Characterising the bacterial gut microbiome of probiotic-supplemented very preterm infants

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Abstract

Background:

The gut microbiome plays a critical role in the healthy development, immunity and metabolism of infants. Preterm birth disrupts microbiome development and can contribute to acute and chronic disease. To promote microbial and infant development, and to mitigate the risk of disease, premature infants may be treated with probiotics. Here we used 16S rRNA high throughout sequencing to characterize the bacterial microbiome of probiotic-supplemented premature infants. The study aimed to identify and understand variation in bacterial gut flora, including changes from admission to discharge, and the effect of several clinical variables using a combination of univariate and mixed effects analyses.

Results:

Infants born <32 weeks gestation and <1500 g were recruited in North Queensland, Australia, with faecal samples collected at admission (n = 71) and at discharge (n = 63). Our research builds on previous research and supports significant changes over time in the preterm infant microbiome, and in response to several variables. Univariate analysis showed admission and discharge samples had significantly different microbial populations, with Staphylococcus enriched at admission and Enterobacter, Lactobacillus, Clostridium sensu stricto 1 and Veillonella at discharge. From the mixed effects modeling we observed significantly lower alpha diversity in infants diagnosed with either sepsis or retinopathy of prematurity (ROP), and those that only received formula milk. Chorioamnionitis, preeclampsia, sepsis, necrotizing enterocolitis and ROP were also all associated with differential abundance of several taxa.

Conclusions:
Our study builds on previous research and supports significant changes in the preterm microbiome over time and in association with several factors. The fact that several associations were observed, and some in ways that counter previous work, highlights the complexity of microbiome ecology.

**Key Words:** microbiome, preterm, premature, neonate, infant.

**Background**

The gut microbiome composition of preterm infants is significantly different to those born full term, and is characterised by lower diversity (1, 2), high inter-individual variation (3-5) and fewer commensal microbes. Despite high inter-individual variation, preterm infants typically have reduced levels of common commensals like *Bifidobacterium* (3, 5), *Lactobacillus* (3, 6) and *Bacteroides* (4, 5), and higher levels of pathogens like *Klebsiella pneumoniae* (7) and *Clostridium difficile* (5). However, the gut microbiome is dynamic and changes significantly over time as the infant grows (8). Although reduced levels of common commensal organisms and diversity can persist for months (9, 10), maybe years (11), choreographed abrupt changes in composition (12, 13) and increases in diversity (10) mean that eventually the preterm gut microbiome composition becomes more similar to that of full-term infants.

This developing microbiome plays a significant role in infant development and is integral to immune (14) and metabolic health (15). As the infant grows and develops, the gut microbiome develops in parallel. A symbiotic relationship exists between infants and their microbes enabling cross-talk between microbes, the gut epithelium, and gut-associated lymphoid tissue. This crosstalk aids development of innate immune defences and promotion
of pathogen recognition. It also regulates gene expression for promotion of epithelial turnover, mucus biosynthesis and production of antimicrobial compounds (16), as well as increased peristalsis (17, 18).

Shifts in the composition and organism dominance result from environmental changes and major colonising events. Colonisation occurs via different routes and may be influenced by several crucial factors, including delivery and diet. There is some evidence to suggest inoculation beginning in utero via maternal-fetal translocation (19, 20), but this route of inoculation is still up for debate (21). Thus delivery is the first major colonising event with the mode of delivery contributing significantly to the observed between individuals (22, 23). Vaginally delivered infants have higher abundance of vaginally derived, beneficial microbes such as Lactobacillus (22, 24), and caesarean born infants have greater abundances of skin dwelling microbes such as Staphylococcus (10, 22). As for diet, breast milk and formula also produce significantly different microbial communities (8, 25), due to the presence of both microbiomes and pre-biotics such as human made oligosaccharides (HMOs) in breast milk (26). Although maternal skin and vaginal microbes colonise infants during birth and feeding, these microbes may only be transient with maternal gut microbes, passed through birth or lactation proving to be more persistent (27).

As much of the microbial inoculation is occurring through maternal-infant exchange, maternal health and medical interventions can also influence the developing infant microbiome. Interventions like antibiotics (28) and diseases like chorioamnionitis (23), a bacterial infection occurring before or during labour, have been shown to influence the infant microbiome previously. So it is possible that other maternal microbiome-altering diseases, like type 2 diabetes (29) and preeclampsia (30), a pregnancy disorder characterised by high blood pressure, also have the potential to disrupt the infant microbiome. A dysbiotic infant microbiome, which is characterised by an imbalance between commensal and pathogenic
microbes, resulting from maternal-infant transfer could have severe consequences for infant health and development (31).

Microbial dysbiosis puts preterm infants at a high risk of acute infection (32, 33), chronic disease (34, 35) and developmental abnormalities (36, 37). The increased risk in disease is a consequence of the breakdown in the symbiotic relationship between infants and colonising microbes during development. Delayed colonization by commensal microbes could result in increased sensitivity leading to irregular immune responses resulting from intolerances to normal flora (38, 39). Additive to this is an imbalance between commensals and pathogens that may induce intestinal inflammation and cytokine production (40), resulting in acute pathologies like necrotizing enterocolitis (NEC) and sepsis, developmental disorders, like retinopathy of prematurity (ROP) (41), and eventually chronic diseases like asthma (42).

Despite differing aetiologies, the gut microbiome has been implicated in the pathologies of NEC, sepsis and ROP, with associations with either low diversity or taxonomic abundance (43-46). All are very different conditions. NEC affects 4-11% of preterm very low birth weight infants with 20-30% overall mortality (33) and is characterised by intestinal inflammation and subsequent necrosis of the bowel. Sepsis is a systemic response to blood-stream infection that affects 20% of preterm infants (32). ROP on the other hand is a potentially blinding disease caused by abnormal development of retinal blood vessels (47).

Preterm infants are disproportionately affected by disease, and more likely to undergo a myriad of treatment regimens that can also impact the developing microbiome. Treatment with antibiotics, a staple in preterm neonatal care, and probiotics, an emerging preventative strategy can both alter the developing microbiome. Antibiotics have been linked to microbial
dysbiosis (48), whilst probiotics have been shown to promote the growth of commensal microbes and increases in diversity (49-51), as well as reducing disease incidence (52).

Despite ever-accumulating research in the field of the preterm infant gut microbiome, there is still a lot to be explored, especially when considering the heterogeneity in the literature. This prospective observational study using 16S rRNA high throughput analysis of faecal and meconium samples aimed to characterise the bacterial gut microbiome of preterm infants. Specifically, we set out to characterise changes in a probiotic-supplemented cohort of preterm infants from admission to discharge, and to examine the impact of several key variables, both maternal and infant.

Methods
Study population
16S rRNA high throughput sequencing was used to characterise the bacterial microbiome, down to the genus, of infants receiving probiotic supplementation and born into the Townsville Hospital and Health Service’s (THHS) Neonatal Intensive Care Unit (NICU). The THHS Neonatal intensive care unit (NICU) is the only level six tertiary referral unit outside southeast Queensland, Australia. Thus, all babies being born at <29 gestation weeks in North Queensland are referred here. North Queensland is affected disproportionately by preterm birth, with the North West experiencing the highest rate (12%) of pre-term births (53), and the Torres and Cape the highest proportion (11.7%) of low birth weight (LBW) infants (53). North Queensland (NQLD) also has a large indigenous population, whose infants are more likely to be born prematurely (13%) and represent one out of ten premature births in Queensland (53). When considering the increasing prevalence of premature birth in the NQLD, 5% over the last decade (53), the burden that preterm birth places on NQLD families and the healthcare system is significant.
Study design and ethics

Ethics was obtained from the Human Research Ethics Committee from the THHS, and recruitment commenced in October of 2017, and continued until October of 2018. Inclusion criteria was infants born <32 weeks’ gestation and admitted to the NICU at the THHS. The exclusion criteria were no parental consent, gestational age of >32 weeks and contraindication to enteral feeds. The probiotic Infloran™ (54) is administered via enteral feeds and to all infants born <32 weeks gestations and <1500 g at the THHS NICU. Recruitment was conducted by a neonatal nurse/research assistant who works at the NICU, and sample collection by NICU nurses using collection kits’ (biohazard bag, sterile swab and storage container). After collection, samples were sent via a pneumatic tube system to Pathology Queensland and stored at -80°C. Infant and maternal clinical information was also collected for downstream analysis.

Sequencing and bioinformatics

In brief, the protocol used in this study included sample storage at -80°C (55), an extraction kit that includes mechanical lysis (56), use of the Illumina MiSeq platform (57), targeting of the V3/V4 regions (58) and use of the SILVA reference database (58). DNA extraction was conducted using the Bioline ISOLATE Fecal DNA Kit (59), with modifications made in consultation with the manufacturer to optimise DNA yield. This included increased beta-mercaptoethanol (from 0.5 to 1% to increase DNA solubility and reduce secondary structure formation), addition of an extra wash step (to improve purity) and decreased elution buffer volume (to increase final DNA concentration). For library preparation we followed the Illumina metagenomics library preparation protocol (60), using the Index Kit v2 C (61), along with Platinum™ SuperFi™ PCR Master Mix (62). The MiSeq
Reagent Kit V3 (61) was used in combination with the Illumina MiSeq System, targeting the V3 and V4 regions with the 785F/800R primer combination for sequencing.

Pre-analytical bioinformatics were conducted in R Studio Version 3.6.1 (63). To process the raw reads produced by sequencing into interpretable abundances, an amplicon sequence variants (ASV) table, a pipeline was adapted from Workflow for Microbiome Data Analysis: from raw reads to community analyses (64), which along with the subsequent analyses can found under Additional File 1. DADA2 (65) was used for quality filtering and trimming, demultiplexing, denoising and taxonomic assignment (with the SILVA Database), and the microDecon package (66) used to remove homogenous contamination from samples using six blanks originating in extraction.

Statistical analysis

Exploring changes in composition and diversity from admission to discharge

For statistical analysis, a phyloseq object was created using the package Phyloseq (67). Taxa were then filtered by prevalence (threshold = 0.01), agglomerated at the genus level and then normalized through Total Sum Scaling. The data were then explored through Principle Coordinate Analysis (PCoA) plots using a Bray-Curtis dissimilarity matrix. Permutational analysis of variance (PERMANOVA) was then conducted for community-level comparisons between Admission and Discharge samples to observe group-level differences based on the Bray-Curtis dissimilarity matrix, using the adnois() function of the package Vegan (68). Alpha diversity indices, Shannon Index and Observed (richness), were then calculated on filtered, non-agglomerated data, and a comparison was made between Admission and Discharge samples using a Wilcoxon Rank Sum Test, with adjusted p-values accounting for False Discovery Rate using the Benjamini-Hochberg procedure (69). To identify individual microbes whose abundance changed significantly from admission to
discharge, data that was filtered and agglomerated at the genus level, but not transformed, were then normalized and modeled (negative-binomial) with *DESeq2* (70). Then with the *DESeq()* function, a Wald Test with the Benjamini-Hochberg multiple inference correction was performed to determine significant differentially abundant taxa.

**Exploring the effect of clinical variables on alpha diversity and taxonomic abundance**

Lastly, associations between several clinical variables and community structure were explored. The relationship between clinical variables and both Shannon Diversity and taxonomic abundance were assessed using multivariant linear regression models. For exploring the relationship with Shannon diversity, a mixed effects linear regression model was created using the package *lme4* (71), with a gaussian distribution and using the restricted maximum likelihood estimation. Continuous predictors were scaled and centered to avoid convergence issues and multicollinearity assessed using the *AED* package (72). Gestation and birth weight were found to be collinear and thus birth weight was removed from the model. Thirteen predictors: mode of delivery, feeding type, gestation, antenatal antibiotics, antenatal infections, NEC, sepsis, chorioamnionitis, neonatal antibiotics, death, prolonged membrane rupture, preeclampsia, diabetes and retinopathy of prematurity were included in the initial model. To control for high amounts of inter-individual variation in the microbiome of preterm infants (1), individual’s identification (unique record number – *URN*) was included as a random factor. As we wanted to assess the influence of clinical variables at both admission and discharge, this was included as an interaction variable (labelled Type). The resulting model *Shannon ~ (13 Parameters) * Type + (1|URN)*, assesses the effect of the 13 predictors on Shannon diversity for both types of samples, Admission and Discharge, whilst accounting for the individual, represented here by *URN*. 
Backwards selection (69) was then implemented to simplify the model by comparing Akaike’s Information Criterion (AIC) scores between regression models and removing predictors that were not contributing to the model. The process was repeated until the least complex adequate model was identified, when no more predictors could be removed without significant effects. The final model was $\text{Shannon} \sim (\text{Sepsis} + \text{Feeding Type} + \text{Chorioamnionitis} + (\text{Mode of Delivery} + \text{Gestation Days} + \text{NEC} + \text{Preeclampsia} + \text{ROP})) * \text{Type} + (1|\text{URN})$. The significance of the fixed effects variables in this final model was then assessed using analysis of deviance (Type II Wald Chi-square test) from the car package (73), and post-hoc pairwise Tukey comparisons (correcting for multiple comparisons) from the emmeans package (74).

For differential taxonomic abundance, two negative binomial generalized linear models were created using the package DESeq2. A combination of previous literature and exploratory analysis, including PCoA plots, PCA and scatterplots, were used for model selection. Again, continuous predictors were scaled and centered, and multicollinearity was assessed. Taxa were agglomerated at the genus level, due to the limited sequencing depth of short amplicon sequencing. To reduce the number of false positives, two separate models were run; one each for admission and discharge samples. The resulting model $\text{Taxonomic Abundance} \sim \text{Sepsis} + \text{Feeding Type} + \text{Chorioamnionitis} + \text{Mode of Delivery} + \text{Gestation Days} + \text{NEC} + \text{Preeclampsia} + \text{ROP}$ was created to assesses the effect of the 9 independent predictors at both time points on all genera present. Low abundance and low frequency taxa were then removed, and a Wald Test with the Benjamin-Hochberg multiple inference correction was then performed to determine significant differentially abundant taxa. More information on the analysis can be found in Additional File 1.
Results

Exploring changes in composition and diversity from admission to discharge

Figure 1. Histograms representing taxonomic distribution (top 20 taxa) of relative abundance for admission and discharge samples at both phylum (A) and genus (B) levels.
Figure 2. A: Principle coordinate analysis plot for admission versus discharge based on Bray-Curtis dissimilarity matrix \( (p<0.01 & R^2 = 0.06) \), B: box plots of alpha diversity for admission versus discharge, C: table of differential abundance testing for admission versus discharge (base value is admission).

85 preterm infants born <32 weeks and <1500g were recruited from the THHS NICU. 134 stool samples were collected, of which 71 were from admission and 63 from discharge. Significant changes in taxonomy were observed (Figure 1), with Enterobacter \( (p<0.01) \), Lactobacillus \( (p<0.01) \), Clostridium Sensu Stricto 1 \( (p<0.01) \) and Veillonella \( (p<0.05) \) all significantly enriched at discharge, and Staphylococcus at admission \( (p<0.01) \) (Figure 2C).

For beta diversity, although there was limited separation between admission and discharge samples, there was clustering that resulted in a significant difference between the two groups based on abundance and phylogeny (Figure 2A, PERMANOVA; \( p<0.01 & R^2 = 0.06 \), homogeneity of variance; \( p = 0.85 \)). The average species diversity within samples (Observed and Shannon) increased from admission to discharge (Figure 2B), but not significantly.

Exploring the effect of clinical variables on alpha diversity and taxonomic abundance

Several maternal and infant variables were significantly associated with the preterm infant gut microbiome. Mixed effects models show that several clinical and environmental variables are significantly associated with both the diversity and taxonomic composition within samples. Significant pairwise differences in diversity were observed for feeding type, sepsis and ROP (Figure 3), and chorioamnionitis, sepsis, NEC, ROP and feeding type were all associated with changes in taxonomy (Table 1).
Figure 3. Boxplots of alpha diversity (Shannon Index) for significant analysis of deviance outcomes, with significant Tukey’s pairwise comparisons designated by lower case letters, (where a is significantly different from b) on linear mixed effects model. Annotation for Feeding Type: B: Breastmilk, B/F: Breastmilk and Formula & F: Formula. A: Box plot comparing alpha diversity at admission and discharge between different modes of delivery, B: Box plot comparing alpha diversity between different diets, C: Box plot comparing alpha diversity at admission and discharge between infants with and without preeclamptic mothers, D: Box plot comparing alpha diversity between sepsis diagnoses, E: Box plot comparing alpha diversity at admission and discharge between ROP diagnoses.
| log2FoldChange | lfcSE | padj  | Genus     | Variable                  | Sample    |
|---------------|-------|-------|-----------|---------------------------|-----------|
| 3.04          | 0.97  | 0.04  | Staphylococcus | Chorioamnionitis:Yes      | Admission |
| 13.92         | 2.79  | <0.01 | Bifidobacterium | Sepsis:Yes               | Admission |
| -17.23        | 3.99  | <0.01 | Pseudomonas  | Sepsis:Yes               | Admission |
| -19.18        | 3.79  | <0.01 | Diaphorobacter | Sepsis:Yes               | Admission |
| -14.29        | 2.34  | <0.01 | Bifidobacterium | NEC:Yes                  | Admission |
| 4.57          | 0.97  | <0.01 | Staphylococcus | ROP:Yes                  | Admission |
| -4.11         | 1.40  | 0.03  | Streptococcus  | Chorioamnionitis:Yes     | Discharge |
| -29.19        | 2.40  | <0.01 | Escherichia/Shigella | Preeclampsia:Yes | Discharge |
| -3.06         | 0.86  | <0.01 | Bifidobacterium | Feeding:Formula          | Discharge |
| -4.01         | 1.36  | 0.01  | Klebsiella    | Feeding:Formula          | Discharge |

Table 1. The significant differentially abundant taxa at the genus level obtained from DESeq2 analysis, with log2FoldChange for the variable listed compared to the base value.

Mode of delivery and diet

Both the mode of delivery and type of milk the baby received had significant associations with diversity, but only mode of delivery was associated with differential taxonomic abundance. Diversity was significantly higher in cesarean born infants at discharge than those born vaginally, relative to the difference observed at admission (Figure 3A; $\chi^2 = 4.18$, df = 1, $p<0.05$). However, subsequent post-hoc analysis showed no significant pairwise comparisons within the delivery variable. The type of milk the infant received also had a significant effect
(Figure 3B; $\chi^2 = 7.29$, $df = 2$, $p<0.05$), with subsequent post-hoc pairwise comparisons finding a significant difference between formula-fed infants ($\bar{x} = 2.10 \pm 0.17$) and those breastfed ($\bar{x} = 1.56 \pm 0.11$) (Figure 3B; $p<0.05$). For differential abundance, infants who were fed only breastmilk had significantly higher abundances of both *Bifidobacterium* (Table 1; $p<0.01$) and *Klebsiella* (Table 1; $p<0.01$) relative to those only fed formula. Pregnancy complications

Both preeclampsia and chorioamnionitis had a significant impact on the infant gut microbiome. Of the two complications, only preeclampsia influenced infant microbial diversity. A significant difference exists at discharge between infants whose mothers were diagnosed with preeclampsia ($\bar{x} = 1.68 \pm 0.27$) and those infants whose mother did not have the disease ($\bar{x} = 1.83 \pm 0.10$) ($\chi^2 = 4.96$, $df = 1$, $p = 0.03$), relative to the difference observed at admission (Figure 3C). However, no significant pairwise differences were found in subsequent analyses for diversity.

Both preeclampsia and chorioamnionitis also significantly influenced taxonomy (Table 1). In infants whose mothers were diagnosed with Chorioamnionitis before or during labor, *Staphylococcus* was significantly higher at admission ($p<0.05$) and *Streptococcus* significantly lower at discharge ($p<0.01$). For infants whose mother was diagnosed with Preeclampsia there were no differences at admission, but significantly lower *Escherichia/Shigella* ($p<0.01$) at discharge.

Neonatal complications

Three neonatal complications, ROP, NEC and sepsis, were found to significantly impact the developing preterm gut microbiome. Both sepsis (Fig 3D; $\chi^2 = 4.73$, $df = 1$, $p = 0.03$) and ROP (Fig 3E; $\chi^2 = 11.68$, $df = 1$, $p = <0.01$) significantly influenced diversity, with infants who were diagnosed with sepsis having significantly lower diversity ($\bar{x} = 1.10 \pm 0.17$) than
infants who did not ($\bar{x} = 1.84 \pm 0.09$) have the disease. For ROP, subsequent post-hoc analysis found pairwise differences between infants who were diagnosed with the disease ($\bar{x} = 1.25 \pm 0.18$) and those who did not have ROP, both at admission ($\bar{x} = 2.04 \pm 0.18$) (Figure 3E; $p<0.01$), and at discharge ($\bar{x} = 1.71 \pm 0.10$) compared to admission ($\bar{x} = 2.04 \pm 0.18$) (Figure 3E; $p<0.01$).

Both sepsis and ROP, along with NEC also significantly influenced the abundances of taxa. Sepsis had an impact at admission, with *Pseudomonas* ($p<0.01$) and *Diaphorobacter* ($p<0.01$) significantly lower and *Bifidobacterium* significantly enriched ($p<0.01$) in patients diagnosed with the disease. *Bifidobacterium* was significantly lower at admission in infants diagnosed with NEC ($p<0.01$), and *Staphylococcus* significantly enriched in infants diagnosed with ROP ($p<0.01$).

**Discussion**

The aim of this study was to understand and identify variation in gut flora development in a unique cohort of probiotic-supplemented preterm infants. Specifically, we set out to assess how the bacterial microbiome differs between two time points in the hospital, admission and discharge, and the effect of several clinical variables (both maternal and infant) in a cohort of preterm infants from North Queensland, Australia. To do so, we utilised 16S rRNA high throughput sequencing. We then conducted univariate comparisons to examine the difference between the infant microbiome at admission and discharge, and mixed effects models to explore the influence of several clinical variables, including Sepsis, Feeding Type, Chorioamnionitis, Mode of Delivery, Gestation, NEC, Preeclampsia and ROP.

**Exploring changes in composition and diversity from admission to discharge**
Although median alpha diversity increased between admission and discharge, the difference was not significant. As previously mentioned, other work has shown the preterm infant microbiome changes significantly with time, eventually becoming more similar to that of full-term infants. Our contrary findings could be due to a large spread of diversity scores at admission, and a reduction in diversity for some infants. While the cause of this large variation in diversity during admission is unclear, the decrease in diversity could be for several reasons, one of which is antibiotics, as past research has demonstrated a negative relationship between antibiotics and the developing microbiome, including an impact on diversity (23). However, as a large proportion of premature infants generally receive antibiotic therapy (75), and 94% of our samples came from antibiotic treated infants it is not possible to make this comparison.

For taxonomic abundance, significant differences in abundances of *Staphylococcus*, *Enterobacter*, *Lactobacillus*, *Clostridium Sensu Stricto 1* and *Veillonella* were observed between admission and discharge samples. Overall, several taxa were found to dominant at either admission or discharge, with *Staphylococcus* the most abundant genus at admission. In healthy newborns, colonisation usually begins with oxygen-tolerant microbes (76), like *Staphylococcus*, that consume oxygen shifting the environment from aerobic to anaerobic (77). This in turn allows the colonisation of strict anaerobes like *Clostridium* (76). The preterm infant microbiome typically has a greater abundance of these facultative anaerobes (6, 22), in combination with fewer aerobes (78) and delayed colonisation of obligate-anaerobes (13), like *Bifidobacterium* (5). It is therefore not surprising to see *Staphylococcus* in higher abundance at admission, which has also been observed previously (22). As time progresses, most other microbes also increase in absolute abundance (5), due to further colonisation and replication of microbes.
At discharge from the NICU, several taxa appear to dominate, with *Bifidobacterium*, *Lactobacillus* and *Enterobacter* found in high abundance across most of the cohort. Significant differences in abundances between admission and discharge were found for *Lactobacillus* and *Enterobacter*, but not for *Bifidobacterium* ($p = 0.11$). This is surprising considering preterm infants are known to experience delayed and limited colonization of common commensals like *Bifidobacterium* and *Lactobacillus* (9, 22, 79). Although changes in *Bifidobacterium* did not reach a level of significance in our cohort, it is worth noting that 99 of 134 samples contained the genus, in a cohort of infants born <32 weeks gestation, that were also receiving a probiotic (Infloran™) containing both *Lactobacillus acidophilus* and *Lactobacillus bifidus* (*Bifidobacterium bifidum*). So, although significant changes were not observed, the presence of *Bifidobacterium* in the majority the cohort suggests the probiotic may be having an impact.

When comparing variation of microbial communities between samples using beta diversity, there appears to be limited separation by sample type (admission/discharge) (*Figure 2A*). However, there is still a significant association between when the sample was collected (admission or discharge) and beta diversity, which is unsurprising considering microbial populations change significantly over time during infancy. However, the poor $R^2$ suggests that although when the sample was collected is associated with beta diversity, there is still a lot of variation unexplained in the model. This is likely due to environmental variables that also influence the developing infant gut microbiome.

**Exploring the effect of clinical variables on alpha diversity and taxonomic abundance**

Mixed effects modelling was used to explore the impact of several clinical variables on alpha diversity (Shannon Index) and taxonomic differential abundance. Some of these variables have previously been implicated in shaping the gut microbiome, and others associated with
disease. Our data builds on previous findings, but does not support all previously made observations, highlighting the complexity of gut microbiome ecology.

Mode of delivery and diet

In contrast to previous work, we observed no significant pairwise differences in diversity or taxonomy between vaginally and caesarean delivered infants at admission or discharge. Typically, caesarean born infants bypass the vaginal route of inoculation, resulting in greater diversity (23), with fewer or delayed colonisation of *Lactobacillus* (22), *Bifidobacterium* (9, 22) and *Bacteroides* (80-82), and higher than normal amounts of skin dwelling microbes, such as *Staphylococcus*. The inconsistency between our results and the literature may be due to other confounding variables, such as prematurity itself or supplementation with probiotics, which has been demonstrated to alter *Bifidobacterium* and *Lactobacillus* populations in preterm infants (49). If probiotic treatment is driving the disparity between our results and previous work, then probiotic supplementation may be able to correct for the differences normally seen between vaginally and caesarean born infants.

For diet, we observed significantly lower alpha diversity and higher abundances of *Bifidobacterium* at discharge in breastfed infants, relative to those solely formula fed. This supports previous work that has found breastfed infants have lower diversity (25) but more commensal microbes (22, 83), including different *Staphylococcus* (84), *Lactobacillus* and *Bifidobacterium* species (22). The higher presence of *Bifidobacterium* in infants only fed breastmilk is likely due to its presence, as well as the presence of HMOs in breastmilk (85-87). The fact that *Bifidobacterium* was only significantly higher in infants that were solely breastfed highlights the importance of breastfeeding and suggests that supplementing formula with some level of breastmilk may not be enough to correct for the microbial imbalances associated with formula feeding.
Klebsiella was also found to be significantly higher in breastfed infants. The genus
Klebsiella contains well known pathogen species, such as Klebsiella pneumoniae, previously
associated with NEC (88). The transfer of this pathogen from mother to infant via breastmilk
has also been implicated in sepsis in clinical observations (89). However, as pathogens like
K. pneumoniae only constitute a small proportion of the genus Klebsiella, and Klebsiella is
also a member of normal gut flora there is little need for concern. Additionally, despite
clinical reports linking maternal-infant translocation of microbes to breastfeeding, breastmilk
is the most cost effective preventative intervention from infection (90). In fact, the presence
of microbes, specifically commensal microbes like Bifidobacterium and Lactobacillus could
by why breastfeeding reduces the risk of diseases like sepsis (91).

Pregnancy complications

Infants whose mother was diagnosed with chorioamnionitis had higher abundances of the
genus Staphylococcus at admission, but fewer Streptococcus at discharge. A significant
relationship between chorioamnionitis and the altered infant gut microbiome has also been
observed previously for differential abundance of different taxa (92), as well as a close to
significant difference in diversity (25). As chorioamnionitis is a bacterial infection of the
membrane surrounding the fetus, occurring before or during labor, translocation of pathogens
from the membrane to the fetus may occur. Unfortunately, the translocation and resulting
increased abundance of Staphylococcus may be why exposure to chorioamnionitis increases
the risk of preterm infants to adverse neonatal outcomes (92), like sepsis, which has also been
associated with Staphylococcus (93, 94).

For infants whose mother was diagnosed with preeclampsia, Escherichia/Shigella was
significantly lower at discharge, and although no significant pairwise comparisons were
found, preeclampsia did appear to have some effect on alpha diversity at discharge as
determined by analysis of deviance. As preeclampsia can alter the maternal microbiome (30),
and a large proportion of infant microbial colonization is from a maternal route it is unsurprising that preterm infants whose mothers were diagnosed with preeclampsia can have significantly different microbiomes. Additionally, there was also a close to significant decrease in alpha diversity ($p = 0.08$) from admission to discharge for infants whose mother was diagnosed with the disease, which may explain why several individuals experience a reduction in diversity from admission to discharge.

Despite our results, work by Stewart et al, *The Environmental Determinants of Diabetes in the Young (TEDDY)* study, does not support preeclampsia influencing the infant gut microbiome at the genus level, but only at species (8). This could be for several reasons. Firstly, other clinical or environment variables affecting the infant either directly or through the mother across the two cohorts could drive the difference. For example, the gut microbiome can influence the pharmacokinetics of treatments like antihypertensives used in the treatment of preeclampsia (95), and so it’s possible that drug-microbe interactions may also be altering microbial populations passed onto the infant through microbial maternal-infant translocation. Additionally, our cohort and that from the TEDDY study are vastly different, with the TEDDY study including both full- and pre-term children who seroconverted to islet cell autoantibody positivity or developed type 1 diabetes (and matched controls) from 3 to 46 months of age. Our cohort was entirely premature infants who at discharge may have only been 3 months old, and as preeclampsia is associated with preterm birth (96), our cohort had a larger proportion of infants born to preeclamptic mothers (18% compared to 4%). Thus, it could be that preeclampsia has a greater impact on preterm infants, or that preeclampsia only has a significant effect in the early months of life, when the mother is still the dominant colonising route for microbes.

Neonatal complications
We found that sepsis significantly influences the abundance of *Bifidobacterium*, *Pseudomonas* and *Diaphorobacter*. Multi-omics approaches have previously linked sepsis to the gut microbiome (97), with other research showing associations between sepsis and low diversity (12), as well as higher abundances of *Staphylococcus* (93, 94), and lower abundances or absence of commensal microbes like *Bifidobacterium* (38, 97). Although we also observed differences in *Bifidobacterium*, the directional effect is counter to what was observed previously. However, it is worth noting that of the eight infants diagnosed with sepsis, only three had *Bifidobacterium* in their sample. So, despite reaching statistical significance, this finding may not be clinically relevant. Furthermore, we did also observe higher *Staphylococcus*, it just did not reach a level of significance at either admission or discharge.

For NEC, we observed significantly lower abundances of Bifidobacterium, but in contrast to previous work, no enrichment of any taxa. As previously mentioned, *Bifidobacterium* is a common commensal microbe, that is also found in the probiotic Infloran™. It is uncommon in preterm infants born <33 weeks gestation (79), and has previously been shown to be protective against NEC (98). Although our work does not support previous evidence of a single infectious pathogen, the plethora of microbes that have previously been associated with NEC (39, 99, 100), in combination with studies showing reduced commensal microbes (101, 102) and diversity (103, 104), suggests the aetiology is far more complicated than just the presence of a single pathogen. Rather, the disease appears to result from microbial dysbiosis, that includes reduced commensal microbes like *Bifidobacterium*.

We also observed significant enrichment of *Staphylococcus* (of the *Staphylococcaceae* family) at admission for infants diagnosed with ROP, as well as significantly lower diversity. An association between the gut microbiota and ROP has only
been explored once before, by Skondra et al (44). They observed significant enrichment of
the family *Enterobacteriaceae* in preterm infants with the disease at 28 weeks postmenstrual
age (44). The discrepancy in our results is not necessarily a product of error, but rather, as
seen with NEC, due to the complex aetiology characterised by more than just the presence of
a particular group of taxa. This complexity makes it difficult to hypothesise the specific role
that the microbiome could be playing in ROP. However, if a role is established there is
potential for the microbiome to become a target for intervention, and thus this should be the
target of further research.

**Limitations**

Limitations of our work include low sequencing depth and only sampling in early infancy.
The use of 16S metabarcoding limited our detection power to the genus level, resulting in no
identification of species or functional genes. Additionally, only collecting samples at
admission and discharge means we have no insight into the longevity of the differences
observed, which may impact their clinical significance. Our future work will use a
combination of 16S metabarcoding and shotgun metagenomic techniques to both characterize
species and genes and to explore if the differences observed in this study, and others, persist
in the long-term.

**Conclusion**

This prospective observational study used 16S rRNA high throughout sequencing to
characterize the bacterial microbiome of probiotic-supplemented infants. It aimed to identify
and understand variation in bacterial gut flora between two time points and as the result of
several clinical variables. Our study builds on previous research and supports significant
changes in the preterm microbiome over time and associations with several factors.
Admission and discharge samples had significantly different microbial populations, with *Staphylococcus* enriched at admission and several other taxa at discharge. Clinical conditions (sepsis and ROP), and formula feeding significantly lowered alpha diversity, and along with chorioamnionitis, preeclampsia and NEC, significantly affected community composition. The fact that several associations were observed, and in some contexts in ways that counter previous work, highlights the complexity of microbiome ecology.

**Abbreviations**

ASV: amplicon sequence variant  
NEC: necrotising enterocolitis  
ROP: retinopathy of prematurity  
rRNA: ribosomal ribonucleic acid  
K: Klebsiella  
B: breastmilk  
F: formula  
THHS: Townsville Hospital and Health Service  
NICU: neonatal intensive care unit  
PCoA: principle coordinate analysis  
PCA: principle component analysis  
URN: unique record number  
AIC: Akaike’s Information Criterion  
PERMANOVA: permutational analysis of variance  
ASV: amplicon sequence variant  
PCR: polymerase chain reaction
Declarations

Ethics approval and consent to participate

The research was performed in accordance with the Declaration of Helsinki and ethics approval was obtained from the Human Research Ethics Committee from the Townsville Hospital and Health Service (HREC/17/QTHS/7). Informed consent was obtained from parents/legal guardians of all subjects through the signing of a Parental Information Sheet and Consent Form (PICF).

Consent for publication

Not applicable.

Availability of data and materials

The sequencing dataset generated and/or analysed during the current study are available through the International Nucleotide Sequence Database Collaboration at the National Center for Biotechnology Information (NCBI) repository, https://www.ncbi.nlm.nih.gov/bioproject/687291.

BioProject ID: PRJNA687291.

Additional Files

All additional materials can also be found at:

https://github.com/JacobAFW/NICU_Microbiome_Study.
Additional File 1:

- Format: .pdf.
- Title: Complete workflow.
- Description: The full workflow for the analysis, from raw fastq files through to statistical analysis.

Additional File 2:

- Format: .doc.
- Title: Additional analysis outputs.
- Description: Outputs from the analysis that were not included in the manuscript but that may be of interest (e.g. Post-Hoc Tukey’s analysis).

Additional File 3:

- Format: .csv.
- Title: Metadata.
- Description: Metadata used for the analysis.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded through the Townsville Hospital and Health Service with a Study, Education and Research Trust Account (SERTA) research grant. The funding body had no role in the design of the study or collection, nor the interpretation of data and writing.
Authors' contributions

DR provided oversight and management of the project. DR, YK, RH, DW and RN were all involved in the study design. JW performed the DNA extraction, library preparation, sequencing, bioinformatics and statistical analysis, with guidance and technical input from RH and DR. KS contributed to the statistical analysis. JW prepared the manuscript, with input, editing and approval from DR, CM, YK, RH, RN, KS and DW.

Acknowledgements

Helena Mcinnes
Nicole Dionysius
Dr. Tiffany Kosch
Harrison Jaa-Kwee
Sandra I. Villamil

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