

1 Uncovering microbial populations in the lumen of neonatal enteral feeding tubes utilising 16s  
2 rRNA sequencing.

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22

## 23 Abstract

24 Gastrointestinal microbiome is increasingly implicated in the morbidity associated with being  
25 born preterm. Enteral tubes (ET) are essential for the nutritional care of preterm infants.  
26 Limited culture-based studies have suggested they are colonised by high densities of  
27 microorganisms. Microbial DNA was extracted from 60 ETs retrieved from infants in a  
28 tertiary neonatal unit and analysed by 16s rRNA sequencing of the V4 variable region.  
29 Relative abundance analysis on dominant microorganisms demonstrated that compared to  
30 breast milk, formula significantly increased abundance of *Streptococcus spp* and significantly  
31 decreased *Enterococcus spp* and Enterobacteriaceae. Vaginal birth was also associated with  
32 significantly increased relative abundance of *Streptococcus*. This study more accurately  
33 demonstrates the extent of microbial diversity in neonatal ETs, with feeding regime  
34 significantly influencing colonisation patterns. Colonisation with unwanted organisms, as a  
35 result of specific care regimes, could result in disruption of the fragile infant gut microbiome,  
36 with implications for long-term morbidity.  
37

## 38 Background

39 Despite advances in neonatal medicine that have seen improvements in survival rates of  
40 infants born at the extremes of prematurity over the past two decades, there is still a  
41 considerable burden of morbidity. In particular, necrotising enterocolitis (NEC), a severe and  
42 devastating disease primarily affecting the gastrointestinal (GI) tract of premature infants,  
43 inducing inflammation and bacterial invasion of intestinal walls<sup>1</sup>, remains a concern in the  
44 preterm population, especially those born with low birth weight. While the precise  
45 pathogenesis of NEC is not fully understood, important factors include intestinal ischaemia,  
46 gut colonisation by pathologic bacteria, and excess protein substrate in the intestinal lumen<sup>2</sup>  
47 3. Similarly, nosocomial infection remains a significant cause of morbidity and mortality  
48 within the extremely preterm population.

49

50 Recently there has been increasing interest in the role of host intestinal microbiome in the  
51 pathogenesis of NEC and susceptibility to invasive bacterial infection. The hypothesis that  
52 the development of a normal commensal flora is fundamental to lifelong health and disease  
53 susceptibility is now firmly recognised<sup>4 5</sup>. Perturbations to the maternal, or early infantile,  
54 microbiome have been linked to autoimmune and metabolic disorders, such as Type 1 and 2  
55 diabetes<sup>6</sup>. It has also been demonstrated that infants exposed to prolonged courses of  
56 antibiotics have increased incidence of NEC, among other severe outcomes<sup>7 8</sup>. This could  
57 result from antibiotics hindering or suppressing the development of a 'normal' microbial  
58 flora, instead allowing for selection of more resistant pathogenic bacteria, increasing risk of  
59 morbidity and mortality. In addition, routine use of probiotics in an attempt to modify

60 bacterial colonisation in preterm infants has been shown to significantly reduce the risk NEC  
61 and improve feed tolerance<sup>9</sup>.

62

63 Preterm infants are exposed to a variety of factors that result in them having abnormal  
64 intestinal flora compared to infants born at term; they are more likely to be born by caesarean  
65 section, are often treated with broad spectrum antibiotics and spend prolonged periods in a  
66 hospital environment where they are exposed to a variety of nosocomial organisms. In  
67 addition, they often have indwelling medical devices such as central venous catheters and  
68 enteral feeding tubes, which further influence their exposure to microorganisms. Compared to  
69 term-born infants, preterm infants have abnormal faecal colonisation, with a paucity of  
70 normal enteric bacterial species, and delayed bacterial colonization<sup>10 11</sup>. Feeding with  
71 maternal breast milk (MBM) is the most effective preventative mechanism against  
72 development of NEC<sup>12 13</sup>, and one reason for this may be that it promotes the growth of non-  
73 pathogenic bacterial species while reducing the pathogen load<sup>14</sup>. MBM has also been shown  
74 to block bacterial lectins<sup>15</sup>, significantly reduce the risk of infection<sup>16</sup>, improve feed  
75 tolerance and have longer term benefits on growth and neurodevelopment<sup>17</sup>. Many of these  
76 effects may also be mediated through alterations in intestinal bacterial flora. Whilst MBM is  
77 beneficial, it is not always available and so alternative feeds must be utilised; with many  
78 studies demonstrating that these alternative feeds can impact microbiome development<sup>18 19</sup>.  
79 Feed type therefore clearly has implications for microbiome development and outcomes in  
80 preterm infants.

81

82 Early research into the understanding of the microbiome relied mainly on culture-based  
83 analysis of microbes; however, more recently developed techniques are revealing that  
84 culture-based analysis alone has led to a severe underrepresentation<sup>20</sup>. A growing wealth of  
85 literature suggests that, for many bodily and environmental sites, the greater proportion of  
86 microbes present may in fact be unculturable by traditional recovery techniques. A study  
87 using molecular based methods to gain an understanding into the human intestinal microbial  
88 flora demonstrated that 80% of the microbes uncovered were unculturable, with 60% being  
89 novel at the time<sup>21</sup>. 16s rRNA sequencing is rapidly providing more accurate representations  
90 of microorganisms within a given environment. Such methods are also able to identify  
91 species that may not be recoverable by culture-based analysis, such as viable but non-  
92 culturable (VBNC) species.

93

94 Previous studies have analysed alpha (diversity of microbial communities within one site)  
95 and beta (dissimilarities in microbial diversity between two sites) diversity of faecal samples  
96 to assess impact of common infant characteristics and care regimes (feed types, antibiotic  
97 prescription etc). Given the links between feed type, bacterial colonisation and outcomes in  
98 preterm infants, we sought to accurately portray the microbial communities found within  
99 neonatal feeding tubes, utilising modern molecular analysis techniques, as well as gain  
100 insight into how care practices in early life influence their dynamics. This novel approach  
101 could potentially lead to more personalised medicine, providing vital information to  
102 clinicians and enabling tailoring of care practices to differently vulnerable infant populations.

103

## 104 **Methods**

### 105 **Ethics**

106 Ethical approval was obtained (NHS Research and Ethics Committee: East of Scotland  
107 Research Ethics service, Reference 17/ES/0142). As per the ethical approval, informed  
108 consent for tube collection was not required as samples analysed were those designated for  
109 disposal, no human genetic material was collected and no patient identifiable information was  
110 collected, stored or analysed.

111

### 112 **Setting and population**

113 All tubes were collected from a tertiary Neonatal Intensive Care unit offering surgical and  
114 other speciality services. All nasogastric or orogastric feeding tubes removed from infants  
115 within the unit as part of their normal care regime between April and June 2018 were  
116 included unless they were damaged upon removal/collection or were removed within an  
117 emergency situation where there was inadequate time for appropriate sample collection.  
118 Clinical data collected included date of tube removal, tube characteristics (including length,  
119 diameter and material type), duration of placement, reason for removal, type of tube  
120 (nasogastric or orogastric), gender of infant, gestational age (GA) of infant at birth, GA at  
121 tube removal, mode of delivery, feed type, nutritional supplement use, antibiotic exposure  
122 and mode of ventilation. Feed type was categorised into MBM, donor breast milk (DBM),  
123 infant formula (IF) and nil by mouth (NBM). GA at birth and at time of tube removal was  
124 categorised according to World Health Organisation definitions as extremely preterm (< 28  
125 weeks), very preterm (28 to 32 weeks), moderately preterm (32 to 37 weeks) and term (> 37  
126 weeks).

127

## 128 **Sample processing and DNA extraction**

129 After removal from infants, feeding tubes were placed directly into sterile bags, sealed and  
130 stored at 4°C within the unit's designated research refrigerator preceding collection. Samples  
131 were collected and transported to a containment level 2 category laboratory. After  
132 sterilisation of the external surfaces of the tubes, sterile Dulbecco's Phosphate-buffered saline  
133 (DPBS) was flushed through the tubes and collected. Tubes were cut into approx. 1 cm  
134 segments. Segments were vortexed within tube flush to dislodge all microbes. Cell  
135 suspension was removed and centrifuged to pellet cells. Genomic DNA was isolated from  
136 entire pellet with the QIAmp Ultraclean production (UCP) pathogenic mini kit (Qiagen,  
137 Germany) following manufacturers guidelines with the following modification for optimal  
138 DNA extraction: QIAmp pathogen lysis tubes (L) for mechanical lysis in a TissueLyser LT  
139 for 15 mins at 50hz, as outlined in Pre-treatment of Microbial DNA protocol, before DNA  
140 extraction following manufacturers Sample Prep (Spin protocol) guidelines. Total DNA  
141 eluted into 30µl buffer AVW (RNase-free water with 0.04% NaN<sub>3</sub> (sodium azide)) and yield  
142 measured by NanoDrop™ spectrophotometer before being stored at -80°C. Once 60 samples  
143 had been reached, they were transferred to an external company (Environmental Genomics  
144 Facility, University of Southampton, UK) for 16s rRNA sequencing.

145

## 146 **Analysis of amplicon sequencing data**

147 The V4 region of the 16S rRNA gene was amplified from all 60 samples using fusion primers  
148 515F:*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA*  
149 and 806R:  
150 *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT*,  
151 which consist of the V4 region primers<sup>22</sup> ligated to Illumina Nextera adaptor consensus

152 sequences (indicated in italics). PCR reactions were carried out in 25  $\mu$ l volumes, consisting  
153 of 12.5  $\mu$ l NEBnext Q5 HiFi Hotstart mastermix, 12.5 ng genomic DNA, and 1  $\mu$ l forward  
154 and reverse primers (10  $\mu$ M), and amplified using the following conditions: 95°C for 3 min,  
155 followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final  
156 extension of 7 min at 72°C. Amplicons were cleaned using 0.8 x volume AMPure XP beads  
157 (Beckman Coulter Ltd, UK) and dual indexed using a Nextera XT v2 Index Kit (Illumina,  
158 United States) using a further 8 PCR cycles. The resulting amplicon libraries were pooled and  
159 sequenced on an Illumina MiSeq instrument, using a MiSeq v3 Reagent Kit (Illumina) and 2  
160 x 300 bp paired end sequencing. Sample demultiplexing was carried out on-instrument by the  
161 MiSeq control software.

162

163 Qualitative Insights into Microbial Ecology 2 (QIIME2 version 2018.8) was used for analysis  
164 of 16s rRNA gene amplicon sequences. Demultiplexed reads were trimmed using Cutadapt  
165 version 1.17.23 to remove residual adapters and primers, and to remove low-quality 3' bases  
166 (quality threshold 20). Reads less than 250 bp following trimming were discarded. Denoising  
167 was carried out using the DADA2.24 plugin within QIIME2. Taxonomy was assigned to the  
168 resulting amplicon sequence variants (ASVs) using the naïve-Bayes machine learning  
169 classifier method implemented in QIIME2's q2-feature-classifier plugin<sup>25</sup> (taxonomic  
170 assignment against Greengenes 13\_8 99% classifier). Keemei plugin was utilized for  
171 metadata file validation<sup>26</sup>.

172

173 Diversity analysis included both alpha and beta diversity. Alpha diversity was assessed using  
174 both species richness (Faith's Phylogenetic Diversity (PD)) and community evenness (Pileu's  
175 evenness), combined with Kruskal-Wallis total and pairwise analysis. Beta diversity (extent



176 of change in community composition) was assessed using non-parametric multivariate  
177 PERMANOVA pairwise analysis. QIIME biom convert function provided summary  
178 information of relative abundance of taxonomic groups for each taxonomic level. Analysis of  
179 relative abundance of main bacterial genus: *Enterobacteriaceae*, *Staphylococcus*,  
180 *Streptococcus*, *Enterococcus* and *Neisseria*, grouped relative to patient information was  
181 conducted. Data was tested for normality using Shapiro-Wilk tests. For non-normally  
182 distributed data, Kruskal-Wallis H tests were conducted for each. For groups with significant  
183 differences Dunn's post hoc pairwise multiple comparisons were conducted. Significance  
184 was set at  $p < 0.05$ .

185

## 186 Results

### 187 Infant characteristics

188 Infants were included in the study with a mean (standard deviation, SD) GA at birth of 29.86  
189 (5.14) weeks. 60 tubes were collected from 30 infants throughout the study with a mean (SD)  
190 of 2 (1.2) tubes per infant and range 1-6 tubes. For summary statistics refer to Table 1.  
191 Predominant characteristics of the samples were: male, born extremely premature although  
192 within the moderately premature category at tube removal, not receiving antibiotics,  
193 ventilated, receiving vitamin or iron supplements but receiving MBM, with a 6 french gauge  
194 nasogastric tube placed for 7 days and routinely removed.) For further patient information  
195 refer to supplementary materials Figure S1.

196

197

198 Table 1. Summary patient information for all 60 samples included within study.

Variable	n (n=60)	%
Sex		
F	25	42%
M	35	58%
GA at Birth*		
Extremely Preterm	24	40%
Very Preterm	21	35%
Moderately Preterm	6	10%
Term	9	15%
GA at Removal		
Extremely Preterm	3	5%
Very Preterm	13	22%
Moderately Preterm	29	48%
Term	15	25%
Route of Birth		
Vaginal	36	60%
Ceasarian Section	24	40%
Antibiotic Prescription		
Y	18	30%
N	42	70%
Vitamin Supplement		
Y	24	40%
N	36	60%
Iron Supplement		
Y	12	20%
N	48	80%
Overall Ventilation		
Y	18	30%
N	42	70%
Ventilation Type		
Invasive	9	15%
Non-Invasive	9	15%
None	42	70%

\*GA classed according to WHO: <28 weeks = Extremely preterm. 28-32 weeks = Very Preterm. 32-37 week = Moderate to Late preterm. >37 weeks = Term.

\*\*Invasive types: SIMV (4), PC-SIMV (5), PC-AC (15), PC-AC + VG (3), PC-AC + Nitric (1), Optiflow (1). Non-Invasive: CPAP (14), Bubble-CPAP (4), Nippy-CPAP (1), High-Flow (4 and Vapotherm (6)). None: All infants not receiving ventilation, Low-flow O2 (3) and Nasopharyngeal Airway (1).

Abbreviations: GA : Gestational Age. SIMV : Synchronized Intermittent-Mandatory

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Ventilation. PC : Pressure Controlled. AC : Assist Controlled. CPAP : Continuous Positive Airway Pressure. VG : Volume Guaranteed.

199

## 200 [Common bacterial phyla](#)

201 The most dominant phylum throughout the entire sample population was *Firmicutes*,  
202 followed closely by *Proteobacteria*. *Actinobacteria* also displayed prominently in many  
203 samples. Nearly all the *Firmicutes* present were *Bacilli* - more specifically of the order  
204 *Lactobacillales* and *Bacillales* with minor populations of *Gemellales* and *Clostridiales*  
205 present. The *Proteobacteria* populations were comprised of predominantly  
206 *Gammaproteobacteria* followed by *Betaproteobacteria* and within these *Enterobacteriales*  
207 and *Neisseriales* respectively.

208

## 209 [Alpha diversity analysis](#)

210 The Alpha-group-significance command was used to analyse microbial compositions in  
211 relation to sample metadata. Faith's PD analysis of community richness was measured for all  
212 categorical patient variables, demonstrating that categorical variables associated with overall  
213 significant differences in community richness were: GA category at birth ( $p = 0.007$ ), mode  
214 of delivery ( $p = 0.021$ ), where infants born via caesarean section displayed a lower  
215 community richness than those born vaginally, and feed type ( $p = 0.049$ ).

216

217 Pairwise analysis (Kruskal-Wallis) of diversity comparing GA categories revealed significant  
218 differences between extremely preterm infants compared to very preterm infants ( $p = 0.002$ ),  
219 and term infants compared to very preterm ( $p = 0.009$ ). Extremely preterm infants had the  
220 highest microbial richness (Faith's PD value), whilst very preterm infants had the lowest. For  
221 main feed type, although there was overall group significance, pairwise analysis did not

222 detect significant differences between subgroups. Infants fed DBM had the highest average  
223 community richness, while being fed IF resulted in lowest species richness.  
224  
225 Pileu's evenness (measure of community evenness) was then calculated. Variables associated  
226 with significant differences in evenness were GA category at removal ( $p < 0.001$ ), mode of  
227 delivery ( $p = 0.023$ ), antibiotic exposure ( $p = 0.033$ ) and vitamin usage ( $p = 0.005$ ). Similar  
228 to community richness, those born vaginally also displayed higher community evenness than  
229 those born via caesarean section. Those receiving antibiotics had decreased evenness  
230 compared to those not receiving treatment, whereas those who were receiving vitamin  
231 supplements had increased community evenness.

232

### 233 **Beta Diversity**

234 PERMANOVA analysis was utilised to test the distances between samples from within a  
235 subgroup in relation to clinical data, in order to ascertain if samples from within categorical  
236 subgroups were more similar to one another or to the other subgroups. Clinical variables  
237 found to have significant differences between their subgroups were; main feed type ( $p =$   
238  $0.002$ ), GA at birth ( $p = 0.005$ ), GA at tube removal ( $p = 0.007$ ), antibiotic exposure ( $p =$   
239  $0.013$ ) and gender ( $p = 0.038$ ), though mode of delivery was not significant ( $p = 0.06$ ).  
240 Categorical variables with more than two subgroups were analysed against one another using  
241 pairwise PERMANOVA permutation tests. For main feed type all feed subgroups were found  
242 to be significantly different from one another: DBM versus IF, MBM and NBM ( $p = 0.003$ ,  
243  $0.002$  and  $0.042$  respectively), IF versus MBM and NBM ( $p = 0.050$  and  $0.029$  respectively)  
244 and MBM versus NBM ( $p = 0.015$ ). For GA at birth, there was a significant difference seen  
245 in extremely preterm infants compared to very preterm ( $p = 0.007$ ). For GA at tube removal,  
246 there were significant differences between extremely preterm compared to both moderately

247 preterm and term infants ( $p = 0.013$  and  $0.039$  respectively), and for moderately preterm  
248 versus term ( $p = 0.013$ ).

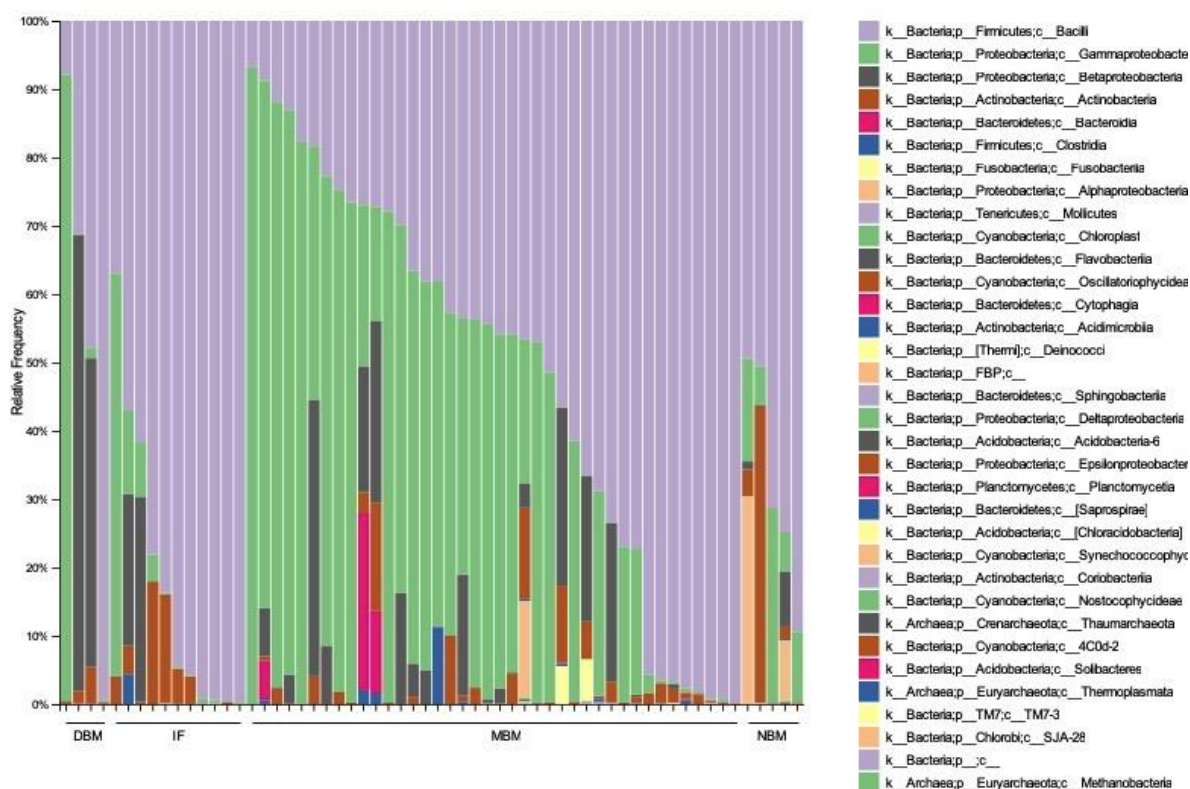
249  
250 Multiple multivariate response linear regressions were run utilising an increasing amount of  
251 patient variables. Summary  $r^2$  values, indicating the percentage of community variation the  
252 regression model can explain, increased with increasing numbers of patient variables  
253 included, however this also decreased the  $r^2$  values of each individual covariate. Some over  
254 fitting occurred in the expanded model as the fold 0-cross validation prediction accuracy was  
255 higher than within model error, however, all other cross folds, and all within the reduced  
256 model, were lower.

257

258 Summary  $r^2$  values for expanded and reduced models were  $0.4792$  and  $0.3249$  respectively,  
259 suggesting that even the expanded model only accounted for around 48% of community  
260 variation. For the expanded model the most influential variable was a GA category at birth of  
261 term, followed closely by a GA category at tube removal of term. Whereas for the reduced  
262 model, it was a GA category at birth of moderately preterm, followed closely by term. In the  
263 expanded model all variable categories appear to be responsible for variation of around 1-2%,  
264 and this is similar in the reduced model at around 1-3%.

265

## 266 Relative abundance of dominant phyla



267

268 Figure 1. Taxonomic plot displaying relative frequency for all 60 samples split based on main  
269 feed category, where; DBM= donor breast milk, IF= infant formula, MBM= maternal breast  
270 milk and NBM= nil by mouth.

271

272 Visual inspection of Figure 1, displaying relative frequency of species at a higher taxonomic

273 (Class) level split based on main feed type, provides some insight into the significant

274 differences observed. Analysis of relative abundance of main bacterial family or genus:

275 *Enterobacteriaceae*, *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Neisseria*, grouped

276 relative to feed type was conducted. Shapiro-Wilk normality tests suggested data was not

277 normally distributed and so Kruskal-Wallis H tests were conducted for each. For groups with

278 significant differences Dunn's post hoc pairwise multiple comparisons were conducted with

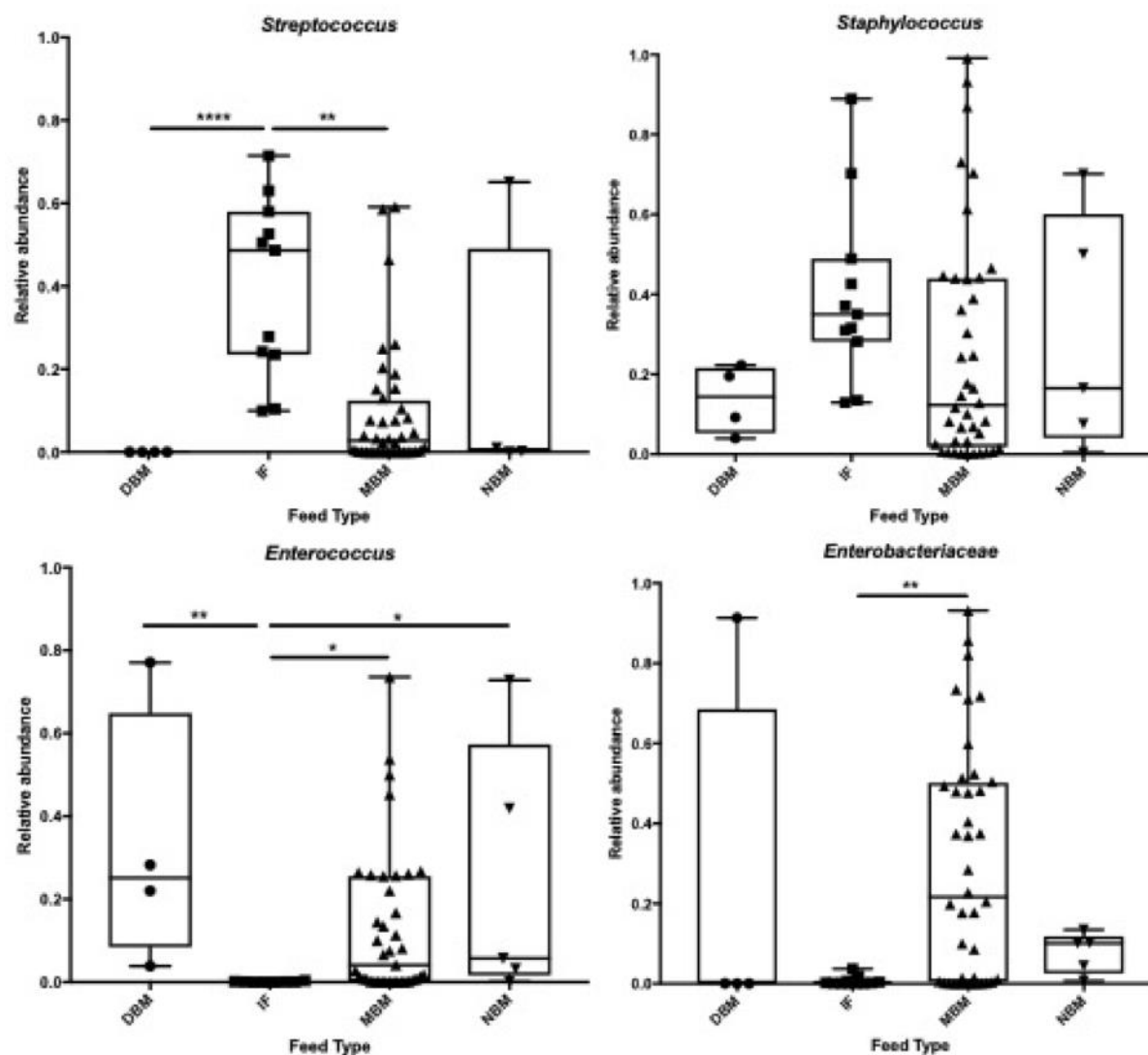
279 confidence levels set at  $p < 0.05$ . Refer to Table 2, for relative abundance data summary.

280

281 Table 2. Mean, standard deviation (SD) and range, for relative abundance of main  
282 microorganism; *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Neisseria* and  
283 *Enterobacteriaceae*. Relative abundance of total microorganisms is capped at 1, where 1 is  
284 complete dominance of the entire sample. Samples with relative abundance 0 (absent from  
285 sample) of a given microorganism were removed from results for this table.

Microorganism	Relative Abundance	
	Mean (SD)	Range
<i>Staphylococcus</i>	0.272 (0.272)	0.00044 to 0.991
<i>Enterococcus</i>	0.136 (0.203)	0.00005 to 0.771
<i>Streptococcus</i>	0.148 (0.211)	0.0002 to 0.715
<i>Neisseria</i>	0.085 (0.145)	0.00004 to 0.668
<i>Enterobacteriaceae</i>	0.221 (0.284)	0.00003 to 0.931

286  
287 Overall significant differences between feeding groups was found for relative abundance of  
288 *Enterococcus* ( $p = 0.002$ ), *Streptococcus* ( $p < 0.001$ ) and *Enterobacteriaceae* ( $p = 0.004$ ).  
289 Dunn's multiple comparison test was used to distinguish statistically significant differences  
290 between feed subgroups. For *Streptococcus*, the IF subgroup was found to have significantly  
291 higher relative abundance than the DBM ( $p < 0.001$ ) and MBM ( $p < 0.001$ ). Whereas for  
292 *Enterococcus*, the IF subgroup was found to have significantly lower relative abundance from  
293 all other feed subgroups ( $p < 0.04$  for all), as can be seen in Figure 2. For  
294 *Enterobacteriaceae* only the IF and MBM subgroups were found to be statistically  
295 significantly different ( $p = 0.003$ ), with MBM being significantly higher. There were no  
296 significance differences in relative abundance between feeding groups for *Staphylococcus* or  
297 *Neisseria*.  
298



299

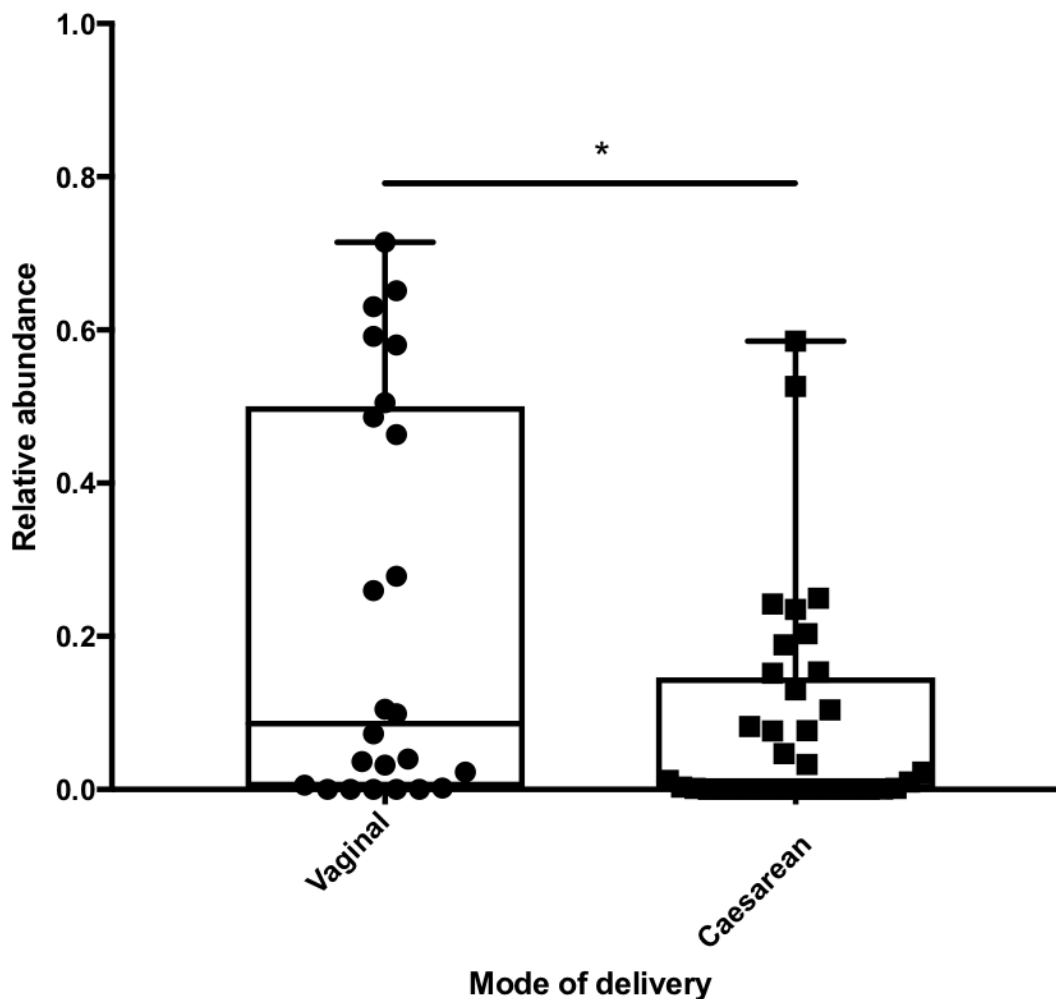
300 Figure 2. Box plots for relative abundance classified to family level for 4 most abundant  
301 profiles; *Streptococcus*, *Staphylococcus*, *Enterococcus* and *Enterobacteriaceae*, against main  
302 feed type. Relative abundance of total microorganisms is capped at 1, where 1 is complete  
303 dominance of the entire sample. Samples with relative abundance 0 (absent from sample) of a  
304 given microorganism were not removed from results. p-values as indicated by ns =  $p > 0.05$ ,  
305 \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$  and \*\*\*\* =  $p \leq 0.0001$ .

306

307 Analysis of main family of phylum was conducted for other patient characteristics.

308 *Streptococcus* relative abundance plotted against mode of delivery (Figure 3) was found to be  
309 statistically significant ( $p = 0.037$ ).





310

311 Figure 3 Displays relative abundance of *Streptococcus* split by mode of delivery. p-values as  
312 indicated by ns =  $p > 0.05$ , \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$  and \*\*\*\* =  $p \leq$   
313 0.0001.

314

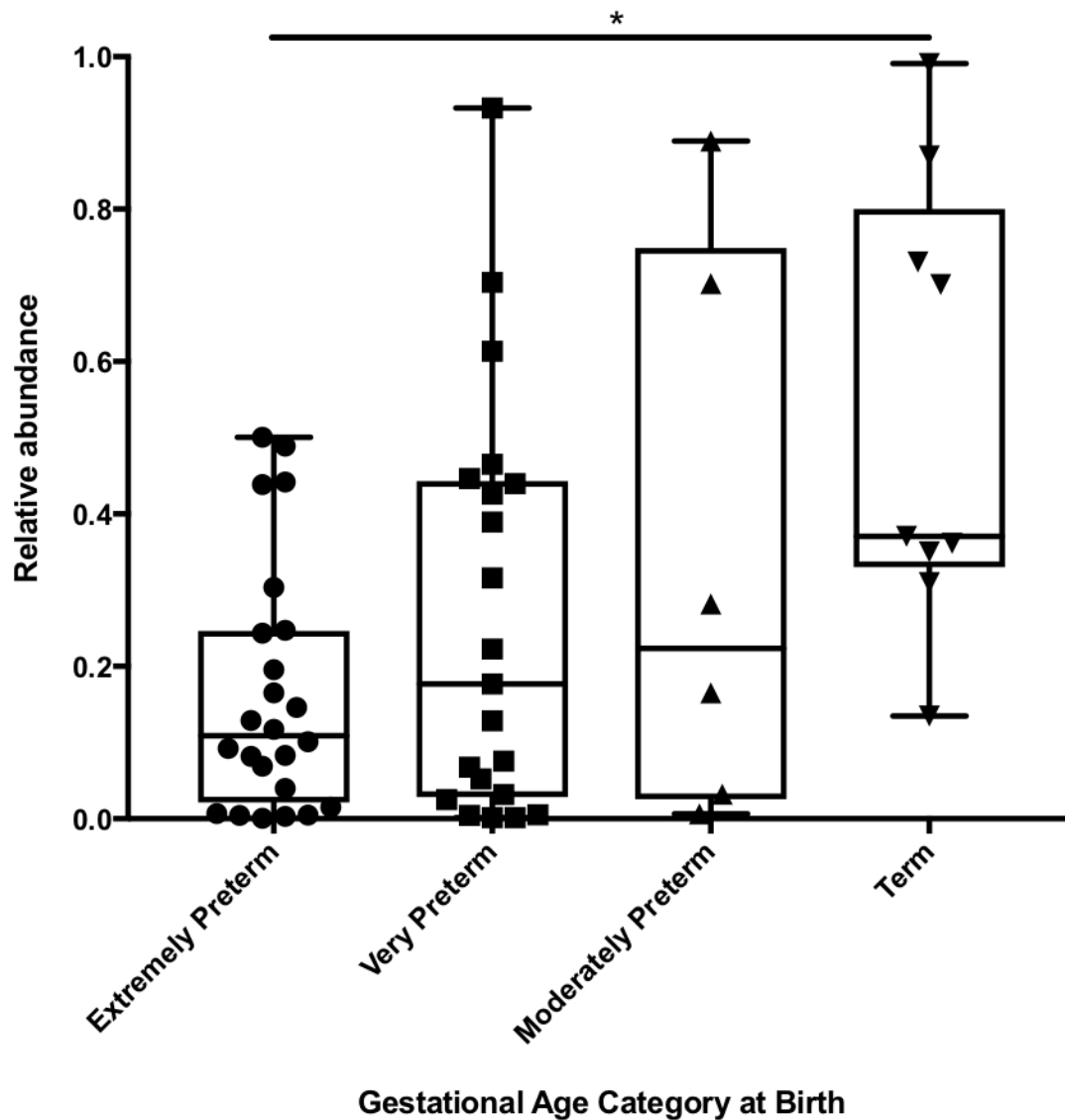
315 The analysis of both GA categories at birth and tube removal revealed only relative  
316 abundance of *Staphylococcus* against GA at birth ( $p = 0.020$ ) was statistically significant.

317 Dunn's multiple comparison analysis demonstrated that it was the extremely preterm and  
318 term categories that differed significantly ( $p = 0.012$ ). From Figure 4 it can be seen that

319 relative abundance of *Staphylococcus* increased with increasing GA at birth. Although not  
320 significant ( $p = 0.914$ ), GA at tube removal also appeared to follow the same trend. Other

321 patient characteristics (e.g. antibiotic exposure or vitamin usage) were not found to

322 significantly impact relative abundance when plotted individually.



323

324 Figure 4. Graph displaying relative abundance of *Staphylococcus* against gestational age  
325 category at birth. Relative abundance capped a 1, where 1 is complete sample dominance and

326 0 is absence of sequences from sample. p-values as indicated by ns =  $p > 0.05$ , \* =  $p \leq 0.05$ ,

327 \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$  and \*\*\*\* =  $p \leq 0.0001$ .

328

## 329 Discussion

### 330 Diversity analysis

331 Previous research has suggested links between levels of microbial diversity and human health  
332 and disease<sup>27</sup>. Alpha diversity analysis results from the study reported here demonstrates the  
333 significant influence of mode of delivery on microbial communities found within the feeding  
334 tubes. This is in line with previous work, utilising infant faeces, demonstrating that vaginally  
335 born infants have a greater abundance of species, where their microbiota is seeded by the  
336 vaginal and faecal flora of the mother, whereas caesarean born infants microbiota is more  
337 influenced by the local environment and the mother skin microbiota<sup>28</sup>. Another dominant  
338 influencer on alpha diversity was GA category at birth, and at tube removal. This is in  
339 keeping with previous observations that the microbiome of many bodily sites matures over  
340 the first few months of life, with preterm infants displaying altered development and  
341 communities<sup>29 30</sup>.

342

343 Beta diversity analysis demonstrated the pivotal impact of feeding regime upon the  
344 maturation of specific microbial communities, a known influence on the neonatal gut  
345 microbiome, as analysed through faecal samples<sup>31</sup>. However, it has been suggested that  
346 faecal samples underrepresent the biodiversity of the gastrointestinal system<sup>32</sup>. Others have  
347 analysed stomach contents, aspirated through feeding tubes<sup>33</sup>, but without knowing the exact  
348 contents of the feeding device; this also introduced bias. The placement of enteral feeding  
349 tubes directly into the stomach, provides researchers analysing them a unique insight into the

350 dynamics of the gut microbiome, without the influence of other bodily sites. The contents of  
351 neonatal feeding tubes provide a truly representative sample of the neonatal gut microbiome,  
352 as a result of aspiration checks, prior to each feeding, collecting microorganisms from within  
353 this niche and depositing them upon an abiotic surface, away from the influence of the host  
354 immune system.

355

### 356 [Abundance analysis](#)

357 Abundance analysis of the main microorganisms' present demonstrated how the tubes that  
358 were exposed to infant formula had altered populations, with increased relative abundance of  
359 *Streptococcus*, but decreased abundance of *Enterococcus* and *Enterobacteriaceae*. Those  
360 born vaginally also displayed significantly increased relative abundance of *Streptococcus*  
361 within the tubes compared to those delivered by caesarean section. When combined these  
362 suggest that an initial increased risk of seeding by *Streptococcus* during vaginal birth could  
363 be exacerbated by *Streptococcus* overgrowth upon infant formula feeding, as well as  
364 suppression of potentially beneficial populations. This is especially concerning given the  
365 association of *Streptococcus* with neonatal early-onset sepsis and meningitis<sup>34 35</sup>.

366 Conversely, infants displaying early colonisation with *Enterococcus* are less likely to develop  
367 allergy later in life<sup>36</sup>, perhaps through suppression of inflammatory responses<sup>37</sup>. Larger  
368 cohort-controlled studies should be conducted to assess the specific impact of vaginal birth  
369 and subsequent formula feeding as a risk factor for *Streptococcus* related morbidity and  
370 mortality within the neonatal population. This could lead to the creation of specific  
371 prophylactic care regimes or vigilance schemes, aimed at reducing risk within a  
372 subpopulation.

373

## 374 Implications

375 Despite the relative lack of knowledge on the pathogenesis of NEC, it is clear that bacteria  
376 play, at least, a contributory role<sup>38 39</sup>. Bacterial lipopolysaccharide is able to induce  
377 activation of TLR-4 and therefore stimulate the inflammatory environment associated with  
378 NEC<sup>40</sup>. Specific populations of bacteria may also dominate the gastrointestinal environment  
379 and secrete toxic metabolites and endotoxins, worsening inflammation and increasing the risk  
380 of potential intestinal damage. Research into specific populations of bacteria associated with  
381 NEC have yielded contrasting results, with many identifying bacteria often found in healthy  
382 controls<sup>41-43</sup>. These studies have led some to conclude that it may not be one specific  
383 pathogenic species contributing, but instead the culmination of many species distorting the  
384 normal microbial environment<sup>44</sup>. Colonisation of enteral feeding tubes, as a result of early  
385 infant seeding with potentially pathogenic microorganisms, translocation up the tubes as a  
386 result of aspiration, or introduction via contaminated external sources, could result in  
387 unwanted pathogenic overgrowth within this important niche. Due to the direct placement of  
388 these tubes into the infant stomach, any pathogens would be able to not only continually seed  
389 their fragile microbiome, leading to dysbiosis, but could potentially invade the host tissue  
390 following any device related damage to the gut mucosa.

391

392 Given the variability in causative organisms for neonatal infection<sup>45</sup>, investigations into  
393 potential sources of infection should utilise techniques that can accurately portray all  
394 microorganisms. Previous studies investigating contamination have relied on limited culture-  
395 based analysis<sup>46</sup>. As previously mentioned, culture-based analysis alone can lead to severe  
396 underrepresentation of microbial diversity, which in a clinical scenario could lead to

397 inaccurate targeted therapies. Despite their limitations, culture analysis of contaminated  
398 enteral feeding tubes still demonstrate that high levels of pathogenic colonisation frequently  
399 occur<sup>47</sup>. The use of modern molecular techniques to fully describe the microbial populations  
400 in neonatal enteral feeding tubes is a particular strength of the present study. Increasingly,  
401 recent molecular based analysis studies are showing the importance of minor microbial  
402 populations on microbiota formation<sup>48</sup>, and future work should focus on utilising the most  
403 accurate methods.

404

#### 405 **Limitations**

406 Although a substantial study, sample numbers meant we were not able to adequately control  
407 for all cofounders. This was highlighted by the multiple multivariate response linear  
408 regression analysis in which all infant characteristics were shown to contribute to community  
409 variation to a similar degree. This was most likely a result of the sample size splitting certain  
410 populations into very minor subgroups.

411

#### 412 **Conclusions**

413 The results demonstrate that early nutrition significantly influences colonisation patterns, and  
414 that a combination of vaginal birth and subsequent infant formula feeding may be a specific  
415 risk factor for *streptococcal* contamination of enteral feeding tubes. To further elucidate the  
416 impacts of early life feeding and care regimes impact microbiome development, a large-scale  
417 observational study should be conducted to analyse the impact of infant formulae feeding on  
418 vaginally born infants' infection and morbidity, specifically those attributed to *Streptococcus*.  
419 Future studies should also be conducted utilising larger cohort sizes to more accurately  
420 account to confounding patient variables. The therapeutic utilisation of probiotic

421 microorganisms to modify early stage colonisation, as well as restrict potentially pathogenic  
422 microorganism overgrowth, of formula fed infants, to more closely resemble those fed breast  
423 milk, also provides an exciting avenue for future research on tube colonisation. Overall this  
424 study demonstrates the feasibility of 16s rRNA sequencing as a tool for uncovering  
425 previously undiscovered microbial populations within the neonatal population.

426

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439

#### 440 [Conflicts of interest](#)

441 All authors involved within this study certify they have no conflicts of interests (financial and  
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