Characterising the Bacterial Gut Microbiome of Probiotic-supplemented Very Preterm Infants

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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*, *Colstridium 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, mucous 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

98 microbes, resulting from maternal-infant transfer could have severe consequences for infant
99 health and development (31).
100 Microbial dysbiosis puts preterm infants at a high risk of acute infection (32, 33),
101 chronic disease (34, 35) and developmental abnormalities (36, 37). The increased risk in
102 disease is a consequence of the breakdown in the symbiotic relationship between infants and
103 colonising microbes during development. Delayed colonization by commensal microbes
104 could result in increased sensitivity leading to irregular immune responses resulting from
105 intolerances to normal flora (38, 39). Additive to this is an imbalance between commensals
106 and pathogens that may induce intestinal inflammation and cytokine production (40),
107 resulting in acute pathologies like necrotizing enterocolitis (NEC) and sepsis, developmental
108 disorders, like retinopathy of prematurity (ROP) (41), and eventually chronic diseases like
109 asthma (42).
110 Despite differing aetiologies, the gut microbiome has been implicated in the
111 pathologies of NEC, sepsis and ROP, with associations with either low diversity or
112 taxonomic abundance (43-46). All are very different conditions. NEC affects 4-11% of
113 preterm very low birth weight infants with 20-30% overall mortality (33) and is characterised
114 by intestinal inflammation and subsequent necrosis of the bowel. Sepsis is a systemic
115 response to blood-stream infection that affects 20% of preterm infants (32). ROP on the other
116 hand is a potentially blinding disease caused by abnormal development of retinal blood
117 vessels (47).
118 Preterm infants are disproportionately affected by disease, and more likely to undergo
119 a myriad of treatment regimens that can also impact the developing microbiome. Treatment
120 with antibiotics, a staple in preterm neonatal care, and probiotics, an emerging preventative
121 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.

147 **Study design and ethics**

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

159

160 **Sequencing and bioinformatics**

161 In brief, the protocol used in this study included sample storage at -80°C (55), an
162 extraction kit that includes mechanical lysis (56), use of the Illumina MiSeq platform (57),
163 targeting of the V3/V4 regions (58) and use of the SILVA reference database (58).
164 DNA extraction was conducted using the Bioline ISOLATE Fecal DNA Kit (59),
165 with modifications made in consultation with the manufacturer to optimise DNA yield. This
166 included increased beta-mercaptoethanol (from 0.5 to 1% to increase DNA solubility and
167 reduce secondary structure formation), addition of an extra wash step (to improve purity) and
168 decreased elution buffer volume (to increase final DNA concentration). For library
169 preparation we followed the Illumina metagenomics library preparation protocol (60), using
170 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

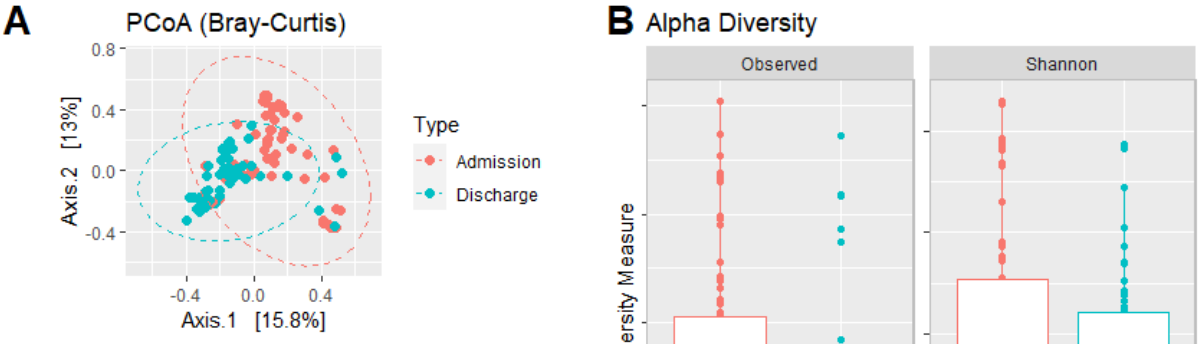
196 discharge, data that was filtered and agglomerated at the genus level, but not transformed,
197 were then normalized and modeled (negative-binomial) with *DESeq2* (70). Then with the
198 *DESeq()* function, a Wald Test with the Benjamini-Hochberg multiple inference correction
199 was performed to determine significant differentially abundant taxa.
200
201 **Exploring the effect of clinical variables on alpha diversity and taxonomic abundance**
202 Lastly, associations between several clinical variables and community structure were
203 explored. The relationship between clinical variables and both Shannon Diversity and
204 taxonomic abundance were assessed using multivariant linear regression models. For
205 exploring the relationship with Shannon diversity, a mixed effects linear regression model
206 was created using the package *lme4* (71), with a gaussian distribution and using the restricted
207 maximum likelihood estimation. Continuous predictors were scaled and centered to avoid
208 convergence issues and multicollinearity assessed using the *AED* package (72). Gestation
209 and birth weight were found to be collinear and thus birth weight was removed from the
210 model. Thirteen predictors: mode of delivery, feeding type, gestation, antenatal antibiotics,
211 antenatal infections, NEC, sepsis, chorioamnionitis, neonatal antibiotics, death, prolonged
212 membrane rupture, preeclampsia, diabetes and retinopathy of prematurity were included in
213 the initial model. To control for high amounts of inter-individual variation in the microbiome
214 of preterm infants (1), individual's identification (unique record number – *URN*) was
215 included as a random factor. As we wanted to assess the influence of clinical variables at both
216 admission and discharge, this was included as an interaction variable (labelled Type). The
217 resulting model *Shannon ~ (13 Parameters) * Type + (1|URN)*, assesses the effect of the 13
218 predictors on Shannon diversity for both types of samples, Admission and Discharge, whilst
219 accounting for the individual, represented here by URN.

220 Backwards selection (69) was then implemented to simplify the model by comparing
221 Akaike's Information Criterion (AIC) scores between regression models and removing
222 predictors that were not contributing to the model. The process was repeated until the least
223 complex adequate model was identified, when no more predictors could be removed without
224 significant effects. The final model was *Shannon ~ (Sepsis + Feeding Type +*
225 *Chorioamnionitis + (Mode of Delivery + Gestation Days + NEC + Preeclampsia + ROP)) **
226 *Type + (1|URN).* The significance of the fixed effects variables in this final model was then
227 assessed using analysis of deviance (Type II Wald Chi-square test) from the *car* package
228 (73), and post-hoc pairwise Tukey comparisons (correcting for multiple comparisons) from
229 the *emmeans* package (74).
230 For differential taxonomic abundance, two negative binomial generalized linear
231 models were created using the package DESeq2. A combination of previous literature and
232 exploratory analysis, including PCoA plots, PCA and scatterplots, were used for model
233 selection. Again, continuous predictors were scaled and centered, and multicollinearity was
234 assessed. Taxa were agglomerated at the genus level, due to the limited sequencing depth of
235 short amplicon sequencing. To reduce the number of false positives, two separate models
236 were run; one each for admission and discharge samples. The resulting model *Taxonomic*
237 *Abundance ~ Sepsis + Feeding Type + Chorioamnionitis + Mode of Delivery + Gestation*
238 *Days + NEC + Preeclampsia + ROP* was created to assesses the effect of the 9 independent
239 predictors at both time points on all genera present. Low abundance and low frequency taxa
240 were then removed, and a Wald Test with the Benjamin-Hochberg multiple inference
241 correction was then performed to determine significant differentially abundant taxa. More
242 information on the analysis can be found in *Additional File 1*
243

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.



C

Differential Abundance

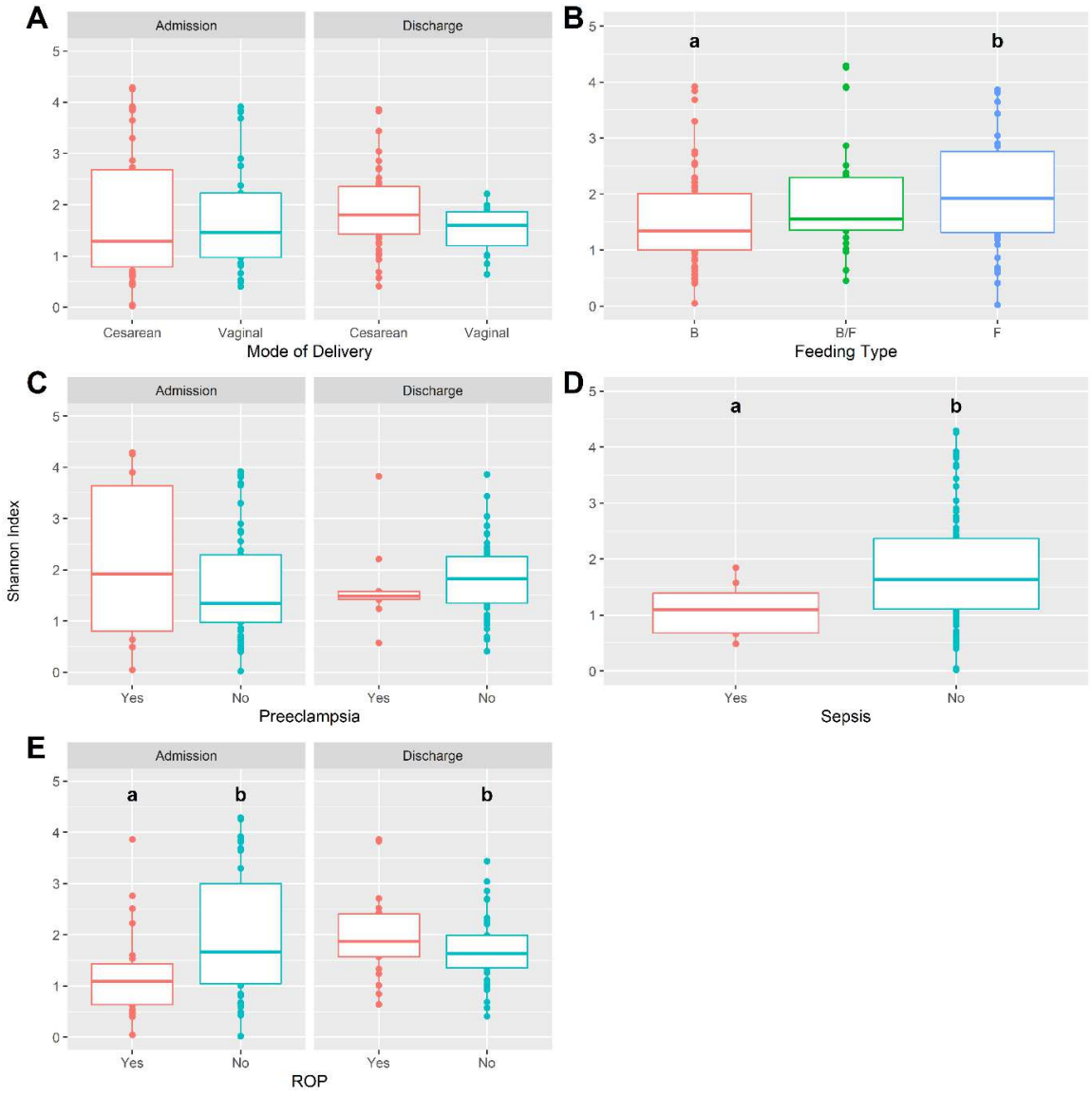
Genus	padj	lfc	lfcSE
Staphylococcus	<0.01	-7.06	0.58
Enterobacter	<0.01	6.07	1.10
Lactobacillus	<0.01	5.19	1.00
Clostridium_sensu_stricto_1	<0.01	6.11	1.35
Veillonella	<0.01	3.74	1.06

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).



273

274 *Figure 3. Boxplots of alpha diversity (Shannon Index) for significant analysis of deviance*
275 *outcomes, with significant Tukey's pairwise comparisons designated by lower case letters,*
276 *(where a is significantly different from b) on linear mixed effects model. Annotation for*
277 *Feeding Type; B: Breastmilk, B/F: Breastmilk and Formula & F: Formula. A: Box plot*
278 *comparing alpha diversity at admission and discharge between different modes of delivery,*
279 *B: Box plot comparing alpha diversity between different diets, C: Box plot comparing alpha*
280 *diversity at admission and discharge between infants with and without preeclamptic mothers,*
281 *D: Box plot comparing alpha diversity between sepsis diagnoses, E: Box plot comparing*
282 *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 log2FoldChage 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

295 (Figure 3B; $\chi^2 = 7.29, df = 2, p<0.05$), with subsequent post-hoc pairwise comparisons
296 finding a significant difference between formula-fed infants ($\bar{x} = 2.10 \pm 0.17$) and those
297 breastfed ($\bar{x} = 1.56 \pm 0.11$) (Figure 3B; $p<0.05$). For differential abundance, infants who
298 were fed only breastmilk had significantly higher abundances of both *Bifidobacterium* (Table
299 1; $p<0.01$) and *Klebsiella* (Table 1; $p<0.01$) relative to those only fed formula.
300 Pregnancy complications
301 Both preeclampsia and chorioamnionitis had a significant impact on the infant gut
302 microbiome. Of the two complications, only preeclampsia influenced infant microbial
303 diversity. A significant difference exists at discharge between infants whose mothers were
304 diagnosed with preeclampsia ($\bar{x} = 1.68 \pm 0.27$) and those infants whose mother did not have
305 the disease ($\bar{x} = 1.83 \pm 0.10$) ($\chi^2 = 4.96, df = 1, p = 0.03$), relative to the difference observed
306 at admission (*Figure 3C*). However, no significant pairwise differences were found in
307 subsequent analyses for diversity.
308 Both preeclampsia and chorioamnionitis also significantly influenced taxonomy
309 (Table 1). In infants whose mothers were diagnosed with Chorioamnionitis before or during
310 labor, *Staphylococcus* was significantly higher at admission ($p<0.05$) and *Streptococcus*
311 significantly lower at discharge ($p<0.01$). For infants whose mother was diagnosed with
312 Preeclampsia there were no differences at admission, but significantly lower
313 *Escherichia/Shigella* ($p<0.01$) at discharge.
314
315 Neonatal complications
316 Three neonatal complications, ROP, NEC and sepsis, were found to significantly impact the
317 developing preterm gut microbiome. Both sepsis (Fig 3D; $\chi^2 = 4.73, df = 1, p = 0.03$) and
318 ROP (Fig 3E; $\chi^2 = 11.68, df = 1, p = <0.01$) significantly influenced diversity, with infants
319 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^{TM}) 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

391 disease. Our data builds on previous findings, but does not support all previously made
392 observations, highlighting the complexity of gut microbiome ecology.

393 Mode of delivery and diet

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

405 For diet, we observed significantly lower alpha diversity and higher abundances of
406 *Bifidobacterium* at discharge in breastfed infants, relative to those solely formula fed. This
407 supports previous work that has found breastfed infants have lower diversity (25) but more
408 commensal microbes (22, 83), including different *Staphylococcus* (84), *Lactobacillus* and
409 *Bifidobacterium* species (22). The higher presence of *Bifidobacterium* in infants only fed
410 breastmilk is likely due to its presence, as well as the presence of HMOs in breastmilk (85-
411 87). The fact that *Bifidobacterium* was only significantly higher in infants that were solely
412 breastfed highlights the importance of breastfeeding and suggests that supplementing formula
413 with some level of breastmilk may not be enough to correct for the microbial imbalances
414 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^{TM}. 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

DNA: deoxyribonucleic acid

NQLD: North Queensland

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.

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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.

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Figures

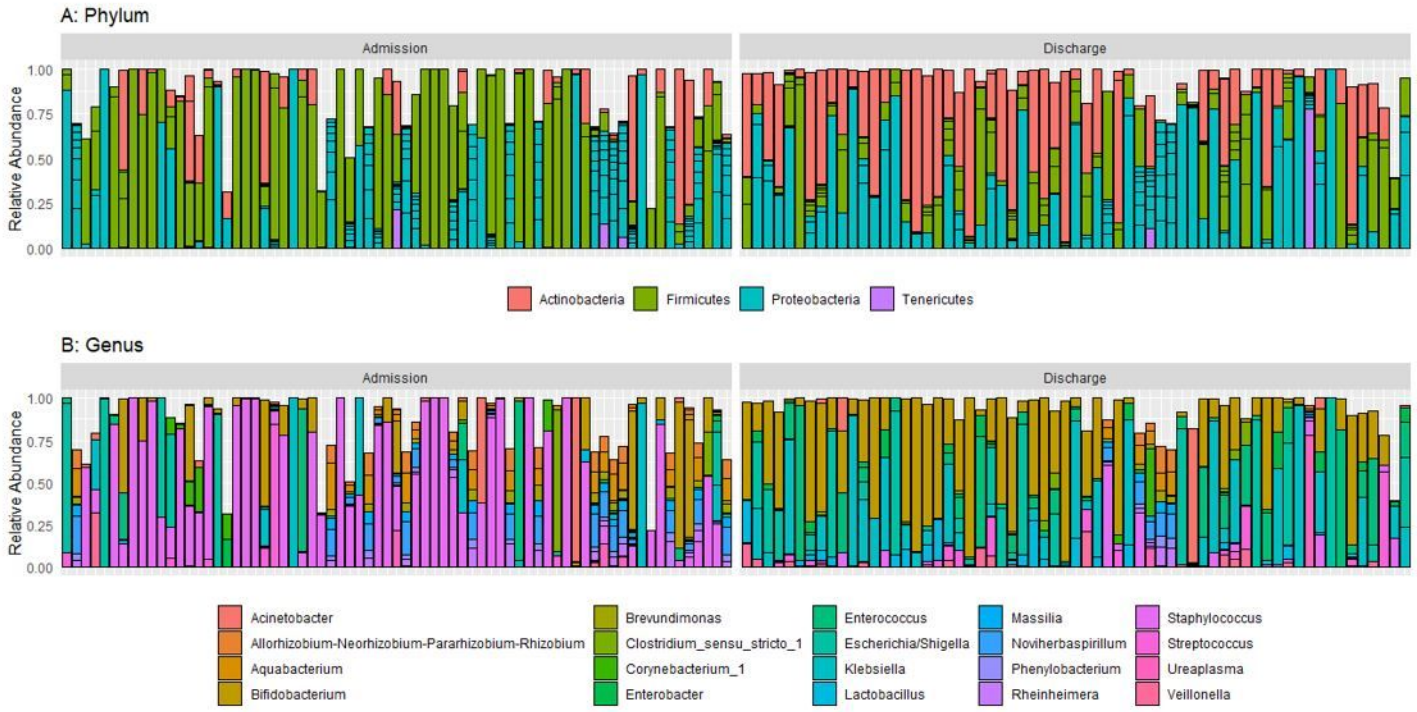
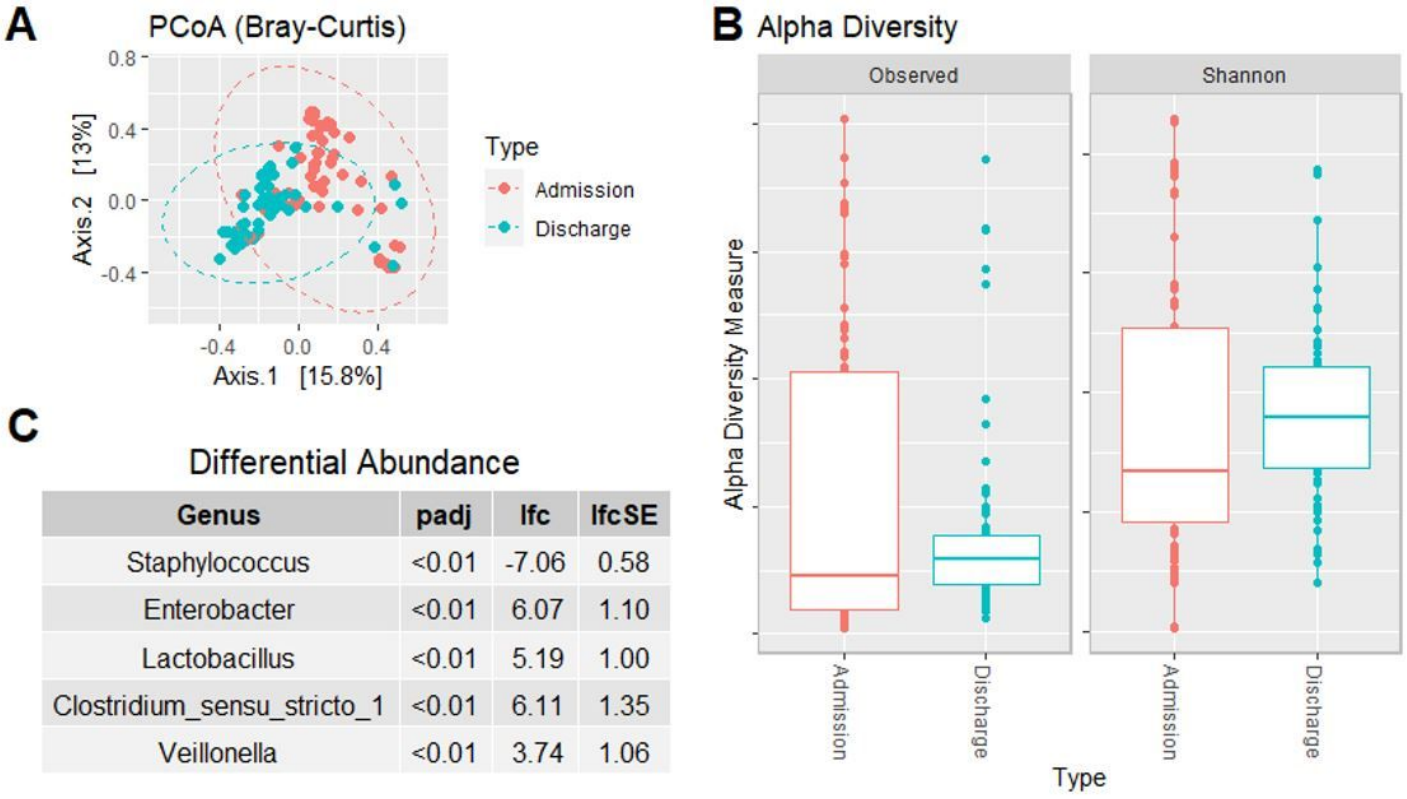


Figure 1

Histograms representing taxonomic distribution (top 20 taxa) of relative abundance for admission and discharge samples at both phylum (A) and genus (B) levels.



C

Differential Abundance

Genus	padj	lfc	lfcSE
Staphylococcus	<0.01	-7.06	0.58
Enterobacter	<0.01	6.07	1.10
Lactobacillus	<0.01	5.19	1.00
Clostridium_sensu_stricto_1	<0.01	6.11	1.35
Veillonella	<0.01	3.74	1.06

Figure 2

A: Principle coordinate analysis plot for admission versus discharge based on Bray-Curtis dissimilarity matrix ($p<0.01$ & $R2 = 0.06$), B: box plots of alpha diversity for admission versus discharge, C: table of differential abundance testing for admission versus discharge (base value is admission).

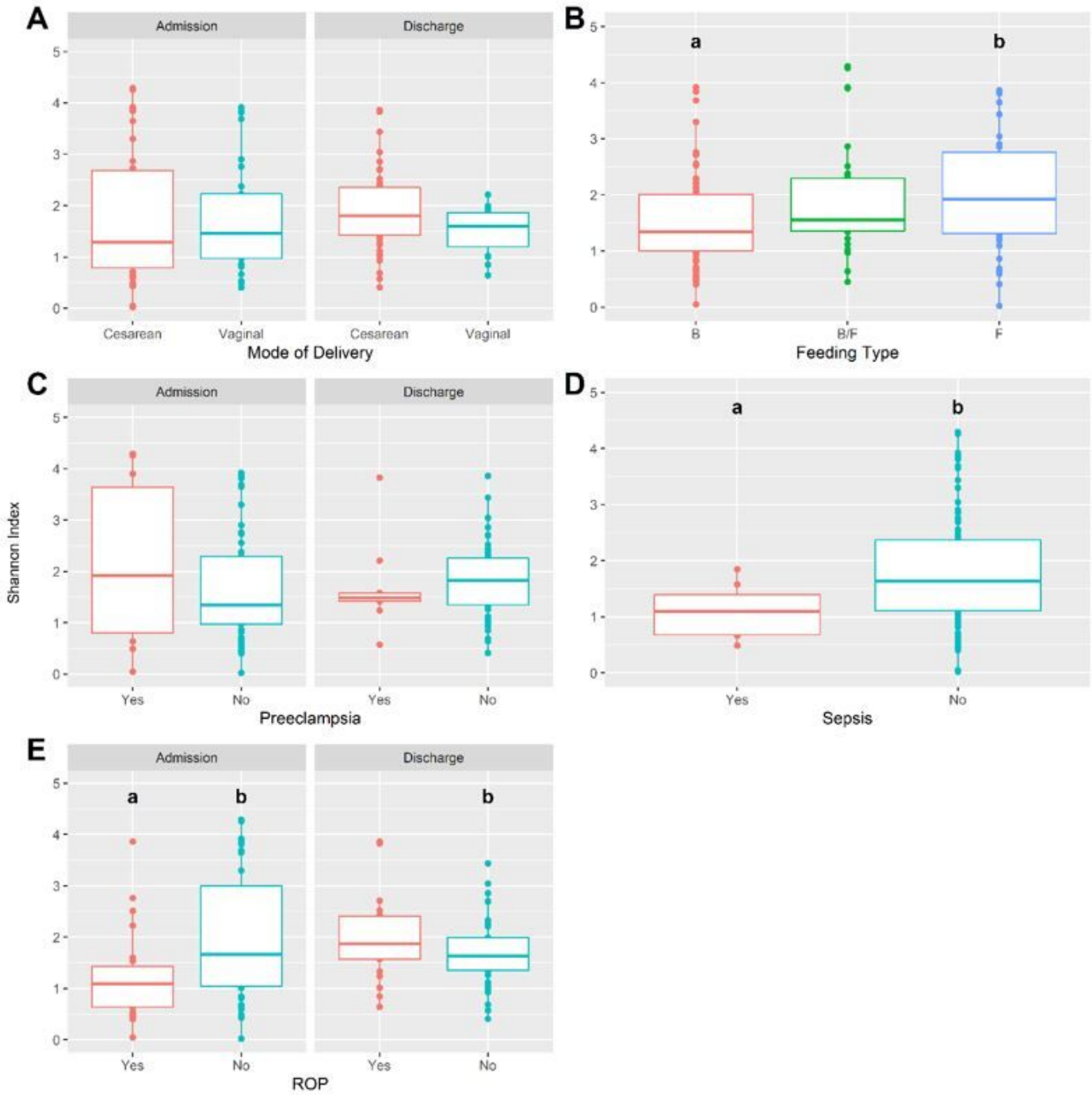


Figure 3

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.

Supplementary Files

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

- AdditionalFile1.pdf
- AdditionalFile2.docx
- AdditionalFile3.csv