Effects of Early Life NICU Stress on the Developing Gut Microbiome

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Abstract

Succession of gut microbial community structure for newborns is highly influenced by early life factors. Many preterm infants cared for in the NICU are exposed to parent-infant separation, stress and pain from medical care procedures. The purpose of the study was to investigate the impact of early life stress on the trajectory of gut microbial structure. Stool samples from very preterm infants were collected weekly for 6 weeks. NICU stress exposure data was collected daily for 6 weeks. V4 region of the 16S rRNA gene was amplified by PCR and sequenced. Zero-inflated Beta regression model with random effects was used to assess the impact of stress on gut microbiome trajectories. Week of sampling was significant for: *Escherichia*, *Staphylococcus*, *Enterococcus*, *Bifidobacterium*, *Proteus*, *Streptococcus*, *Clostridium_butyricum* and *Clostridium_perfringens*. Antibiotic usage was significant for *Proteus*, *Citrobacter* and *Clostridium_perfringens*. Gender was significant for *Proteus*. Stress exposure occurring 1 and 2 weeks prior to sampling had a significant effect on *Proteus* and *Veillonella*. NICU stress exposure had a significant effect on *Proteus* and *Veillonella*. There was an overall dominance of Gammaproteobacteria. Findings suggest early life NICU stress may significantly influence the developing gut microbiome, with important NICU practice and future microbiome research considerations.

Keywords

preterm infants; NICU; stress; chronic stress; early life stress; gut microbiome

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1. Introduction

Gastrointestinal and neurodevelopmental systems are highly vulnerable in preterm infants. Some of the most devastating complications experienced by preterm infants include diseases that originate in these systems (i.e. necrotizing enterocolitis and intraventricular hemorrhage) (Bolisetty et al., 2014; Christian et al., 2015; Fitzgibbons et al., 2009; Kidokoro et al., 2014; Stoll, Hansen, Bell, & et al., 2015). How stress alters the gastrointestinal system remains largely unknown. Interestingly, a powerful bidirectional system of communication exists between the gut and brain, but we know little about this in preterm infants.

An important factor influencing health is the commensal gut microbiota that coexist in a mutualistic relationship with the host (Sampson & Mazmanian, 2015). Of particular interest is the general enterotype of commensal and potentially virulent microbial species and their relative proportions, that may play a role in general health as well as mental health (La Rosa et al., 2014; Liu, 2017; Sharon et al., 2014). Preliminary relationships are emerging that link the composition of the gut microbiome to increased risk for anxiety and depression (Kaplan, Rucklidge, Romijn, & McLeod, 2015). For former preterm infants, anxiety and depression are some of the internalizing behaviors they are at-risk for in adulthood (Mathewson et al., 2017; Pyhälä et al., 2017).

After birth, newborns are colonized with trillions of bacteria, the specific composition of bacteria contributing to the programming of gut function and development, epithelial barrier function, immune system and overall gut homeostasis (Hooper, 2004). For healthy term infants, the major first source of colonizing bacteria is of maternal origin (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2016; Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015). Previous research has shown a developmental sequence of phyla blooms occurs after birth, ultimately leading to one's microbial signature throughout adulthood (Lan, Kriete, & Rosen, 2013). NICU infants are often deprived of the "normal colonization" process, resulting in potential delays in the normal taxa succession (Bentley et al., 2016; Lax & Gilbert, 2015; Raveh-Sadka et al., 2016; Shin, Whon, & Bae, 2015). This delay can lead to a state of dysbiosis with short and long-term pathological implications.

Clinicians and researchers have been hopeful that this newer area of science will lead to discoveries about the underlying pathology of preterm infant illness and disease. However, studies investigating the developing microbiome of preterm infants have revealed conflicting and sometimes counterintuitive results. For the first 3 months of life, Brown et al. (2018) examined the gut microbiome of 35 preterm infants and found high variability within infants. In subsequent sampling, some infants had microbial community abundance that was unique from earlier samples as those from other infants and no species were strongly associated with necrotizing enterocolitis (NEC) (Brown et al., 2018). Dobbler et al. (2017) identified low microbial diversity and chaotic succession for infants who developed NEC. The Norwegian Microbiota Study reported duration of NICU care to be a significant mediator of diversity, but not breastfeeding or history of antibiotic use (Dahl et al., 2018). After adjusting for antibiotic and breastmilk exposure and delivery mode, Chernikova et al. (2018) found differences in diversity based upon birth gestational age, with extremely preterm infants having less diversity than moderate-late preterm infants (Chernikova et al.,

2018). Questions remain if this association is because of inherent gut maturation, also leading to immune effects, or influences from the NICU environment.

Early life stress, an environmental exposure, has the potential to leave lasting effects. While some stressors during the lifespan may promote growth and adaptation, other stressors may become biologically embedded, to the extent that they alter future health trajectories. For many hospitalized preterm infants, early life exposure to cumulative and intense stressors may be perceived by the infant as potentially toxic. Toxic stress is defined as strong, frequent or prolonged activation of the body's stress response systems, without the buffering protection of parental support, which has the potential to alter health trajectories. Of particular importance to the developing preterm infants is brain development and future regulation of stress (Jensen, Dickie, Schwartz, & et al., 2015; Shonkoff et al., 2012; Westfall & Nemeroff, 2015). Toxic stress has been investigated in a number of populations, however little is known about how toxic stress effects the developing preterm infants (Barker, 1998; Gunnar, Wewerka, Frenn, Long, & Griggs, 2009). A number of studies now suggest that stress can affect the gut microbiome and lead to dysbiosis, as well as interrupt activation of the innate immune system in response to stress (Dantzer, Cohen, Russo, & Dinan, 2018; Karl et al., 2018).

Despite the patient-focused manner caregivers seek to provide care, the NICU is largely a medically focused environment driven by protocols and caregiver schedules. The environment places preterm infants under a great deal of stress as infants are exposed to prolonged separation from parents, chronic and extreme stressors, and painful life-saving medical procedures (D'Agata, Young, Cong, Grasso, & McGrath, 2016). Furthermore, preterm infants are unable to articulate to caregivers their burden of stress. The cumulative and multifaceted experiences of high-level stress, coupled with the preverbal stage of development and inability to self-advocate, potentially leads to toxic stress exposure. The frequent or prolonged activation of the body's stress response systems, in the absence of buffering protection of a supportive adult relationship, leads to alterations in the child's biologic functioning (Gunnar et al., 2009; Shonkoff et al., 2012). Neurodevelopmental health trajectories are of utmost concern for infants as this period of brain development has been identified for infants and young children as a functionally sensitive period in which experience and environmental stimuli exert great influence (Sale, Berardi, & Maffei, 2014).

An alternative, yet highly relevant, factor of influence is the commensal gut microbiota that coexists in a mutualistic relationship with its host (Sampson & Mazmanian, 2015). The bidirectional axis between the highly complex systems of gut-brain has been suggested to significantly influence health (Clarke et al., 2014; De Palma, Collins, Bercik, & Verdu, 2014; M. W. Groer et al., 2014; Lu et al., 2015; Stilling et al., 2015). Animal studies have demonstrated the gut microbiome exerts an influence on systems outside of the gastrointestinal tract, including brain circuits that control stress response, anxiety-like behavior and cognitive function (Arboleya et al., 2016; Clarke et al., 2013; Gilbert et al., 2016; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011; Sudo et al., 2004).

Compelling evidence has demonstrated that intense experiences may become biologically embedded, potentially affecting outcomes. Care provided in the NICU, however well

intended, is an intense early life experience for many infants who require medical care after birth. Currently, a gap in the literature exists about the relationship between early life stress from routine NICU care and longitudinal microbial communities for very low birth weight (VLBW) infants. We hypothesized that the microbial community of the preterm infant gut would be altered by the NICU stress experience. To understand how early life NICU stress exposure may contribute to preterm infant gut microbiome development, we conducted a retrospective, longitudinal data analysis of stool samples sequenced for microbial abundance and diversity and NICU stress exposure during the first 6 weeks of life.

2.   Methods

2.1   Participants

This study was conducted using secondary data. VLBW infant participants were part of a larger longitudinal study of long-term growth, health and neurodevelopmental effects (Groer, Ashmeade, Duffy, Morse, & Zaritt, 2016; Groer, Ashmeade, Louis-Jacques, Beckstead, & Ji, 2016; Ho et al., 2018). Infants were admitted to the Neonatal Intensive Care Unit at Tampa General Hospital in Tampa, Florida between 2011 and 2015. Exclusion criteria for entry into the parent study included birth weight greater than 1500 grams, major congenital anomalies, moribund infants and infants of HIV-infected mothers. The study was approved by the University of South Florida Institutional Review Board. Infants were enrolled after informed consent was obtained from their parent or guardian. A total of 82 VLBW infants were eligible to participate in this study.

2.2   Stress Data

Stress data were collected using the Neonatal Infant Stressor Scale (NISS), a ranked quantitative instrument used to collect information concerning daily stress experiences of infants during care in the NICU (D'Agata et al., 2017; Newnham, Inder, & Milgrom, 2009). This instrument measures interventions performed, not infant response. Common NICU interventions are ranked in categories on a 4-point scale as *a little stressful*, *moderately stressful*, *very stressful* and *extremely stressful*. Each procedure attempt is counted as one stress event. Examples of NICU care procedures and their corresponding scoring include: intubation attempt is *extremely stressful* and weighted with a score of 5; suctioning attempt is *very stressful*, score of 4; diaper change is *moderately stressful*, score of 3; and aspiration of nasogastric tube is *a little stressful*, score of 2.

For preterm infants who receive NICU care, care is both individualized and standardized. Individualized interventions are directed at the specific illness experienced by the infant, while standardized interventions are interventions performed more routinely, by virtue of being hospitalized. Examples of standardized interventions may include daily weight, diaper changes and position changes. For many preterm infants, typical care also includes some type of respiratory support and gavage feedings. Many of these interventions are performed repeatedly each day and at regular intervals. Higher intensity interventions, such as intubation, chest tube insertion, blood draws and suctioning, occur with greater variability, thus tend to be more individualized.

To develop a *core score* of standardized interventions, those interventions typically performed routinely on VLBW infants, we analyzed approximately 850 days of previously collected NISS data from NICU infants in another state (D'Agata et al., 2017). From 850 days of NISS data, we extrapolated and averaged the *a little stressful* and *moderately stressful* category data (D'Agata et al., 2017). This provided a *core score* of standardized and lower intensity stress interventions to then add to the higher intensity interventions collected from each infant's electronic medical record. From the electronic medical record, we collected the actual attempted and completed higher intensity stress events, *very stressful* and *extremely stressful* categories. These actual daily events were then added to the *core score* measure to arrive at a daily stress score. The *core score* of 109 reflected an average of 40 daily stress events.

To validate the core score, we randomly selected 15 participants from the current parent study and collected the same *a little stressful* and *moderately stressful* data over 42 days (approximately 630 days of data) to compare findings. Despite the comparison of data for these two cohorts being 2 different states in different regions of the United States, the same *core score* was identified.

Stress data were collected daily from NICU admission through 6 weeks of care or discharge, whichever came first. The total daily stress scores were totaled by week for weekly stress scores. The weekly stress scores were used as an independent variable in the analyses.

2.3.   Microbiome Data

Stool samples were collected for 6 consecutive weeks from the diapers of VLBW infants while they were admitted to the NICU. The purpose of sample collection in the parent study was to study the succession of the microbiome, and the relationships of microbiome to early and later health outcomes. Weekly stool samples were collected from these infants and preserved at -80° C for microbiome analysis. Samples available for this study primarily included weeks 3–6 as inadequate sample volumes were available for weeks of life 1 and 2 after other stool assays were done in the parent study (Groer, Ashmeade, Louis-Jacques, et al., 2016).

The Mo Bio Power Fecal DNA extraction kit (Qiagen) was used to extract total DNA from 100–250 mg of thawed stool samples. The V4 region of the 16S rRNA gene was amplified by polymerase chain reaction and isolated, then the amplicon library was generated following a standardized protocol using Illumina Miseq (Illumina, San Diego, CA, USA) at Argonne National Laboratory (Chicago, IL). Two hundred base pairs DNA sequence reads were demultiplexed, merged, and imported into QIIME™ for identification of bacterial operational taxonomic units (OTUs). Miseq data were reported to the genus level, with some OTUs reported at species level. Species was used to differentiate genus with same naming convention.

2.4   Statistical Analysis

We report samples on a weekly basis. Stool sample collection was attempted at least once per week while infants were hospitalized in the NICU however, some samples were collected inadvertently several times in one week and no sample collected in the subsequent

week. Figure 1 shows the missing data pattern of the microbiome compositional data by week. Due to the large amount of missing data in the first two weeks of enrollment, we focused our study of the longitudinal bacterial relative abundances at the genus level from week 3 to week 6, which had the maximum number of infants (116 samples with 29 infants) with complete data. We then considered the subset of infants which had only one missing value between week 3 and week 6 exclusively. The Little's MCAR test was applied to the subset of infants and showed that the microbiome compositional data at each week were missing completely at random (MCAR), where the probability of missingness does not depend on the values of data (Little, 1988). Under this assumption and because genera abundances are fairly stable across time, cubic spline interpolation approach (Steinway, Biggs, Loughran, Papin, & Albert, 2015) was applied to infants with only one missing value between week 3 to week 6 exclusively. After the imputation, we had a total of 188 samples with 47 infants.

Exploratory data analysis was conducted to describe characteristics of each baseline variable (antibiotic, infant gender, birth weight, and gestational age), and the time-dependent variable (cumulative weekly stress) during each week, using summary statistics for the 47 infants.

After removing the low abudant genus, 21 bacterial genera remained (Kostic et al., 2015). Since the relative abundances of each bacterial genera were bounded in [0, 1], highly skewed and zero inflated (Figure 2), the zero-inflated Beta regression model with random effects (ZIBR) was used to assess the impact of stress on the trajectories of the gut microbiome compositional data, adjusting for the baseline covariates effects (Chen & Li, 2016). ZIBR model is a mixture of the Binary model and Beta models. The Binary part models the presence of the bacterial genera and the Beta part models the non-zero relative abundance of the bacterial genera. A subject-specific random effect is specified in each part of the model to capture the dependence of the longitudinal abundance measures over time. For both parts of the model, we considered the time effect (week3 as baseline, week4, week5, week6), antibiotic usage, infant gender, birth weight, gestational age, stress 2 weeks prior to sample collection ($stress_{t-2}$), and stress one week prior to sample collection ($stress_{t-1}$). $Stress_{t-2}$ and $stress_{t-1}$ represent 7-day total NISS scores from the respective week. For example, to study the relative abundance at week 3 (t=3), we used 7-day total NISS scores at week 1 ($stress_1$) and 7-day total NISS score at week 2 ($stress_2$) as covariates. All the continuous variables were standardized.

We applied the ZIBR to each taxon individually and used the Benjamini-Hochberg approach (Benjamini & Hochberg, 1995) to control the false discovery rate (FDR=5%). P-values are computed for each part (Binary and Beta) of the ZIBR model. To be more specific, p-values from the Binary part measure the strength of association between the presence of genera and covariates, while p-values from the Beta part measure the strength of association between the non-zero relative abundance of genera and covariates (Chen & Li, 2016). In addition, we calculate the overall p-values from ZIBR model, which measure the strength of overall association between genera and covariates by integrating the covariate effects from both Binary and Beta parts. All the analyses were run in SAS version 9.4 and R version 3.4.1 using R package ZIBR.

3. Results

A total of 82 infants were born between 24–37 weeks postmenstrual age and enrolled in the parent study. After adjusting the sample to only those infants with at most one missing sample within weeks 3–6, the sample was reduced to 47 infants. For the 47 infants included in these analyses, there was complete data for all the covariates. Mean birth weight was 1075 grams (range 953–1200), infants were 55% female, mean gestational age at birth was 28 weeks and the majority of infants (72%) were delivered by cesarean section. See Table 1 for additional sample characteristics. The count and percentage of microbiome samples for each week was: week1= 31(37.80), week2= 53(64.63), week3= 69(84.15), week4= 66(80.49), week5= 63(76.83) and week6= 55(67.07).

We report in Table 2 the estimates from the Binary (Panel A) and Beta (Part B) parts of the ZIBR model, respectively. A positive estimate in Panel A, with significant p-value, indicates that the gene is more likely to be present in the stool sample with larger value of the covariate. Similarly, a positive estimate in Panel B, with significant p-value, indicates that the gene is more likely to have larger non-zero relative abundance with larger value of the covariate.

All of the following p-values are FDR-adjusted p-values. In Table 3, we report the overall p-values from ZIBR model. Table 3 shows that stress 2 weeks prior (stress$_{t-2}$) had a significant effect only on *Proteus* (overall p-value = 0.015). In addition, we observed that stress 1 week prior (stress$_{t-1}$) had a significant effect on two genera, including *Proteus* (overall p-value < 0.001) and *Veillonella* (overall p-value = 0.033). Larger stress scores were associated with smaller probabilities of *Proteus* and *Veillonella* being present. However, when *Proteus* and *Veillonella* were present, larger stress scores were associated with larger values of relative abundances for *both genera*.

Figure 3 shows the relative abundance of the significant genera by week. Table 3 shows the overall bacteria genera p-value. The model identified 8 genera for the week effect: *Escherichia* (week6: overall p-value = 0.004); *Staphylococcus* (week5: overall p-value = 0.018; week6: overall p-value = 0.017); *Enterococcus* (week5: overall p-value = 0.010; week6: overall p-value = 0.003); *Bifidobacterium* (week6: overall p-value = 0.004); *Proteus* (week4: overall p-value < 0.001; week5: overall p-value = 0.010; week6: overall p-value = 0.003); *Streptococcus* (week5: overall p-value = 0.044; week6: overall p-value = 0.006); *Clostridium.butyricum* (week6: overall p-value=0.042); *Clostridium.perfringens* (week6: overall p-value = 0.003).

Figure 4 shows the relative abundances of the three genera with significant overall antibiotic effect, by week and antibiotic usage. Antibiotic did not lead to different probabilities of observing *Proteus* (p-value = 1.000), *Citrobacter* (p-value = 0.387) or *Clostridium.perfringens* (p-value = 0.138) but led to different values of non-zero relative abundances: *Proteus* (p-value = 0.012), *Citrobacter* (p-value = 0.012) and *Clostridium.perfringens* (p-value = 0.012), adjusting for the other covariates.

Proteus was the only genus that had a significant overall gender effect (overall p-value = 0.034), see Figure 5. However, if we consider each part of the model separately, gender did

not lead to different probabilities of observing *Proteus* (*p*-value = 1.000) or different values of relative abundances for *Proteus* (*p*-value = 0.057) given *Proteus* was present.

Two genera were significantly affected by the birth weight, including *Klebsiella* and *Proteus* (overall *p*-value = 0.037; overall *p*-value = 0.048). Gestational age was not a significant factor for all the genera considered in this model.

4. Discussion

Stress exposure has been reported to alter biological mechanisms in preterm infants who require neonatal intensive care. Understanding the role NICU stress exposure plays in the developing gut microbiome however requires further investigation. By examining the microbial abundance and diversity of preterm infant stool during the delivery of intensive care, this study sought to explore if higher intensities of stress exposure alter the gut microbiome composition during the first 6 weeks of life. Several differences in bacterial genera during this time period. We found an overall dominance from the Gammaproteobacteria taxon. In particular, sampling weeks 4, 5, 6, antibiotic usage, gender, birth weight and stress exposure during weeks 1 and 2 prior to sampling had significant effects on the dominance of *Proteus*. Other significant effects of Gammaproteobacteria included birth weight on *Klebsiella*, week6 of sampling on *Escherichia*, antibiotic usage on *Citrobacter* (see Table 3).

In this study, we were particularly interested in understanding what role stress exposure from NICU care may play in the developing gut microbiome. Using the NISS, daily stress exposure was summed into weekly totals. Our findings indicate that stress exposure during 1 and/or 2 weeks prior to sampling had a significant effect on both presence and relative abundance of *Proteus* and *Veillonella*, with *Clostridium_perfringens* approaching significance. Furthermore, the estimates indicate that if these genera are present, infants with higher stress exposure tend to have higher relative abundances of *Proteus* and *Veillonella*. In the animal model, stress has been linked to bowel disease (Chow, Tang, & Mazmanian, 2011; Medel-Matus, Shin, Dorfman, Sankar, & Mazarati, 2018; Reber et al., 2016). Additionally, the presence of Proteobacteria pathobionts species have been shown to profoundly influence vulnerability to gut pathology (Chow et al., 2011; Langgartner et al., 2017). In humans, connections are emerging between the microbiota and brain health (Jiang et al., 2018; Pulikkan et al., 2018; Shen et al., 2018; Winter, Hart Robert, Charlesworth Richard, & Sharpley Christopher, 2018). As research continues to elucidate the microbiota-brain-gut axis (Foster, Rinaman, & Cryan, 2017), it will be important to understand how early life environmental stress exposure and dysbiosis effect preterm infant short and long-term neurodevelopment (Groer et al., 2014).

Gammaproteobacteria are Gram-negative rod-shaped facultative anaerobes. There are many potentially virulent organisms in this family (e.g. *Escherichia*, *Klebsiella*, *Serratia*, *Enterobacter* and *Proteus*) and many of these are related to common NICU morbidities (Mithal, Palac, Yogev, Ernst, & Mestan, 2017; Patel et al., 2016). *Veillonella* are Gram-negative commensal obligate anaerobes; however, *Veillonella* has been implicated in intestinal pathology (Bajer et al., 2017). Preterm infant guts are believed to follow a

nonrandom process of convergence to Clostridia by initial dominance of Bacilli followed by a surge of Gammaproteobacteria blooms, with a gradual increase in Clostridia. Zwittink et al (2017) found GA and associated intensity of NICU care (respiratory support and antibiotic treatment) to be associated with *Bifidobacterium* abundance whereas, extremely preterm infants demonstrated lower abundance of *Bifidobacterium* and higher abundance of facultative anaerobes (Zwittink et al., 2017). In addition to the present study's aforementioned dominance of Gammaproteobacteria, our other dominant taxa included Bacilli (*Staphylococcus*, *Streptococcus*, *Enterococcus*) and Clostridia (*Veillonella*, *Clostridium*) genera.

A time series abundance study of a single preterm infant's gut colonization revealed week 3 of life to represent dynamic changes in microbial community structure. Change were marked by dominance of the facultative anaerobe *E-coli* early in week 3, then transitioning to obligate anaerobes (*Streptococcus*, *Clostridium_butyricum* and *Veillonella*) by the end of the same week (Brown et al., 2013). Researchers suggest these changes may reflect fermentation based metabolism, as there were no clinical indicators to account for the observed changes (Brown et al., 2013). In our study, week5 had a significant effect on *Staphylococcus*, *Enterococcus* and *Streptococcus*. Additionally, week6 had a significant effect on *Escherichia*, *Staphylococcus*, *Enterococcus*, *Bifidobacterium*, *Streptococcus*, *Clostridium_butyricum*, *Clostridium_perfringens*. Findings suggest that some of our time effect significance may be due to expected microbial succession.

Our only binary covariates included antibiotics and gender. Infants with antibiotic use had significantly less *Proteus* and significantly more *Citrobacter* and *Clostridium_perfringens* when these genera were present, see Figure 4. Additionally, female infants had significantly less *Proteus* microbes when *Proteus* was present, see Figure 5.

In our study, intertwined effects were noted in which some covariates had an effect on particular OTUs, which may be important to preterm infant health and gut composition. *Proteus* and *Veillonella* were significantly affected by stress exposure (*Clostridium_perfringens* approached significance) while, *Proteus* and *Clostridium_perfringens* were significantly affected by antibiotic usage (*Veillonella* approached significance). When considering opportunities for future causal research, these findings beg the question, did these preterm infants experience more stress because they were sick and required antibiotics, thus creating an environment for potentially virulent bacteria or did antibiotic usage cause an increased risk through overabundance which then is additive with gender, GA and stress? Investigating the functional significance of the dysbiosis will be important for clinical practice. Of note, effects of stress experienced by preterm infants in the NICU have not been well studied. Continued interrogation of what role early life NICU stress, as well as nurturing experiences, play in preterm infant outcomes and how to best measure them are critically important. Other considerations for future research include sample size, stress exposure and missing data suggestions. First, given our small sample size, it will be important to replicate this study in a larger sample size with comparable NICU stress exposure data. Second, investigating if particular NICU stressors or interventions have more of an effect on the presence and abundance of certain OTUs will be important for both research and clinical practice. Third, understanding if there may be a

metabolic interaction between *Proteus* and *Veillonella* will be important when considering bacterial risk. Finally, future studies are needed that develop new statistical methods to handle missing compositional data when the missing completely at random assumption is violated.

In summary, the process of infant gut colonization is dynamic and influenced by many environmental factors. For vulnerable preterm infants who experience high NICU stress exposure, our data suggests these infants also experience increased microbial abundances of *Proteus* and *Veillonella*. As a species of Gammaproteobacteria, dominance of *Proteus* may pose an increased risk for immunocompromised preterm infants. Antibiotic usage, gender, birth weight and week of life were also significant for multiple genera. The factors found to be significant in this study touch almost all NICU infants. Thus, continued investigations of how stress and other environmental factors potentially pose risks to the developing gut microbiome represent exciting opportunities for future research.

Acknowledgements:

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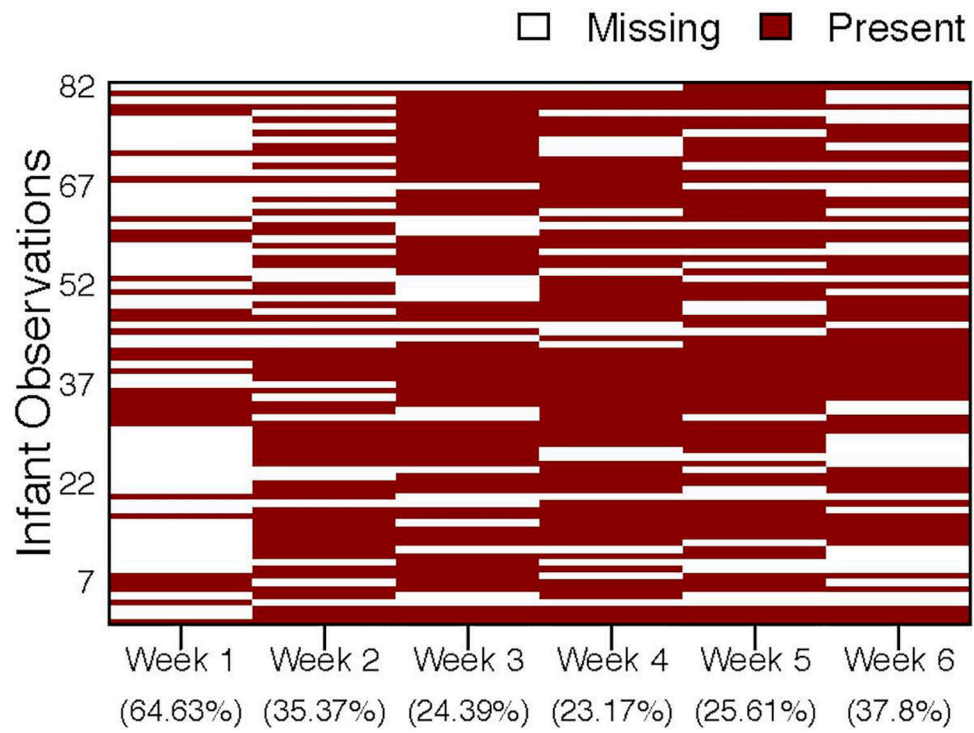
Figure 1.
Plot of missing data pattern and missing data percentage for microbiome data by week, where red color indicates the data are present and white color indicates the data are missing.
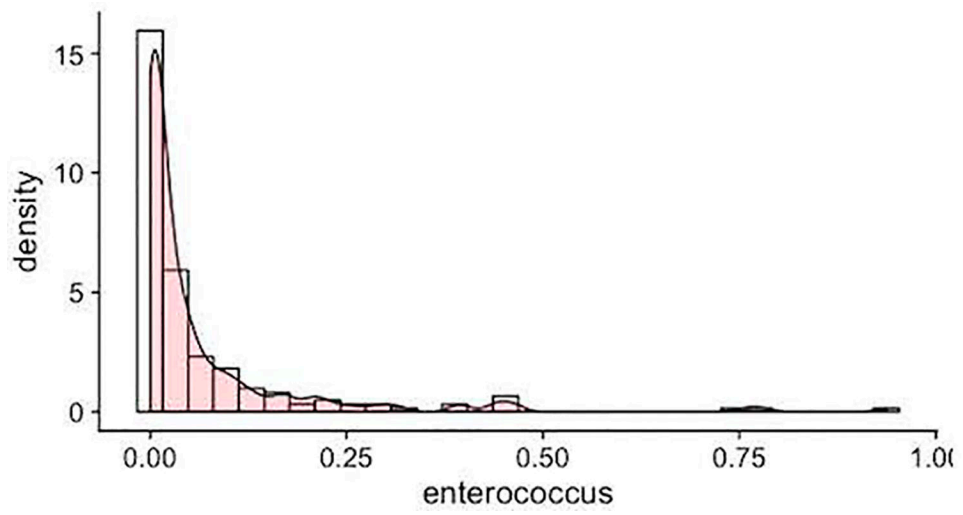
Figure 2.
Example of a genus from the gut microbiome data. Bars represent the histogram and the curve represents the kernel density estimate of Enterococcus.
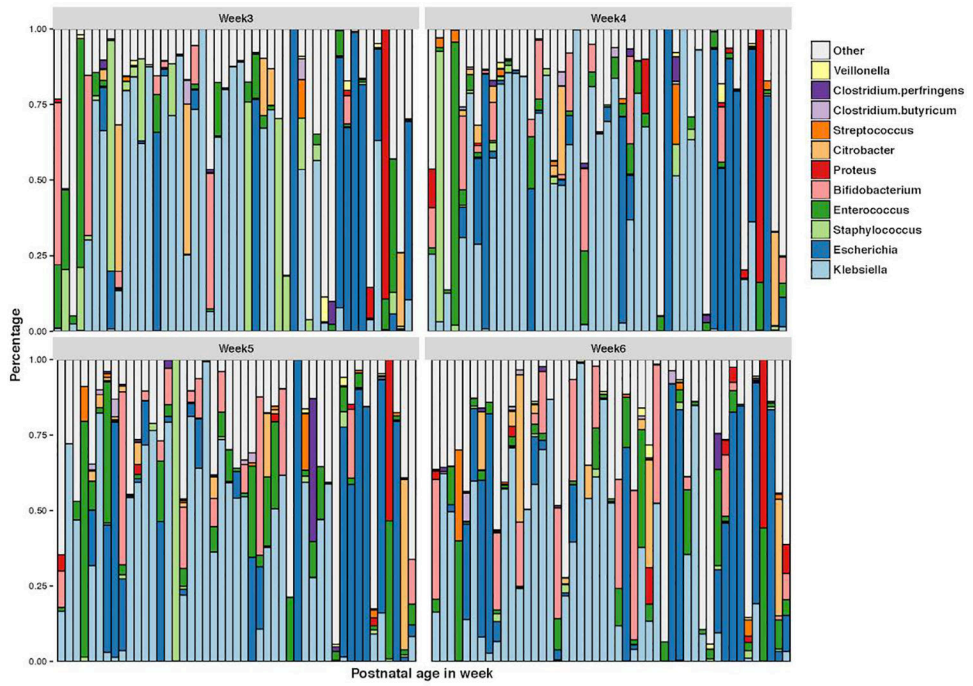
Figure 3.
Relative abundance of significant genera by week of life.
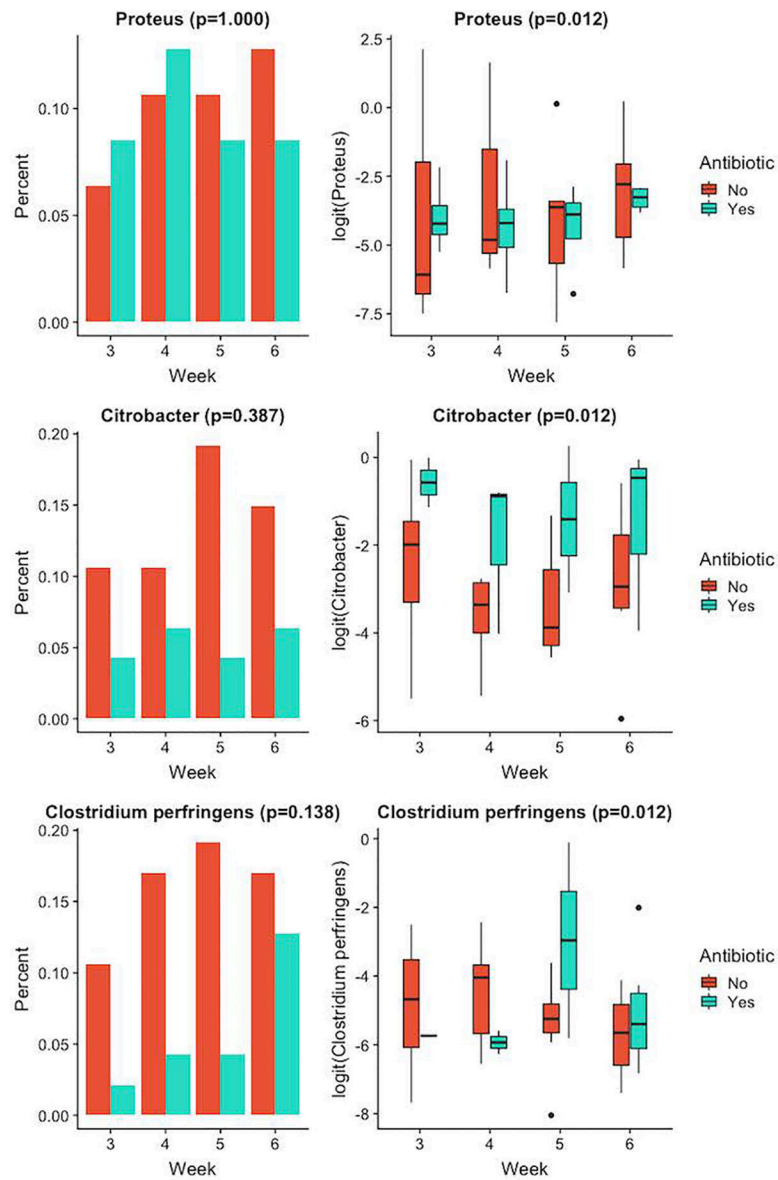
Figure 4.
Relative abundance of genera with significant effect for antibiotic (0= no antibiotic usage, 1= yes antibiotic usage). Left panel shows the percentage of samples in the two antibiotic groups where the genus was present. Right panel shows the logit-transformed non-zero relative abundance in the two groups.
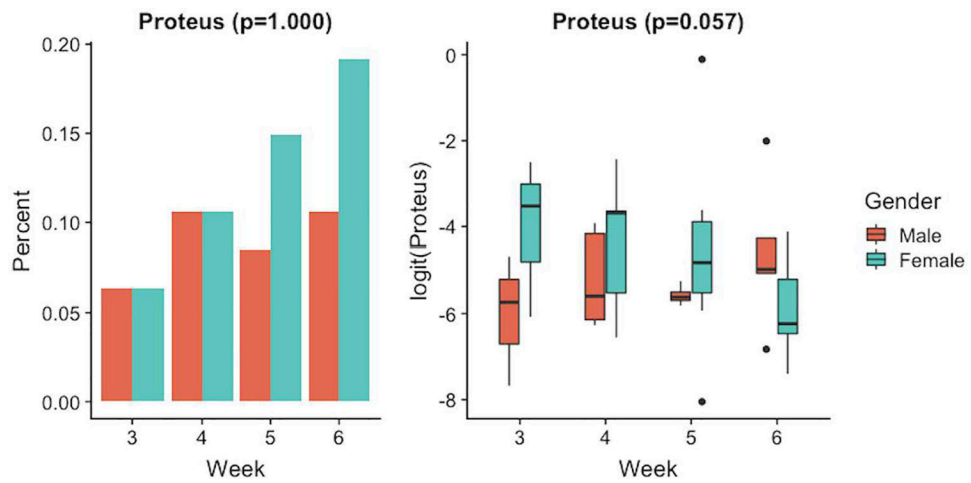
Figure 5.
Relative abundance of genus with significant effect for gender (0= male and 1= female). Left panel shows the percentage of samples in the two gender groups where the genus was present. Right panel shows the logit-transformed non-zero relative abundance in the two groups.

Table 1.

Descriptive statistics of study sample, n = 47. Shown as percentage or median (inter-quartile range).

Delivery Method	Vaginal (0)	13	27.66
	C-section (1)	34	72.34
Antibiotic	No (0)	24	51.06
	Yes (1)	23	48.94
Baby Gender	Male (0)	21	44.68
	Female (1)	26	55.32
Birth Weight (g)		1075.00	(952.50, 1200.00)
Gestational Age (day)		27.60	(26.50, 29.00)
Cumulative Weekly Stress Score	Week 1	937.00	(875.00, 1018.00)
	Week 2	943.00	(847.00, 1026.00)
	Week 3	915.00	(827.00, 977.00)
	Week 4	883.00	(805.00, 975.00)
	Week 5	824.00	(778.00, 923.00)
	Week 6	808.00	(763.00, 878.00)

Table 2.

Panel A. Estimates from the Binary part of ZIBR model for 11 bacterial genera.

Species	Week4	Week5	Week6	Antibiotic	Baby Gender	Birth Weight	Gestational Age	Stress$_{t-2}$	Stress$_{t-1}$
Klebsiella	0.359	0.025	0.818	−1.659	−3.808	4.073	1.174	0.012	−0.485
Escherichia	2.609	3.486	4.802	−1.941	0.259	1.129	−2.368	−0.110	−0.783
Staphylococcus	−0.975	−1.822	−1.591	0.501	−0.176	0.455	−0.042	0.151	−0.111
Enterococcus	1.849	2.594	3.899	−2.285	−2.937	−1.154	0.941	−1.122	−0.304
Bifidobacterium	1.135	2.492	3.052	−3.428	1.210	3.063	−2.644	0.178	−0.782
Proteus	5.092	3.480	5.089	2.217	−5.328	−4.582	2.959	−1.954	−1.841
Citrobacter	0.368	1.549	1.077	−1.446	−4.263	0.619	2.869	−0.228	0.539
Streptococcus	1.287	2.105	2.541	0.176	−1.383	0.598	−0.339	−0.176	0.119
Clostridium. butyricum	1.126	1.534	1.855	−0.075	−1.749	−0.007	1.015	−0.185	0.434
Clostridium. perfringens	1.514	1.858	2.847	−2.633	−0.850	0.842	0.585	0.471	0.169
Veillonella	−0.159	−0.033	1.826	−4.205	−5.926	−0.528	0.364	−0.646	−2.223

Table 2.

Panel B. Estimates from the Beta part of ZIBR model for 11 bacterial genera.

Species	Week4	Week5	Week6	Antibiotic	Baby Gender	Birth Weight	Gestational Age	Stress$_{t-2}$	Stress$_{t-1}$
Klebsiella	0.438	−0.160	−0.408	0.159	0.504	−0.080	−0.102	0.000	−0.018
Escherichia	0.145	0.218	−0.156	0.229	−0.738	−0.381	−1.089	−0.267	−0.020
Staphylococcus	−0.290	−0.218	−0.357	0.061	−0.102	−0.001	−0.005	−0.037	0.092
Enterococcus	0.163	0.531	0.341	−0.425	0.144	0.141	−0.205	−0.053	−0.165
Bifidobacterium	0.286	0.299	0.422	−0.218	0.228	−0.162	0.050	0.292	0.140
Proteus	1.749	0.210	0.642	−3.057	−0.586	−0.086	−0.906	−0.487	1.380
Citrobacter	−0.565	−0.477	−0.388	1.285	0.324	−0.123	0.184	0.066	−0.075
Streptococcus	0.304	0.365	0.032	−0.090	0.490	0.438	−0.221	0.051	0.069
Clostridium. butyricum	−0.006	0.008	−0.211	−0.051	−0.224	−0.098	−0.020	0.184	0.212
Clostridium. perfringens	0.060	−0.089	−1.409	1.847	1.333	−0.525	0.232	−0.286	0.940
Veillonella	−0.328	−0.527	−0.505	−0.448	−0.243	0.210	0.045	0.086	0.047

Table 3.

Overall p-values from ZIBR model for 11 bacterial genera.

Species	Week4	Week5	Week6	Antibiotic	Baby Gender	Birth Weight	Gestational Age	Stress$_{t-2}$	Stress$_{t-1}$
Klebsiella	0.582	0.888	0.273	0.534	0.247	**0.037***	1.000	1.000	0.970
Escherichia	0.424	0.060	**0.004****	0.691	0.575	0.287	0.090	0.693	0.928
Staphylococcus	0.483	**0.018***	**0.017***	0.691	0.934	0.461	1.000	0.990	0.970
Enterococcus	0.424	**0.010***	**0.003****	0.099	0.575	0.237	0.280	0.349	0.896
Bifidobacterium	0.672	0.059	**0.004****	0.136	0.563	0.237	0.255	0.693	0.872
Proteus	**0.000*****	**0.010***	**0.003****	**0.001****	**0.034***	**0.048***	0.104	**0.015***	**0.000*****
Citrobacter	0.868	0.236	0.350	**0.022***	0.331	0.533	0.700	0.990	0.928
Streptococcus	0.483	**0.044***	**0.006****	0.982	0.331	0.287	0.894	0.990	0.970
Clostridium. butyricum	0.612	0.169	**0.042***	0.982	0.221	0.863	0.255	0.867	0.872
Clostridium. perfringens	0.483	0.169	**0.003****	**0.005****	0.113	0.100	0.290	0.532	0.052
Veillonella	0.893	0.499	0.059	0.099	0.221	0.287	0.767	0.773	**0.033***

* p-value < 0.05;

** p-value < 0.01;

*** p-value < 0.001