectories of preterm infants during hospitalization. All non-NEC controls as well as metagenomic data from 92 participants previously published[11,12] were included in this analysis. Each sample was assigned patient-specific constant variables (*e.g.*, sex, race, birthweight, gestational age at birth). We subdivided variables into three categories: maternal/baseline/dietary, antibiotic, and non-antibiotic medications. Variables changing over time were treated as follows; dietary exposures (formula, human milk) were assigned to each sample as the fraction of days of life prior to sample collection exposed. Antibiotics were assigned as binary recent exposure values if received in the 14 days and non-antibiotic medications in the 7 days prior to sample collection (Data S1, S5), previously shown to capture the acute effects of antibiotic exposures in the preterm gut microbiome[12] and non-antibiotic exposures in adults[92]. Administration route was not available on a per-infant basis; however, many medications had predetermined routes for their use in preterm infants or as outlined in the study protocol (Data S18). Maternal exposures and comorbidities were coded as binary values and assigned to each sample on a per-patient basis.

Repeat measures permutational analysis of variance (PERMANOVA) was conducted as previously described[15,21,67] to characterize the impact of the clinical exposome and baseline characteristics on the developing preterm gut microbiome. Patient identity and study source (this study, Gibson et al.[11], Gasparrini et al.[12]) were included as a mandatory blocking factor in all repeat measure PERMANOVA analyses. Variance explained was calculated independently for each variable to avoid issues of variable ordering. Variables were considered to explain a significant portion of the observed variance of taxa or pathways if $P \leq 0.1$. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg method (false discovery rate) in R.

To further identify associations of the NICU exposome on specific taxa and pathway abundances and expression, generalized linear mixed models (GLMMs) were implemented using MaAsLin2 with minimum taxonomic prevalence of 10% and *q*-values ≤0.25

considered significant as per the default[51] (Data S19). For taxonomic changes related to NICU exposures (Figure 3), all variables (Data S5) were included as fixed effects with random effects of hospital site, participant, and study source and only features $q<0.05$ and exposures present in >1% of samples are plotted. Subsequent analyses utilized only variables identified to explain a significant portion of the variance ($P \leq 0.1$) of a dataset (DNA pathways, RNA taxa, RNA functions) by repeat measures PERMANOVA. Day of life and gestational age at birth were included as mandatory fixed effects. Additionally, GLMMs were implemented using the R package nlme to determine significant differences in bacterial replication rates in NEC cases and controls. Significance was determined by comparing a model including NEC status against a null model using ANOVA.

Transcriptional overrepresentation analysis—To identify transcriptionally over-/under-represented taxa compared to their relative abundance in the shotgun metagenomic data, species-specific zero-inflated gaussian models were implemented on arcsine transformed relative taxa abundance data using the glmmADMB package in R. Therefore, relative abundance data for each species generated from both metagenomic and metatranscriptomic reads via MetaPhlAn3 was analyzed. Day of life was included as a fixed effect in all models. Patient ID was included as a random effect. Models took the form: abundance ~ data_type+$\log_{(\text{day of life})}$+(0+$\log_{(\text{day of life})}$|Patient_ID/data_type), with data_type = DNA or RNA. Significant differences between RNA and DNA relative abundance data were determined for each species individually. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg method (false discovery rate) in R.

Machine learning—Three classes of machine learning algorithms (logistic regression, random forest, elastic net) were trained using all microbiome features characterized in this study (metagenomic profiles, metatranscriptomes, bacterial replication rate, community- and MAG-associated virulence profiles) using the R package SiamCat v2.0.0. Features were filtered using a prevalence cut-off of 0.01 and normalized with the method ‘log.std’ (parameter: log.n0=1^{e-05}, sd.min.q=0). To account for repeat sampling cross-validation procedure was blocked by patient. Feature selection was performed with threshold of 100, 50, or 25 features nested within-cross validation and the following parameters: methods.fs=AUC, direction=absolute. Cross-validation was performed with the following parameters: num.folds=10, num.resamples=10.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Antibiotics and non-antibiotic drugs govern infant gut microbiome development
- Antibiotic-associated microbial shifts introduce pathogens and resistance
- Hospitalized preterm infants share isogenic *C. difficile* and *S. epidermidis*
- Microbial development stagnates before late-onset necrotizing enterocolitis

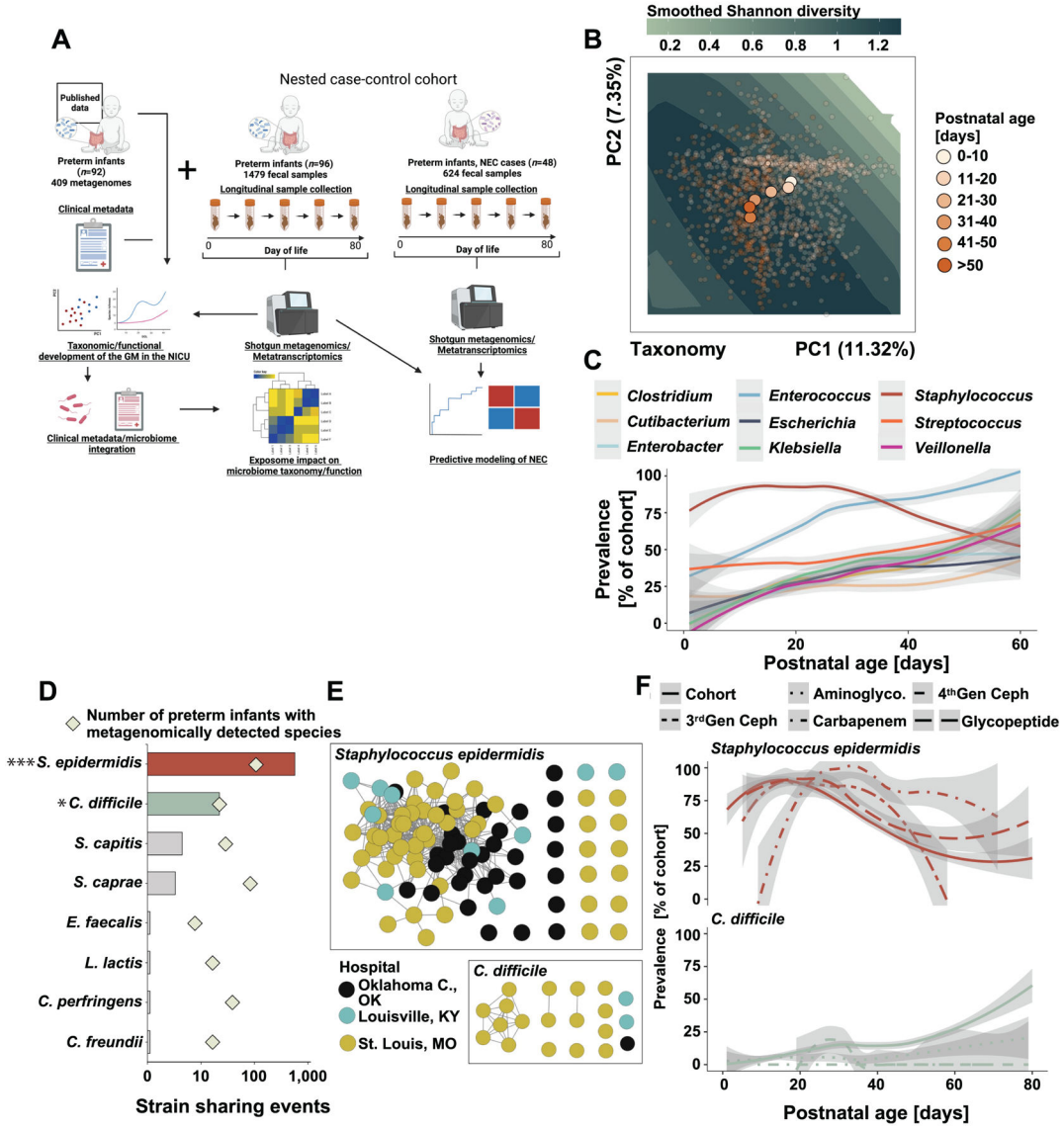

Figure 1 |. Earliest preterm gut microbiota colonization in the NICU.

A) Schematic overview of the study design. Created with BioRender.com. **B)** Principal component analysis of the gut microbiota composition in 1479 samples collected from 96 of preterm infants over the first 80 days of life. Points are colored by postnatal age at sample collection and centroids of each postnatal age bin is plotted. Smoothed Shannon diversity is extrapolated based on the observed data distribution and plotted into the background. **C)** Prevalence over postnatal days for nine selected taxa. **D)** Number of pairwise strain sharing events (ANI>99.999 and breadth >0.5) across unrelated preterm infant pairs for the taxa with most such events. The number of infants metagenomically positive for the selected taxa is plotted as squares. ***, p<0.001 for *S. epidermidis* versus all other listed species. *, p<0.05 for *C. difficile* versus all species listed below it. Pairwise Chi-Square with post-hoc pairwise test with Benjamini-Hochberg adjustment. **E)** Network representation of strain sharing events for the two taxa with most such events across unrelated infant pairs. Each dot

represents a unique preterm infant and is colored by its geographic location. **F)** Impact of antibiotic exposure on the prevalence of *S. epidermidis* (top) and *C. difficile* (bottom) across the entire control cohort over the first 80 days of life. Line patterns correspond to antibiotic category.

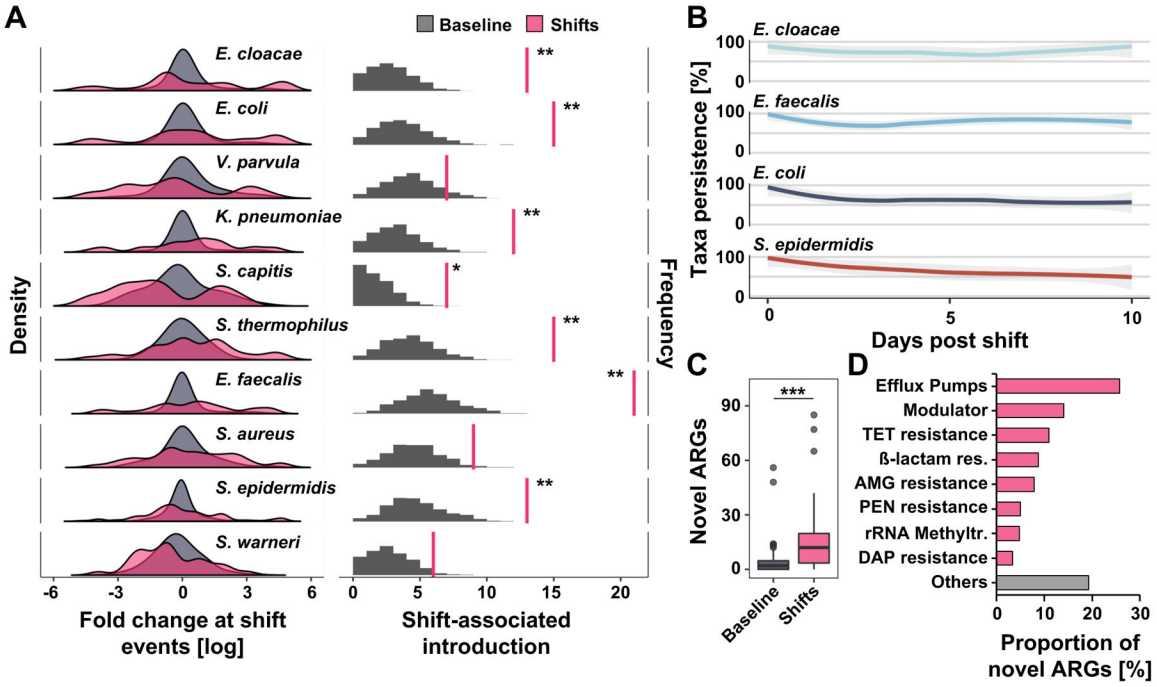

Figure 2 |. Antibiotic-induced microbiome shifts lead to abundance changes and introduction of pathobionts into the preterm infant gut.

A) The left panel demonstrates density (frequency of events, Y axis) for abundance changes (X axis) of selected species associated with shift events (pink) compared to 1000 permutations of baseline non-shift events (gray). Microbiome shifts are defined as Bray-Curtis dissimilarities between consecutive samples collected from the same patient that are ≥ Bray-Curtis dissimilarities between samples from unrelated individuals collected with the same time interval (Figure S3 A and B). Right panel depicts number of shift-associated discrete introduction events when metagenomically absent prior (right pink vertical line) compared to permutation of expected introductions in non-shift baseline samples (gray, n = 262, permutation test, FDR-adjusted, *P=0.05, **P<0.01). Full taxa and statistics appear in data file S19. **B)** Persistence (relative abundance greater than 0%) of selected taxa introduced at shift events over ten post-shift days. **C)** Number of antibiotic resistance genes (ARGs) introduced at shift events and undiscovered in pre-shift samples compared to permuted non-shift samples (n=262, Wilcoxon test P=3.49e^{-07}). **D)** Classification of ARGs introduced at shift events. N=1479 samples from 96 infants.

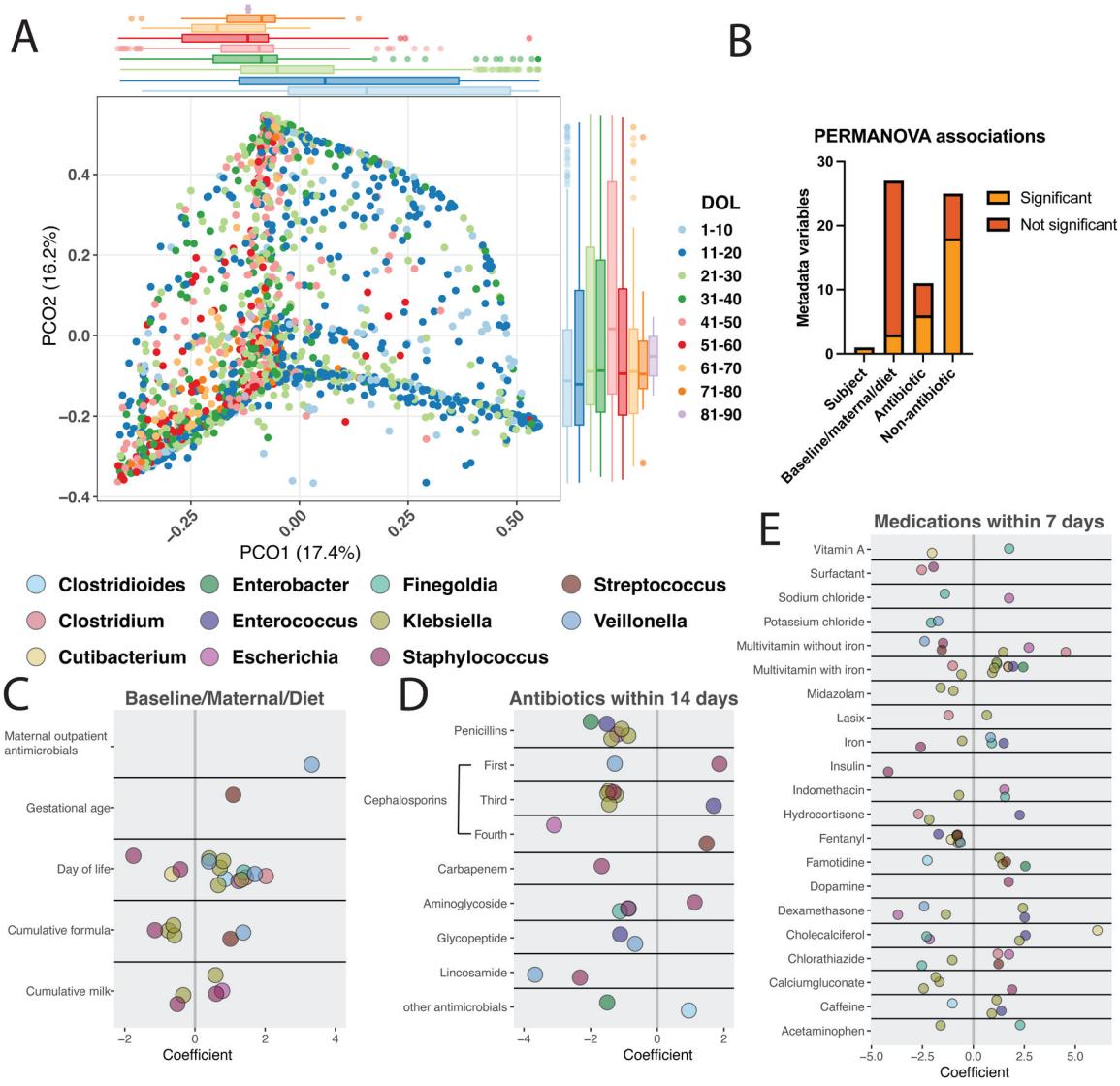

Figure 3 |. Impact of the NICU exposome on the developing preterm gut microbiome.

A) PCoA of Bray-Curtis dissimilarity of 1888 samples from 188 infants during the first 90 days of NICU hospitalization. Boxplots display PCO1 and PCO2 colored by postnatal age with variance explained in parentheses. **B)** Significant ($p<0.1$) and not significant ($p>0.1$) associations by metadata variable class are displayed as determined by repeat measures PERMANOVA. **C-E)** Coefficient of change of each genus corresponding to species significantly ($q<0.05$) changed based on the listed variable using MaAsLin2 with random effects of participant, hospital site, and study (this study, Gibson et al[11], or Gasparrini et al[12]). Exposures affecting less than 1% of samples and fixed effects $q>0.05$ are not displayed, but full associations ($q<0.25$ per MaAsLin2 default) are listed in Data S19.

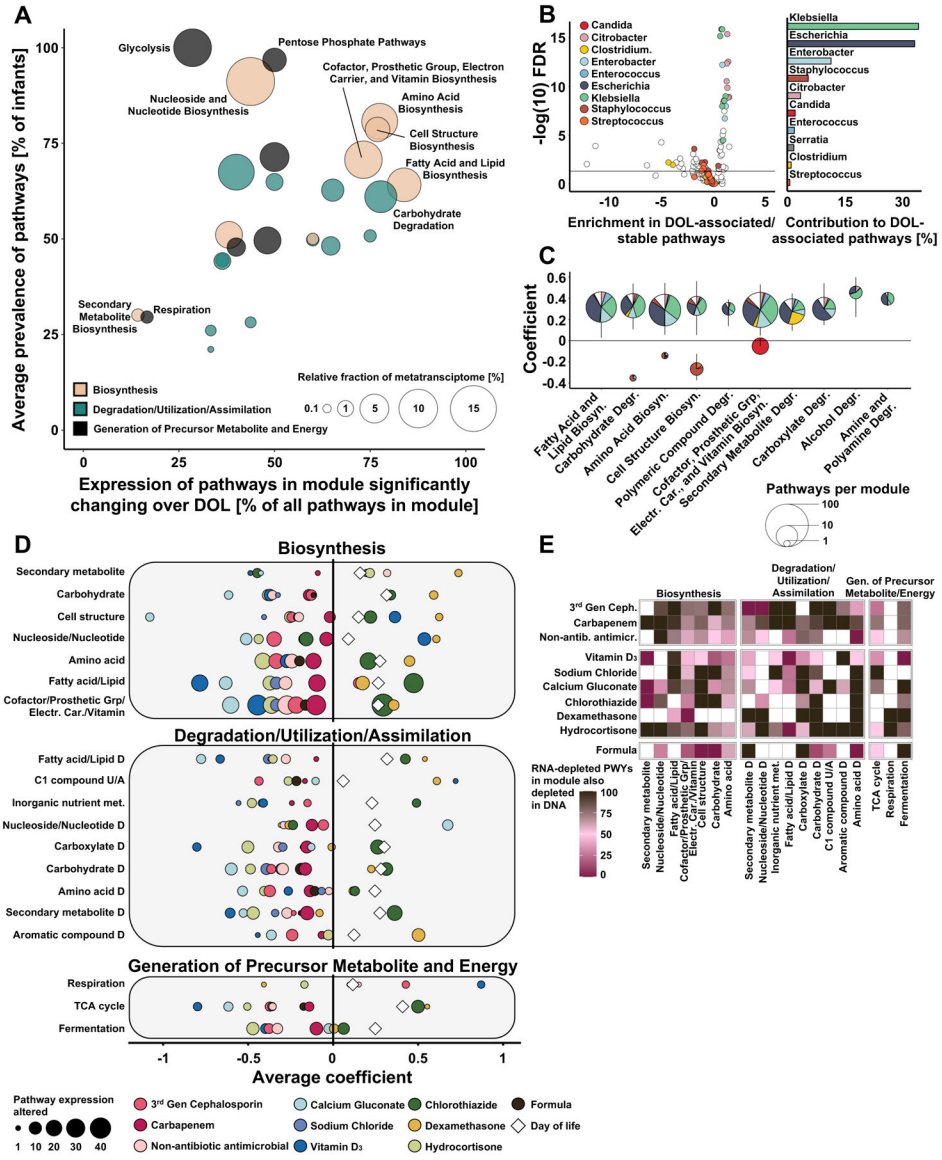

Figure 4 |. Transcriptional trajectories of the gut microbiome are shaped by Enterobacteriaceae and NICU exposures.

A) Characterization of transcriptional activity changes during NICU hospitalization. Pathways are colored by highest level functional categories and significant change over postnatal days is determined with MaAsLin2 (q<0.25). **B)** Species enrichment in pathways with stable or fold change in expression over postnatal days. **C)** Species association with functional categories with significant expression changes over postnatal days of life. **D)** Significant association of NICU exposures with changes in pathway expression. Exposures are indicated by color. Bubble size corresponds to the number of pathways within each functional category with significant expression changes associated with a given exposure. DataS19 has unaggregated and aggregated associations. **E)** Heatmap of pathway expression changes significantly associated with selected exposures indicating presence of concurrent changes in DNA abundance (MaAsLin2 q<0.25). Color scale represents percent change. N= 1381 samples from 95 infants.

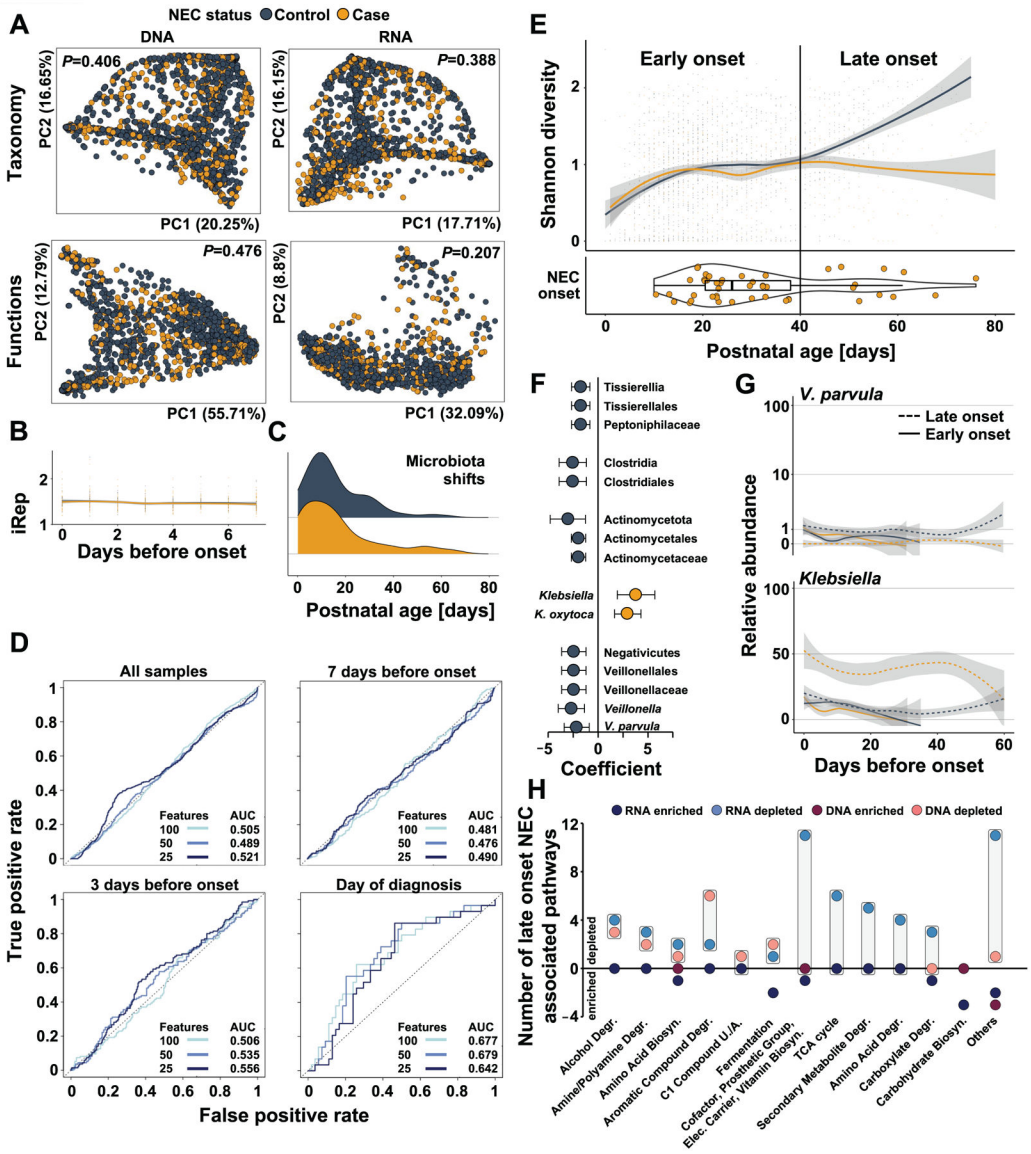

Figure 5 |. Gut microbiome features do not predict NEC onset for all preterm infants but differentiate late-onset cases and controls.

A) Principal coordinate analysis of pre-NEC gut microbiome taxonomic and functional composition in NEC cases (yellow) and matched controls (black). **B)** Bacterial replication rates in samples collected in the weak prior to NEC onset in cases (yellow) and matched controls (black, GLMM $P>0.25$). **C)** Distribution of microbiota shift events prior to disease onset in stool samples collected from NEC cases (yellow) and matched controls (black). **D)** Receiver operating characteristic curves of logistic regression models accounting for repeat measures utilizing all microbiome data collected from all samples, or samples taken in the week before, 3 days before, or at NEC onset. **E)** Shannon diversity of NEC cases (yellow) and matched controls (black) over postnatal days (top). Age of cases at disease onset in postnatal days (bottom). **F)** Taxa significantly associated with case or control status in late onset NEC when accounting for confounding exposures (MaAsLin2, $q<0.25$). **G)** Abundance of *V. parvula* (top) and *Klebsiella* (bottom) over 60 days before onset in early

(solid) and late (dashed) NEC cases (yellow) and matched controls (solid). **H)** Functional pathway expression and abundance associated with late onset NEC. Boxes highlight the difference between the number of DNA-encoded or RNA-encoded pathways in cases vs controls. N=2103 samples from 144 infants.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and virus strains		
Staphylococcus epidermidis from infant stool	This paper	N/A
Clostridioides difficile from infant stool	This paper	N/A
Biological samples		
Fecal samples from preterm infants	This paper, Warner et al.[34], La Rosa et al.[40]	Neonatal Microbiome and Necrotizing Enterocolitis cohort
Chemicals, peptides, and recombinant proteins		
Critical commercial assays		
DNeasy PowerSoil Pro Kit	Qiagen	Cat #47014
Nextera XT kit	Illumina	Cat #20034197
NucliSENS easyMAG system	bioMerieux	Cat #89130–520
Turbo DNase	Thermo Fisher	Cat #AM2238
QIAseq FastSelect – 5S/16S/23S kit	Qiagen	Cat #335925
NEBNext Ultra II Directional RNA Library Prep kit for Illumina	NEB	Cat #E7760
Deposited data		
metagenomes, metatranscriptomes, and metagenomic-assembled genomes	This paper	BioProject PRJNA799247
Fecal metagenomic DNA	Gibson et al.[11]	BioProject PRJNA301903
Fecal metagenomic DNA	Gasparrini et al.[12]	BioProject PRJNA489090
Experimental models: Cell lines		
Experimental models: Organisms/strains		
Oligonucleotides		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Software and algorithms		
Trimmomatic v0.366	Bolger et al.[74]	https://github.com/timflutre/trimmomatic
Deconseq v4.37	Schmieder et al.[75]	https://deconseq.sourceforge.net/
MetaPhlAn3 v3.0	Beghini et al.[76]	https://github.com/biobakery/MetaPhlAn/tree/3.1.0
HUMAnN3 v3.0	Beghini et al.[76]	https://github.com/biobakery/humann
ShortBRED	Kaminski et al.[77]	https://github.com/biobakery/shortbred
MetaSPAdes	Nurk et al.[80]	https://github.com/ablab/spades
Bowtie2	Langmead et al.[81]	https://github.com/BenLangmead/bowtie2
samtools	Li et al.[82]	https://www.htslib.org/
MetaBat2	Kang et al.[83]	https://bitbucket.org/berkeleylab/metabat/src/master/
checkM	Parks et al.[84]	https://ecogenomics.github.io/CheckM/
Quast	Gurevich et al.[85]	https://github.com/ablab/quast
dRep	Olm et al.[87]	https://github.com/MrOlm/drep
mash	Ondov et al.[88]	https://github.com/marbl/Mash
R statistical software v4.0.4 - v4.4.0	R core team	https://www.r-project.org
Rstudio	Rstudio	Posit.co
MaAslin2	Mallick et al.[51]	https://github.com/biobakery/Maaslin2
DirichletMultinomial	Holmes et al.[91]	https://bioconductor.org/packages/release/bioc/html/DirichletMultinomial.html
inStrain	Olm et al.[42]	https://github.com/MrOlm/inStrain
pyani	Pritchard et al.[89]	https://github.com/widdowquinn/pyani
Repeat measures PERMANOVA	Lloyd-Price et al.[21]	https://bitbucket.org/biobakery/hmp2_analysis/src/master/
Other		
Analytic scripts and data	This paper	https://github.com/dantaslab/NICUExposome 10.5281/zenodo.12737979