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# Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter

**Corryn Greenwood**[a,b], **Ardythe L. Morrow**[a,c,d], **Anne J. Lagomarcino**[a], **Mekibib Altaye**[d], **Diana H. Taft**[a,c], **Zhuoteng Yu**[e], **David S. Newburg**[e], **Doyle V. Ward**[f], and **Kurt R. Schibler**[a]

[a] Perinatal Institute, Cincinnati Children's Hospital Medical Center, Dept. Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

[b] Division of Neonatology, Advocate Children's Hospital, Oak Lawn, Illinois, USA

[c] Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

[d] Division of Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

[e] Department of Biology, Boston College, Chestnut Hill, Massachusetts, USA

[f] Broad Institute, Cambridge, Massachusetts, USA

## Abstract

**Objectives—**To determine the impact of empiric ampicillin and gentamicin use in the first week of life on microbial colonization and diversity in preterm infants.

**Study design—**16s rDNA community profiling was used to compare the microbiota of 74 infants born ≤32 weeks gestational age by degree of antibiotic use in the first week of life. The degree of antibiotic use was classified as 0 days, 1-4 days and 5-7 days of antibiotic administration. . All of the antibiotic use was empiric, defined as treatment based solely on clinical suspicion of infection without a positive culture result.

**Results—**Infants who received 5-7 days of empiric antimicrobial agents in the first week had increased relative abundance of *Enterobacter* (p=0.016) and lower bacterial diversity in the second and third weeks of life. Infants receiving early antibiotics also experienced more cases of NEC, sepsis or death than those not exposed to antibiotics.



**CORRESPONDING AUTHOR:** Kurt R. Schibler, MD Professor of Pediatrics Perinatal Institute Cincinnati Children's Hospital Medical Center 3333 Burnet Ave., MLC 7009 Cincinnati, OH 45229 Phone: 513-636-3972, Fax: 513-803-0969 kurt.schibler@cchmc.org.



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**Conclusions—**Early empiric antibiotics have sustained effects on the intestinal microbiota of preterm infants. Intestinal dysbiosis in this population has been found to be associated with elevated risk of necrotizing enterocolitis, sepsis, or death.

## Keywords

diversity; empiric antibiotics; microbiome; necrotizing enterocolitis; preterm infant

Antibiotics are the most commonly prescribed medications administered to infants in neonatal intensive care units (NICUs) (1). Even though the incidence of early-onset sepsis is low, preterm infants often receive empiric antibiotic treatment in the first few days of life when infection is suspected (2, 3). Concerns about occult intrauterine infection precipitating spontaneous premature labor, premature rupture of membranes, and chorioamnionitis often prompt initiation of empiric antibiotic treatment (4). Although initiation of antibiotic treatment for premature infants may be prudent under these circumstances, the duration of treatment is often arbitrary, based not on positive culture results but on the clinician's perception of risk (5). When empiric antibiotic treatment is clinically warranted, many clinicians limit such treatment to two days as the standard. Nevertheless, empiric antibiotic treatment is sometimes continued for 5 days or more based on antepartum factors, such as prolonged rupture of membranes or suspected chorioamnionitis, or for non-specific postnatal signs of infection including need for resuscitation at delivery, respiratory distress, or feeding intolerance. Unfortunately, early antibiotic therapy has the potential to cause harm as well as benefit to the preterm infant by impeding initial microbial colonization (6, 7). The microbial community of preterm infants is known to consist of dramatically fewer beneficial species, lower bacterial diversity, and more pathogens than observed in healthy term infants (8-11), but it is not known to what extent this observation can be attributed to the high use of antimicrobial agents. Even though recent advances in culture-independent sequencing methods have revolutionized our understanding of the microbial communities that live on and within the human body, studies of preterm infants have not yet attempted to examine the impact of antimicrobial use on the gut microbiome.

Unrestricted antimicrobial use can have persistent, unintended consequences, including reduced diversity of the microbial community, and after use is discontinued, recovery of a healthy microbiome is not assured (12). Early empiric antibiotic use in preterm infants is associated with increased risk of necrotizing enterocolitis (NEC), sepsis and death (13-15). Although aberrant early intestinal microbial colonization is thought to contribute to the pathobiology of NEC (8, 11, 16 62), the impact of early empiric antibiotic use on the preterm microbial community is not well studied.

We sought to test the hypothesis that early intensive antibiotic use in preterm infants alters the ontogeny of the microbiota and decrease microbial diversity in a longitudinal study of intestinal colonization in 74 preterm infants. Serial stool samples were collected from days 4 to 23 of life to test any differences in the early establishment of the intestinal microbiome by early and intensive empiric antibiotic therapy.

## METHODS

Study infants were enrolled from three level III NICUs in Cincinnati, Ohio as part of an ongoing cohort study of novel biomarkers for cases of NEC, sepsis, or death in infants ≤32 weeks gestational age. All infants remained free of NEC, sepsis, or death in the first postnatal week and had no identified congenital anomalies. The Institutional Review Boards at the three participating hospitals approved the study. Early empiric antibiotic exposure was defined as antibiotic treatment initiated within the first postnatal day (14). The duration of early empiric antibiotic therapy was defined as the total number of continuous days of administration of antibiotics with sterile culture results. Sepsis was defined as a positive blood, cerebrospinal fluid, urine, or sterile site culture. NEC was defined using modified Bell's stage II or III criteria (17)

In this cohort, early empiric antibiotic use consisted of ampicillin and gentamicin, using standard dosing. Antibiotic exposure groups were defined as no antibiotics (0 days), brief antibiotics (1-4 days of empiric antibiotic therapy), and intensive antibiotics (5-7 days of antibiotic therapy).

Serial stool samples were collected from infants during the first three weeks of life: at week 1 (4-7 days), week 2 (10-16 days), and week 3 (20-23 days of age). Samples were collected from soiled diapers, immediately refrigerated in the NICU, and transported to the laboratory where they remained in the refrigerator until processing with thioglycollate, and storage at -80° C.

As previously described (16) bacterial DNA was extracted from infant stool samples using one of two methods: phenol-chloroform or the QiaAmp DNA stool kit. Bacterial 16S rDNA sequences (Figure 1) were produced by the Broad Institute (Boston, MA) using production protocols established for the Human Microbiome Project (18). The V3 to V5 window of the 16S rRNA gene was amplified and the sequences were determined using the 454 FLX Titanium platform. A total of 1.3 M resulting sequences were processed using a data curation pipeline implemented in mothur for operational taxonomic unit clustering (19), complemented by abundant operational taxonomic unit (20), UCHIME for chimera detection (21) and NEWBLER for assembly-based error reduction (22, 23).

### Statistical Analyses

Sequence data was generated for 256 samples from 81 infants. Samples collected following NEC or sepsis were excluded from analysis. Five infants with only week 1 samples were also excluded. Thus, a total of 74 infants with 239 samples were available for study. Differences among groups were tested using Fisher's Exact test for categorical variables and analysis of variance (ANOVA) for continuous variables. The non-parametric Kruskal-Wallis (KW) test was used to compare differences in diversity between antibiotic groups.

An important metric to describe a microbiome is the diversity of bacterial species identified in that microbiome. We used the QIIME program (24) to calculate the Simpson diversity index, which measures both species richness (number of species present) and evenness of abundance. The entire data set was rarefied to 2200 reads per sample before alpha-diversity

was calculated. This procedure was repeated 5 times. Alpha-diversity metrics were then averaged per sample across these 5 iterations.

In order to account for multiple samples per infant, generalized estimating equations (GEE) *models of alpha diversity* were used to analyze differences in Simpson diversity index between infants with differing levels of antibiotic use. GEE models were run with an exchangeable correlation structure using the *geeglm* function in the *geepack* package in R (25).

The association between early antibiotic use and the relative abundance of the two most common operational taxonomic units were examined using a linear regression model. These two most abundant operational taxonomic units were classified, respectively, as an Enterobacter and a Staphylococcus, and were selected based on initial evidence of change in relation to antibiotic use groups, and being sufficiently abundant to allow robust modeling. The much lower abundance of other operational taxonomic units disallowed modeling due to zero inflation and limited statistical power. Because data from the same individuals tend to be positively correlated, the regression model was modified using GEE. Because the distribution of the antibiotic use was different for different sampling time, we stratified the data related to sample collection time, either week 2 or week 3 of life. We also categorized the duration of antibiotic use into three categories: none (0 days); brief (1-4 days); or intensive (5-7 days). Prior to analysis, raw operational taxonomic unit data was transformed into log scale to conform with the normality assumption of the model. These models were controlled for birth weight, maternal hypertension of pregnancy, delivery mode, and extraction protocol.

As DNA extraction methods can affect the results of 16S rDNA studies, we undertook a series of analyses to identify potential effects in our data (16). Briefly, extraction protocol was associated with neither beta-diversity nor with Simpson index of alpha-diversity. Finally, we included extraction protocol in regression models and found that it did not confound the associations being modeled.

## RESULTS

Of the 74 infants included for study, 13 (18%) had no antibiotics, 48 (64%) had a brief course of antibiotics, and 13 (18%) had intensive antibiotics in the first week of life. Empiric antibiotics were initiated and duration of therapy determined based on clinician's perceived risk of infection. Throughout this study ampicillin and gentamicin were the universally prescribed combination for early empiric treatment. Infants who received intensive antibiotics in the first week of life were delivered at lower birth weights and less mature gestational age, and were more likely to have had premature rupture of membranes (>18 hours prior to delivery) (Table). Also, infants who were born to mothers with hypertension or pre-eclampsia were significantly ($p<0.001$) less likely to receive empiric antibiotics. Intensive antibiotic exposure was significantly higher in the first week of life among 14 case infants who subsequently developed NEC or sepsis or died ($p=0.044$; Table). Cases included NEC alone (n=4); NEC with sepsis (n=2); NEC with death (n=3); NEC with sepsis and death (n=1); and sepsis alone (n=2); and death alone (n=2). However, because all cases of

NEC, sepsis, or death in our study occurred among infants < 29 weeks gestational age, the higher use of antibiotics in this group could be confounding the association. When we examined antibiotic receipt in only subjects < 29 weeks gestation, antibiotic receipt during the first week did not differ significantly between cases compared with controls (p=0.208).

Stool samples were immediately refrigerated in the NICU and transported to the laboratory where they remained in the refrigerator until processing and freezing. The median time to freezing was 27 hours, with 91% of samples frozen by 72 hours. To test for the potential confounding effect of time to freezing we tested bacterial abundance and diversity of specimens processed before and after 24 hours. With the exception of *Lactococcus*, no differences were noted in relative abundance of bacterial genera between samples processed before versus after 24 hours following collection. Likewise, microbial diversity measures were not confounded by time to freezing.

Pie charts of the most common genera are shown in Figure 2 in relation to antibiotic use and day of stool specimen collection. During the first week of life, among infants who received no antibiotics, *Staphylococcus* (41%), *Enterococcus* (26%), and *Enterobacter* (19%) were the most abundant genera. Infants who received a brief course of antibiotics had a greater relative abundance of *Enterobacter* (40%), and had less *Enterococcus* and *Staphylococcus* compared with infants who received no antibiotics. Infants who received intensive antibiotics had a different profile of the most abundant genera in samples from the first week of life, which were *Enterococcus* (34%) and *Clostridium* (33%), followed by *Staphylococcus* (11%) and *Escherichia* (8%). Concurrent with antimicrobial exposure, there was a striking variability in the genera comprising the intestinal microbiota.

During week two of life, the stool of infants who received any antibiotics in the first week of life contained *Enterobacter* as the most common genus. The next three common genera, *Staphylococcus*, *Enterococcus* and *Escherichia*, were similar in relative abundance. For infants who received a brief course of antibiotics in the first week, the most common genera seen in week 2 samples were *Enterobacter* (51%), *Staphylococcus* (17%), and *Escherichia* (12%). Among infants receiving intensive antibiotics in the first week, only four genera - *Enterobacter* (57%), *Escherichia* (21%), *Enterococcus* (15%), and *Staphylococcus* (7%) - comprised >99% of the microbiome. Overall, in the second week of life, there was a trend toward increased abundance of *Enterobacter* among infants exposed to antibiotics compared with infants who did not receive antibiotics.

For samples from week three of life, *Enterobacter* (36%), *Enterococcus* (17%), *Clostridia* (17%), *Veillonella* (16%), and *Escherichia* (11%) were the most common genera present from infants who did not receive antibiotics. A similar pattern was observed for infants who received brief antibiotics, in which *Enterobacter* (48%) and *Enterococcus* (16%) were the most common genera followed by *Escherichia* (13%), *Clostridia* (9%) and *Veillonella* (7%). Among infants who received intensive antibiotics, the most common genera were *Enterobacter* (47%) followed closely by *Enterococcus* (35%).

To examine the statistical significance of these changes in microbiota, GEE models quantified the association between early antibiotic exposure and relative abundance of the

two most abundant operational taxonomic units representing *Enterobacter* and *Staphylococcus*. *Enterobacter* significantly increased with empiric antibiotic use in week 2 with exposure to intensive antibiotics [p=0.016] and brief antibiotics [p=0.006], after controlling for birth weight, maternal hypertension of pregnancy, delivery mode, and extraction protocol. However, *Enterobacter* did not significantly increase in week 3 of postnatal life with more intensive antibiotic use during the first week. Intensive antibiotic use in week 1 was also associated with lower relative abundance of *Staphylococcus* in week 2 [p<0.001], but not through week 3. Brief antibiotics compared with no antibiotics were associated with a higher relative abundance of *Staphylococcus* in week 3 [p=0.004].

Simpson diversity index (Figure 3) was related to antibiotic receipt. For stool samples from week 1, Simpson's diversity index did not differ by antibiotic exposure. By week 2, decreasing diversity was observed after receiving either brief or intensive early antibiotics (p<0.001, Kruskal-Wallis test). By week 3, Simpson's diversity index remained significantly different across treatment groups (p<0.004). To control for potential confounding, the Simpson's index was modeled by GEE to account for multiple samples per individual, and to control for potential confounding factors (birth weight, maternal hypertension of pregnancy, delivery mode, and extraction protocol). Week two samples displayed significantly less diversity in infants who received brief (p=0.035) and intensive antibiotics (p=0.001). Similarly, in week 3, there was less diversity in infants who received brief (p=0.023) and intensive antibiotics (< 0.001).

## DISCUSSION

The data from this study support the hypothesis that intensive early antibiotic administration to premature infants is associated with profound alterations in the intestinal microbiota and potentially, increased risk of NEC, sepsis, or death. A striking reduction in microbial diversity was observed within days of receiving intensive antibiotics and persisted over the 3-week study. More intensive antibiotic use increased representation by *Enterobacter*. Understanding the succession of the microbiota as well as the factors that influence the formation of the microbiota in preterm infants might provide insight into ontogeny of the microbiota and the pathogenesis of life threatening neonatal conditions such as NEC and sepsis. This is a small study and it is possible that results could be confounded by differences between cases and controls in factors such as gestational age. However, our findings are consistent with larger studies of antibiotic use and increased risk of these outcomes (13-15).

Others have reported low bacterial diversity in preterm infants. In a case control study of 20 preterm infants with and without NEC, Wang et al demonstrated low bacterial diversity that was most pronounced among infants who developed NEC (8) with an increase in Proteobacteria around the time of NEC. Our group and others also found that dysbiosis in early colonizing organisms precedes NEC, and domination by Proteobacteria, specifically *Enterobacter*, was found in some infants who subsequently developed NEC (16, 26). Here we report a high relative abundance of *Enterobacter* that is most striking in infants receiving intensive antibiotics. Antibiotic use may select for*Enterobacter*, which include pathogenic species commonly encountered in nosocomial infections in hospitalized patients.

*Enterobacter* species are also frequently resistant to beta-lactams though a constitutive AmpC beta-lactamase (27). Although antibiotic sensitivity patterns and genetic profiles were beyond the scope of this study, it is key to study these residual colonizing species with regard to their degree of antimicrobial resistance. A new plasmid that confers resistance to both beta-lactams and aminoglycosides, the antibiotics used in this study, has been described (27). Animal studies report aggregation of resistance gene clusters, a core antimicrobial resistome, in the gut microbiome of antibiotic-fed swine (28). Drug resistance has been rapidly increasing in sepsis cases, and broad-spectrum antibiotic use strongly increases risk of invasive fungal infection (29, 30).

Increased abundance of *Enterobacter* observed with antibiotic therapy might occur though alternative mechanisms, notably modification of innate immunity. In studies comparing germ-free mice to conventionalized littermates, El Aidy et al characterized the dynamic temporal and region-specific mucosal responses to colonizing microbiota (31). These developmental changes included induction of innate immune function followed by stimulation and expansion of adaptive immune responses and ultimately a more tolerant state of immune homeostasis. Despite reaching a state of homeostasis, RegIII peptide expression remains high in the small intestines but declines to germ free levels in the colon after development of tolerant immune homeostasis. These finding suggest that RegIIIα and RegIIIγ play an important function, keeping microbes at bay in the small intestine. Disrupted expression of RegIII and other innate molecules by antibiotic exposure could potentially adversely impact immune-associated inflammatory pathways. Moreover, Reikvam and coworkers reported that broad-spectrum antibiotic treatment reduced the expression of antimicrobial factors to a level similar to that of germ-free mice and altered the expression of 517 genes in the colonic epithelium (32).

Infants who received a brief course of antibiotics, displayed a decrease in microbial diversity. However, by week 3 the diversity approached that of samples from the first week. The initial short antibiotic exposure could have suppressed the microbiota diversity only temporarily, in contrast to infants who received intensive antibiotics having a sustained reduction in diversity, a change not previously reported.

The infants with no antibiotic exposure displayed the increase in overall diversity reported by others (33). Intensive antibiotic use was significantly greater in infants who were affected by NEC or sepsis, or who died (13-15). It is interesting to speculate that the mechanism underlying this association may be related to antimicrobial killing of protective commensal bacteria with resultant growth of pathogenic bacteria. This mechanism is supported by the reduction of NEC associated with probiotic administration (34, 35).

Other potential determinants of the intestinal microbiota composition include delivery mode and feeding type. *Staphylococcus* is an early colonizer of the infant gastrointestinal tract. The early large abundance of *Staphylococcus* in the no antibiotic group may be a reflection of their predominant (but not statistically significant) delivery mode being cesarean delivery. Common indications for preterm birth by cesarean delivery include pre-eclampsia or eclampsia and intrauterine growth restriction (36), whose influence on infant intestinal microbiota is not known. Enteral feeding practices certainly contribute to variation in

colonization of the gastrointestinal tract. However, in this study variation attributable to enteral feeding practices was probably limited because enteral feeding type and advancement were driven by a standardized feeding protocol for infants weighing 1500 g or less at birth in participating NICUs. Over the time course of this study more than 90% of infants received human milk either mothers-own-milk or donor human milk starting in the first two days of life and advancing to full feeds over about two weeks (37).

*Bifidobacterium* was the most common genera in a cohort of term infants in the Netherlands (38) measured by 16s PCR, and *Bifidobacterium* and *Bacteroides* frequency decreased with oral antibiotic use (mainly amoxicillin). *Clostridium* species increased with oral antibiotic use in hospitalized premature infants, which included late preterm infants 34 to 37 weeks gestational age. Lack of bifidobacteria in our cohort of preterm infants may be a function of greater prematurity, antibiotic use, illness, or possibly that *Bifidobacterium* is not as abundant in premature infants as previously thought, even when they receive human milk. Most infants in our study received either pasteurized donor milk or their own mother's human milk during the study.

Our study includes a larger number of infants relative to previously published studies in premature infants. Limitations of our study include the infrequent early defecation by premature infants in the early days of life, and the few samples obtained may not fully capture the effect of antibiotics on the microbiota. In addition, adverse outcomes including NEC, sepsis and death were too few to determine associations with antibiotic exposure and limited the number of samples included in the study. Antibiotic resistance patterns or genetic activity for resistance were not studied, but remain a promising undertaking for the future.

The duration of early empiric administration of ampicillin and gentamicin to preterm infants correlates with a decrease in intestinal microbial diversity. Among premature infants receiving intensive empiric antibiotics, the reduction in microbial diversity of the gastrointestinal tract continued through the 3 weeks of the study. It is plausible that early intensive antibiotic use may perturb the infant microbiota during a critical period, thus altering the course of ontogeny of the microbiota. This supports the hypothesis that consequent dysbiosis could precede, and perhaps precipitate sepsis and NEC, suggesting a need to assess the long-term impact of antibiotics on the microbiota, as well as their impact on disease outcomes such as NEC.

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Sequence data generated for this work is deposited under the NCBI bioproject ID 63661.

## ABBREVIATIONS (ALPHABETICAL)

| | |
|---|---|
| **ANOVA** | analysis of variance |
| **GEE** | generalized estimating equations |
| **KW** | Kruskal-Wallis |
| **NEC** | necrotizing enterocolitis |
| **NICU** | neonatal intensive care unit |
| **OTU** | operational taxonomic unit |

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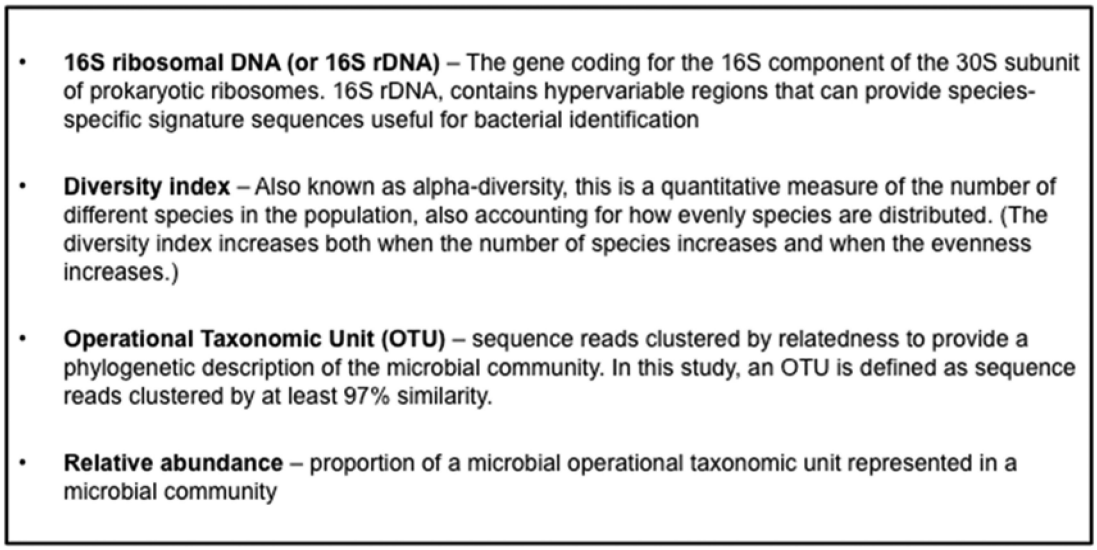


**Figure 1.**
Definitions for metagenomic sequencing and analyses.

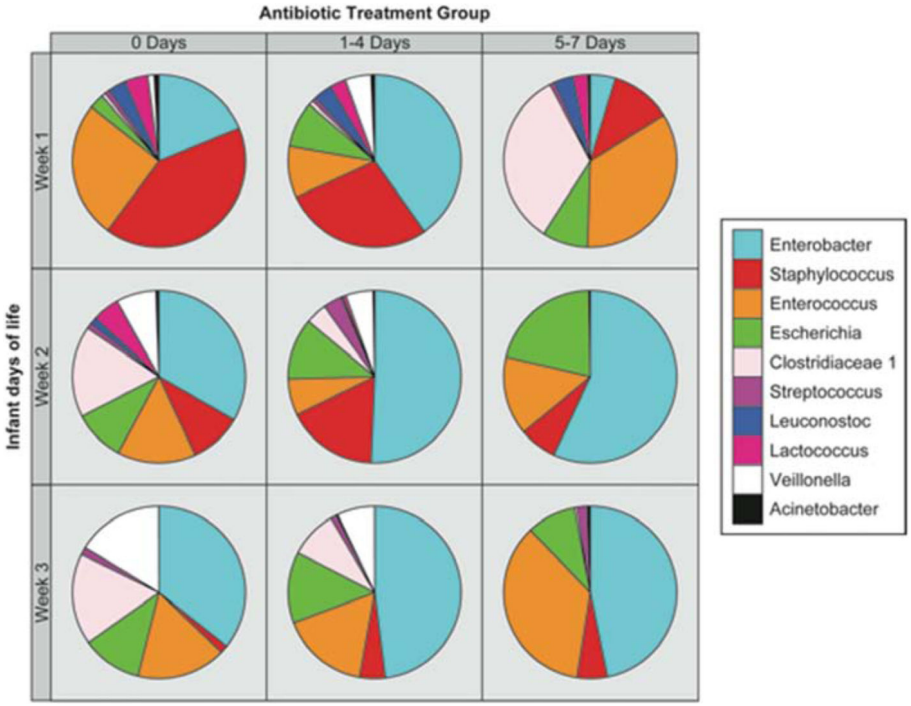


**Figure 2.**

Pie graphs depicting relative abundance of bacterial genera detected in stool specimens from study infants as a function of antibiotic exposure over the first three weeks of life.

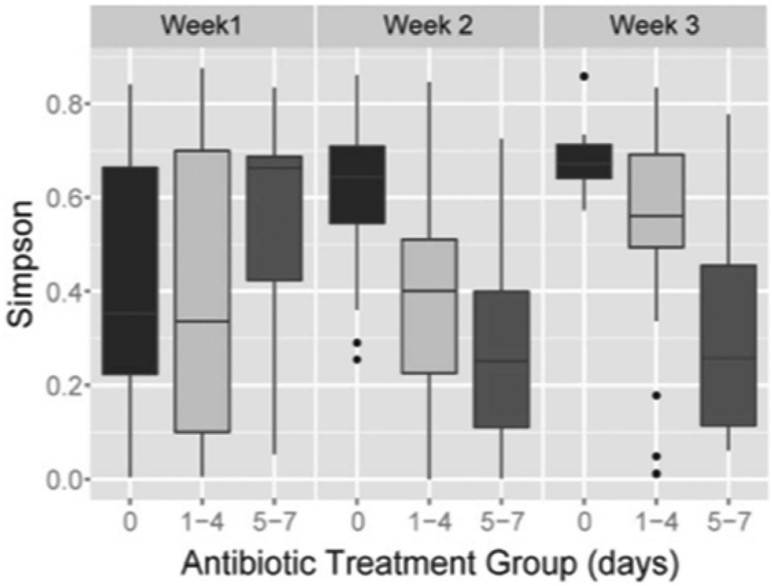


**Figure 3.**

Simpson diversity index depicted in relation to antibiotic receipt (No, 0 days; brief, 1-4 days; or intensive antibiotics, 5-7 days) during the first three weeks of life.

## Table

Demographic and clinical characteristics of study subjects

| | Initial empirical antibiotic therapy, n = infants (% of total study infants) | | | |
|---|---|---|---|---|
| **Variable, description** | **0 Days n = 13 (17.6)** | **1-4 Days, n = 48 (64.8)** | **5-7 Days n = 13 (17.6)** | ***P* value*** |
| **Infant baseline characteristics** | | | | |
| Birth weight, median (range) (g) | 1305 (890-1500) | 1065 (520-1480) | 750 (510-1440) | 0.001 |
| Gestational age, median (range) (wk) | 31 (24-32) | 28 (24-32) | 25 (23-31) | < 0.001 |
| Non-Hispanic black, no. (%) | 3 (23.1) | 10 (20.8) | 7 (53.8) | 0.071 |
| Male, no. (%) | 2 (15.4) | 26 (54.2) | 6 (46.2) | 0.046 |
| Cases, no. (%) | 0 (0) | 9 (18.8) | 5 (38.5) | 0.044 |
| **Maternal and prenatal information** | | | | |
| Prenatal steroids given (any), no. (%) | 13 (100) | 42 (87.5) | 11 (84.6) | 0.553 |
| Hypertension / Pre-eclampsia, no. (%) | 12 (92.3) | 6 (12.5) | 4 (30.8) | < 0.001 |
| Prenatal care (≥ 1 prenatal visit), no. (%) | 13 (100) | 47 (97.9) | 13 (100) | 1 |
| Antepartum hemorrhage, no. (%) | 0 (0) | 12 (25) | 1 (7.7) | 0.069 |
| Multiple birth, no. (%) | 5 (38.5) | 17 (35.4) | 2 (15.4) | 0.417 |
| Antepartum antibiotic therapy, no. (%) | 4 (30.8) | 25 (52.1) | 8 (61.5) | 0.311 |
| **Labor and delivery information** | | | | |
| Cesarean delivery, no. (%) | 12 (92.3) | 31 (64.6) | 8 (61.5) | 0.151 |
| Chorioamnionitis, no. (%) | 0 (0) | 5 (10.4) | 2 (15.4) | 0.443 |
| Delivery room resuscitation, no. (%) | 8 (61.5) | 29 (60.4) | 12 (92.3) | 0.100 |
| Rupture of membranes > 18 hrs, no. (%) | 0 (0) | 9 (18.8) | 7 (53.8) | 0.003 |

**P*-values calculated by Fisher exact test for categorical values (%) and ANOVA for continuous variables (birth weight, gestational age)