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Title: Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics

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Keywords: intestinal microbiota; preterm; infants; antibiotics; intrapartum antimicrobial prophylaxis

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Abstract: Objectives: To assess the process of establishment of the intestinal microbiota in very-low birth-weight preterm infants and to evaluate the impact of perinatal factors, such as delivery mode and perinatal antibiotics, in this process. Study design: We used 16S rRNA gene sequence-based microbiota analysis and quantitative PCR to evaluate the establishment of the microbiota. We also evaluated factors affecting the microbiota establishment, during the first three months of life in preterm infants (n=27) compared with full-term babies (n=13). Results: Immaturity affects the microbiota as indicated by a reduced percentage of the family Bacteroidaceae during the first months of life or by a higher initial percentage of Lactobacillaceae in preterm infants compared with full term infants. Perinatal antibiotics use, including intrapartum antimicrobial prophylaxis, affects the establishment of gut microbiota, as indicated by increased Enterobacteriaceae family organisms in the infants. Conclusion: Prematurity and perinatal antibiotic administration strongly affect the initial establishment of microbiota with potential consequences for later health.

1 **Original Article.**

2 **Title:** Intestinal microbiota development in preterm neonates and effect of perinatal
3 antibiotics

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27 **Abstract**

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32 quantitative PCR to evaluate the establishment of the microbiota. We also evaluated
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39 establishment of gut microbiota, as indicated by increased *Enterobacteriaceae* family
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42 initial establishment of microbiota with potential consequences for later health.

43

44 **Introduction**

45 The microbial colonization of the intestine of newborns starts with facultative
46 anaerobes and continues with strict anaerobic genera, with several factors, such as
47 feeding habits or gestational age, affecting this process¹. This initial microbial
48 colonization results essential for the normal development of the host², with the early
49 neonatal period representing the most important moment for the microbiota-induced
50 host-homeostasis³. However, with the exception of delivery mode and feeding habits,

51 the effect of other factors on the process of microbiota development in the newborn
52 remains poorly understood.

53 Despite the high inter-individual variability, metagenomic and 16S rRNA gene
54 sequencing studies have recently identified the existence of different microbiota
55 enterotypes in humans⁴, and microbiota alterations related with diseases have been
56 observed^{5,6}. In spite of the importance of the initial steps of microbiota establishment
57 for the later well-being, only recently some data on preterm infants became
58 available⁷⁻¹⁴. In many of these works infants suffering necrotizing enterocolitis (NEC)
59 and/or sepsis are compared with those remaining healthy and very often the studies
60 include small cohorts and do not extend beyond the first month of life. In spite of
61 these limitations, the available data indicate that the microbiota of preterm differs
62 from that of full-term infants, suggesting potential targets for microbiota modulation.

63 The fecal microbiota profile of the healthy full-term, vaginally-delivered,
64 exclusively breast-fed (FTVDBF) infant has been considered as the standard for a
65 healthy infant microbiota and recent studies have tried to define its composition^{15,16}.
66 Indeed, the promotion of a microbiota resembling that of the FTVDBF infant has been
67 considered as a target for improving infant formulas¹⁷. Preterm infants present an
68 immature immune system¹⁸ and a compromised gut mucosa¹⁹. These represent a
69 risk for both vertically transmitted infections and late-onset nosocomial infections. In
70 these newborns the process of microbiota establishment is also affected, presenting
71 an increased abundance of Enterobacteriaceae, a delayed colonization by
72 commensal bacteria and a higher colonization by pathogens such as *Klebsiella* sp.,
73 than full-term babies^{7,8,9,20}.

74 The aim of the present study was to assess the process of establishment of the
75 intestinal microbiota in very-low birth-weight (VLBW) preterm infants, compared with

76 that of FTVDBF neonates, and to evaluate the impact of perinatal factors, such as
77 the delivery mode and antibiotics use, in this process. To this end we applied an Ion
78 Torrent 16S rRNA gene sequence-based microbiota analysis and quantitative PCR
79 (qPCR) of specific microbial groups.

80 **Methods**

81 **Volunteers.** The study was approved by the Regional Ethical Committee of Asturias
82 Public Health Service (SESPA) and informed written consent was obtained from the
83 parents. Thirteen caucasian FTVDBF infants, (7 males/6 females) born after an
84 uncomplicated pregnancy, and twenty-seven caucasian very low birth weight (VLBW)
85 preterm infants (12 males/15 females) were recruited at the Neonatology Units of
86 Cabueñes Hospital and Central University Hospital (HUCA) from Asturias (Spain). All
87 full-term infants were vaginally delivered, at a gestational age between 37 and 41
88 weeks (mean 39.2) with birth weights between 3020 and 4160 grams, were
89 exclusively breast-fed and were discharged from the hospital at the third day of life.
90 Preterm infants (7 delivered vaginally and 20 by caesarean section) were born at a
91 gestational age between 24 and 32 weeks (mean 29.6) and their birth weights
92 ranged between 690 and 1,800 grams. None of the infants suffered NEC or
93 presented culture positive early onset infection. With regard to antibiotic
94 administration none of the full-term infants received antibiotics but three mothers
95 received intrapartum antimicrobial prophylaxis (IAP) (in all cases a single dose of
96 ampicillin). Fourteen of the preterm infants' mothers received IAP. One of these
97 mothers received a single dose of penicillin and other mother was administered
98 ampicillin for three days (one dose every six hours). The rest of the mothers received
99 ampicillin plus erythromycin (between two and 24 doses of each antibiotic). Twelve
100 infants received antibiotics at birth (all of them ampicillin plus gentamicin during 5-8

101 days) whilst five extra infants started to receive antibiotics later on, three of them
102 starting before 10 days of life and two infants starting at 12-13 days of life (three
103 cases vancomycin plus amikacin, one vancomycin and one gentamycin plus
104 clindamycin plus teichomycin). Only 5 out of the 27 mother/premature infant pairs did
105 not receive antibiotics, either intra-partum or postnatally, during the sampling period
106 whilst in 9 of the pairs both, mother and infant, received antibiotics. All preterm
107 infants received mixed feeding (infant formula and some sporadic breast-milk
108 administration along the study) and were discharged from the hospital after an
109 average hospital stay of 50 (range 21-93) days.

110 **Sample Collection.** Fecal samples were collected at the hospital at two (between 24
111 and 48 hours of life), 10, 30 and 90 days of age. The first spontaneous or stimulated
112 (after perianal stimulation) deposition was taken in a sterile container, immediately
113 frozen at -20°C and sent, within a week, to the laboratory.

114 **Microbiota analyses by Ion Torrent PGM sequencing of 16S rRNA gene-based**
115 **amplicons.** DNA was extracted from fecal samples as previously described²⁰ and
116 kept frozen at -80°C until analysis. DNA was PCR-amplified, sequenced in a 316 chip
117 at GenProbio Ltd (www.genprobio.com) by using the Ion Torrent PGM system and
118 the Ion Sequencing 200 kit (Life Technologies) and analysed as recently reported²¹
119 using the QIIME software suite. Quality filtering allowed to retain only full length
120 reads with quality >25 that were used to construct *de novo* OTUs using uclust
121 software and 97% sequence identity as threshold. Reference sequences for each
122 OTUs were identified and used for OTUs taxonomic assignment based on a
123 reference dataset from the Ribosomal Database Project. Hierarchical clustering was
124 constructed using the MeV software and the Pearson's correlation as distance
125 metric.

126 **Quantitative PCR Analysis.** For quantification of total fecal bacteria the qPCR
127 conditions and primers described elsewhere²² were used. Quantification of the
128 different bacterial populations assessed was achieved as previously reported²⁰.

129 **Statistical analyses.** Results were analyzed using the SPSS software (SPSS Inc.
130 Chicago, USA). The normality of the data, at each sampling point, was checked
131 using the KS test. For normal variables, one-way anova followed by post-hoc
132 bonferroni's test was used. Some of the bacterial groups showed non-normal
133 distribution, then the differences between groups of infants were analyzed using the
134 non-parametric Kruskal-Wallis test or, in the case of pairwise comparisons, the
135 Mann-Whitney U-test.

136 **Nucleotide sequence accession numbers.** The raw sequences from the samples
137 reported in this article have been deposited in the NCBI Short Read Archive (SRA)
138 under the BioProject ID code PRJNA230470.

139 **Results**

140 **Establishment of intestinal microbiota in VLBW preterm neonates as compared**
141 **with FTVDBF infants.** Ion Torrent sequencing of the PCR products obtained by
142 amplification of the V3-V4 region of the 16S rRNA gene from the 160 fecal samples
143 analysed in this study yielded, after filtering, about $\sim 10^5$ sequences per sample with
144 an average length of 196 bp. We found noticeable differences in the development of
145 the intestinal microbiota composition between preterm and FTVDBF babies (Figure
146 1). Two days-old preterm newborns showed significantly ($p < 0.05$) lower proportions
147 of the families *Bacteroidaceae*, *Clostridiaceae*, *Micrococcaceae*, *Pasteurellaceae* and
148 *Porphyromonadaceae* and higher ($p < 0.05$) of *Bifidobacteriaceae*, *Comamonadaceae*,
149 *Propionibacteriaceae*, *Streptococcaceae*, unclassified Actinobacteria, unclassified
150 Bacilli or unclassified Lactobacillales and specially of *Lactobacillaceae* than FTVDBF

151 infants. At 10 days of age preterm infants displayed a significant reduction ($p < 0.05$) in
152 the percentage of *Bacteroidaceae*, *Bifidobacteriaceae*, *Clostridiaceae*,
153 *Coriobacteriaceae*, *Leuconostocaceae*, *Pasteurellaceae*, *Porphyromonadaceae*,
154 unclassified Actinobacteria and *Veillonellaceae*, and a higher percentage ($p < 0.05$)
155 of the families *Enterobacteriaceae*, *Micrococcaceae* and unclassified
156 Gammaproteobacteria than FTVDBF babies. This situation remained relatively stable
157 during the rest of the study, with significantly ($p < 0.05$) higher proportions of
158 *Enterobacteriaceae* and significantly ($p < 0.05$) lower of *Bacteroidaceae* in preterm
159 infants at 30 and 90 days of age as the main observation during this period.

160 The qPCR results showed that the levels of most microbial groups tended to
161 increase over time, with notable differences in bacterial levels observed between
162 both groups of infants (Figure 2, online). Feces from preterm newborns showed
163 significantly lower levels ($p < 0.05$) of total bacteria at two days of life, but higher at 10
164 days than FTVDBF infants, with an almost identical trend observed for the
165 *Enterobacteriaceae* family. In feces from preterm infants *Enterococcaceae* levels
166 were lower ($p < 0.05$) at two days of age, but significantly higher after 30 days, than in
167 those of FTVDBF infants. Moreover, premature infants showed lower levels of most
168 of the other microbial groups analyzed. These results indicate that the surprisingly
169 high percentage of lactobacilli sequences found in the 16S rRNA profiling analysis in
170 two days-old preterm infants are due to reduced levels of total bacteria, rather than to
171 higher absolute level of lactobacilli in feces from this infant group.

172 **Impact of delivery mode and antibiotics use on the establishment of**
173 **intestinal microbiota.** The mode of delivery was found to have limited effect on the
174 gut microbiota composition of the preterm newborns. No differences were observed
175 on the 16S rRNA profiling data between vaginally delivered preterm infants and those

176 born by caesarean section, whilst the only difference observed by qPCR regarded
177 higher levels of *Bacteroides* in vaginally delivered babies at 10 days of age ($5.31 \pm$
178 1.39 vs. 4.24 ± 0.48 log cells/g, $p < 0.05$).

179 In contrast, antibiotics use was found to have a profound impact on the
180 intestinal microbiota establishment process. When cluster analysis was performed by
181 including preterm infants classified into four groups, depending on mother and infant
182 antibiotic use, as well as full-term infants not exposed to antibiotics as external group,
183 an effect of perinatal antibiotics became evident (Figure 3). IAP was found to have an
184 equal or even higher effect than direct administration of antibiotics to the infant during
185 the first days of life. Interestingly, this effect was not so apparent in the first sampling
186 points, when the only statistically significant differences ($p < 0.05$) were the higher
187 percentage of sequences from *Leuconostaceae* at two days of age and the higher
188 percentage of sequences from *Micrococcaceae* and *Propionibacteriaceae* at 10 days
189 in the infants not exposed to antibiotics, neither directly nor through IAP, than in the
190 other three groups. However, at 30 days of age several statistically significant
191 differences were observed, with the infants not exposed to antibiotics (either directly
192 or via their mothers) showing higher relative abundances of *Comamonadaceae*,
193 *Staphylococcaceae* and unclassified Bacilli than the other three groups ($p < 0.05$). At
194 this time points infants not exposed to antibiotics also presented significantly higher
195 percentage ($p < 0.05$) of *Bifidobacteriaceae*, *Streptococcaceae*, unclassified
196 Actinobacteria and unclassified Lactobacillales and lower ($p < 0.05$) of
197 *Enterobacteriaceae* than both groups of infants whose mothers received IAP
198 (independently on whether or not the infant itself received antibiotics). However,
199 these no-antibiotics exposed infants group did not differ ($p > 0.05$) from the group of
200 infants that received antibiotics but whose mothers did not receive them. After 90

201 days of age, most of these differences have disappeared being *Ruminococcaceae*
202 the only family showing statistically significant differences among groups.

203 It is important to underline that the four groups of infants defined by antibiotics
204 use did not differ statistically at any time point regarding birth-weight or length of
205 hospital stay. At two and 10 days of life the group in which both mothers and infants
206 received antibiotics showed a significantly lower ($p<0.05$) gestational age than those
207 in the group in which neither the mother nor the infant received antibiotics, but no
208 differences were found with the other infant groups. This difference disappeared at
209 later sampling points (30 and 90d) due to new infants receiving antibiotics and,
210 therefore, changing to the antibiotics group.

211 Interestingly, although the number of full-term infants whose mothers received
212 IAP is very limited ($n=3$) the comparison of these babies with the non-antibiotics
213 exposed full-term babies ($n=10$) also suggests a profound effect of IAP in full-term
214 newborns (Figure 4; online), even when these three mothers having IAP received
215 only a single dose of ampicilin.

216 In accordance to that stated above, qPCR analyses did not show any
217 statistically significant differences among groups at 2 or 10 days of age. However, at
218 30 days statistically significant differences ($p<0.05$) were observed for
219 *Staphylococcaceae*, *Enterobacteriaceae* and total bacteria, the levels of the first
220 microorganism being higher in preterm infants not exposed to antibiotics than in the
221 other three infant groups, whilst the contrary was true for *Enterobacteriaceae* and
222 total bacteria. At 90 days of age the only difference observed referred to the higher
223 levels ($p<0.05$) of bifidobacteria in the non-antibiotics exposed infants (data not
224 shown).

225 Discussion

226 Despite the high inter-individual variability, the 16S rRNA profiling analysis
227 evidenced an altered pattern of intestinal microbiota establishment in extreme
228 preterm infants when compared with FTVDBF babies. It is important to underline,
229 however, that very often prematurity is present together with different potential
230 confounding factors which difficult the interpretation of the data. To this regard, our
231 preterm cohort received mixed feeding, with none of the infants being exclusively
232 breast-fed, whilst our control group included exclusively breast-fed babies. Therefore,
233 the potential impact of the different feeding habits cannot be overruled as a factor
234 contributing to explain the differences observed between both groups of infants. In
235 our study the most striking observation was, perhaps, the reduced percentage of
236 sequences belonging to the family *Bacteroidaceae* found in preterm ($\leq 1\%$ of
237 sequences) with regard to that in FTVDBF babies ($\sim 20\%$) during the whole duration
238 of the study. This observation was confirmed by qPCR and it is in agreement with
239 that previously reported for non-VLBW premature babies²⁰. Other noticeable
240 differences regarded the reduced levels of total bacteria and higher relative
241 proportion of *Lactobacillaceae* during the first hours of life, followed by a dominance
242 of *Enterobacteriaceae*, starting during the first days and remaining up to the three
243 months of age, in our VLBW preterm infants. These observations are in agreement
244 with previous reports indicating an increased occurrence of potential pathogenic
245 enterobacteria, as well as a high inter-individual variability in preterm babies^{9,20,23}.

246 Delivery mode and antibiotics treatment are two factors that may affect
247 microbiota composition. In our preterm infants group the delivery mode had a limited
248 effect on gut microbiota composition, in contrast to what has been previously
249 reported for full-term infants²⁴. The only difference observed suggested a delayed

250 colonization by *Bacteroides* during the first days of life in cesarean section (CS)
251 delivered newborns, which has been previously reported in full-term infants²⁵. The
252 use of antibiotics may disrupt the neonatal gut microbiota having profound
253 consequences for later health²⁶. Indeed, recent animal studies demonstrate that
254 antibiotic-mediated disturbance of the intestinal microbiota in the very early life can
255 increase the risk of late-onset sepsis²⁷. Here we have assessed the effect of
256 antibiotics administration, either as IAP to the mother or directly to the infant, upon
257 gut microbiota establishment. Our results indicate an effect of IAP administration.
258 This effect was not immediate as it was hardly detected the first days after delivery
259 but becomes apparent later on. At one month of age infants whose mothers received
260 IAP showed an intestinal microbiota different from that of those infants whose
261 mothers did not receive it. Noteworthy, at this sampling time no statistically significant
262 differences were observed among the four preterm infant groups in background
263 variables such as gestational age, birth-weight or length of hospital stay.

264 Similarly, effects of IAP administration seem to be also present in full-term
265 infants. Although previous studies did not observe any effect of maternal antibiotics
266 consumption during pregnancy upon infant gut microbiota²⁸, others evidenced an
267 effect of maternal perinatal antibiotics use on the fecal microbiota of full-term
268 infants²⁹. Also with full-term infants, other studies have reported that antibiotics
269 administration during the first hours of life caused a reduced level of intestinal
270 *Bifidobacterium* in the immediate days after administration and increased levels of
271 *Enterobacteriaceae* later on^{30,31}. These suggesting a lasting effect of early-life
272 antibiotic administration upon gut microbiota composition, which is in good
273 agreement with our observations. Similarly, incomplete recovery of the gut microbiota
274 after antibiotics administration has been demonstrated in adults³².

275 Our results indicate that immaturity affects intestinal microbiota composition and
276 it accounts for some of the differences observed between preterm and FTVDBF
277 infants, such as the reduced percentage of *Bacteroidaceae* during the first months of
278 life or the higher percentage of *Lactobacillaceae* during the first days of life. However,
279 in some other of the differences observed, such as the increased *Enterobacteriaceae*
280 levels in preterm infants, the antibiotics exposure seems to have a role. To this
281 regard, IAP seems to exert a critical influence in the early intestinal microbiota.
282 Therefore, it may be time for considering the potential deleterious effects upon gut
283 microbiota composition when deciding on antibiotic use. According to the data from
284 the Spanish Society for Neonatology (www.se-neonatal.es) in Spain about half of the
285 mothers of VLBW infants receive IAP. Overall, in developed countries IAP is used in
286 over 30% of total deliveries³³. In spite of not being a clearcut decision pre-partum
287 antibiotics are generally recommended in the case of premature rupture of
288 membranes or when vaginal colonization by group B streptococci is detected,
289 however, they are also frequently used in other clinical situations in which a clear
290 benefit has not been demonstrated³⁴. This, together with the high number of infants
291 receiving antibiotics for what later on results to be a noninfectious cause has raised
292 some concerns³⁵. In addition, recent data suggest that perinatal antibiotics-mediated
293 microbiota disturbance increases the risk for late-onset sepsis and NEC which may
294 constitute a life threatening risk for preterm newborns³⁶. Moreover, very often broad-
295 spectrum antibiotics are used which seems to drastically affect the early gut
296 microbiota establishment process.

297 Given the importance of the microbial gut colonization during the neonatal
298 period it is important to minimize the impact on the early microbiota of any medical
299 intervention. This study identifies alterations on the process of establishment of the

300 intestinal microbiota in preterm infants and points out effects of antibiotics upon this
301 process. These results may be the basis for designing intervention strategies
302 targeting to favor the gut microbiota establishment, and to minimize the impact of
303 medical interventions in early life on this process

304

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308

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419

420 **Figure legends**

421 **Figure 1.** Aggregate microbiota composition at family level in fecal samples from
422 term and preterm infants at the different time points analyzed.

423 **Figure 2. Online only.** Fecal levels (mean \pm SD) of the different microorganisms
424 analyzed by qPCR in samples from term and preterm infants at the different time
425 points analyzed (2, 10, 30 and 90 days). Asterisks indicate statistically significant
426 differences ($p < 0.05$).

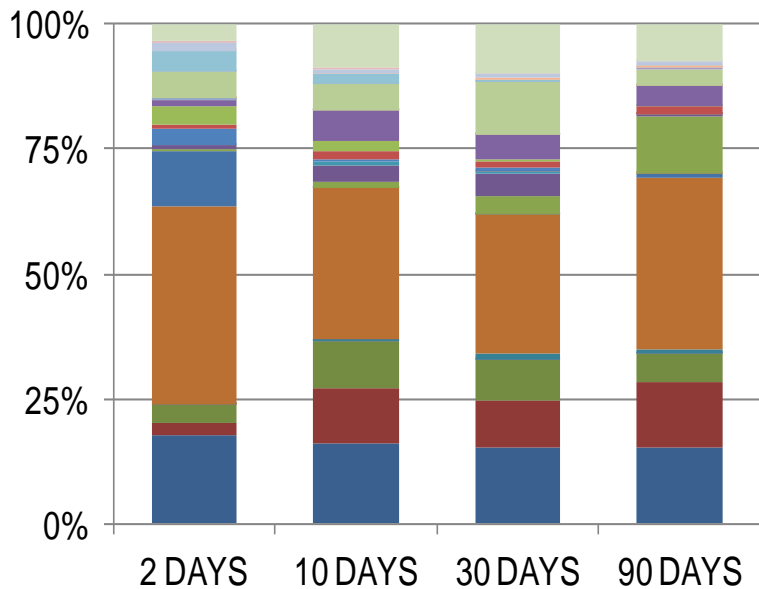
427 **Figure 3.** Hierarchical clustering based on composition, at family level, of samples
428 collected at the different times from term infants not exposed to antibiotics and the
429 four groups of preterm infants classified as a function of the maternal and/or infant
430 antibiotic administration. Every samples' group is associated with its own aggregate
431 representation at family level.

432 **Figure 4. Online only.** Aggregate microbiota at family level, of samples collected at
433 the different time points from term infants whose mother received a single dose of
434 IAP with ampicilin (n=3) and those whose mothers did not receive IAP (n=10).

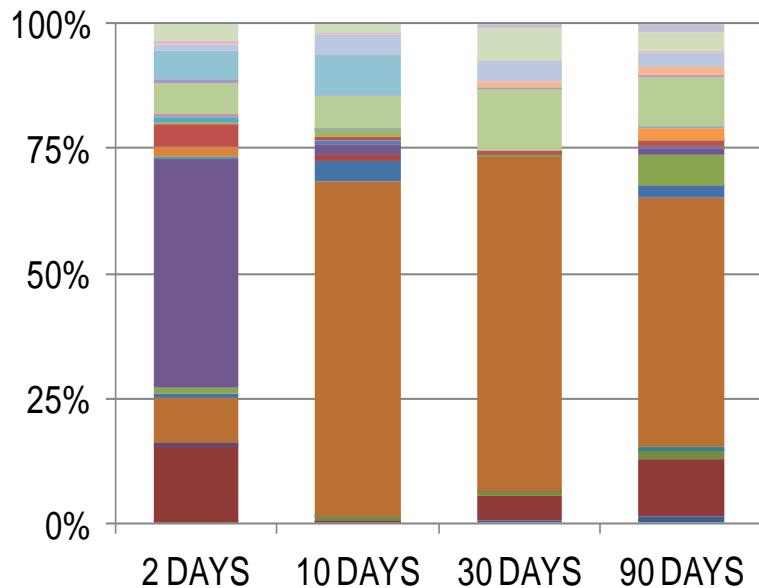
Figure 1

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TERM



PRETERM



- Verrucomicrobiaceae
- Veillonellaceae
- Unclassified Lactobacillales
- Unclassified Gammaproteobacteria
- Unclassified Clostridiales
- Unclassified Bacilli
- Unclassified Actinobacteria
- Streptococcaceae
- Staphylococcaceae
- Ruminococcaceae
- Pseudomonadaceae
- Propionibacteriaceae
- Porphyromonadaceae
- Pasteurellaceae
- Others
- Micrococcaceae
- Listeriaceae
- Leuconostocaceae
- Lactobacillaceae
- Lachnospiraceae
- Fusobacteriaceae
- Enterococcaceae
- Enterobacteriaceae
- Coriobacteriaceae
- Comamonadaceae
- Clostridiaceae
- Bifidobacteriaceae
- Bacteroidaceae

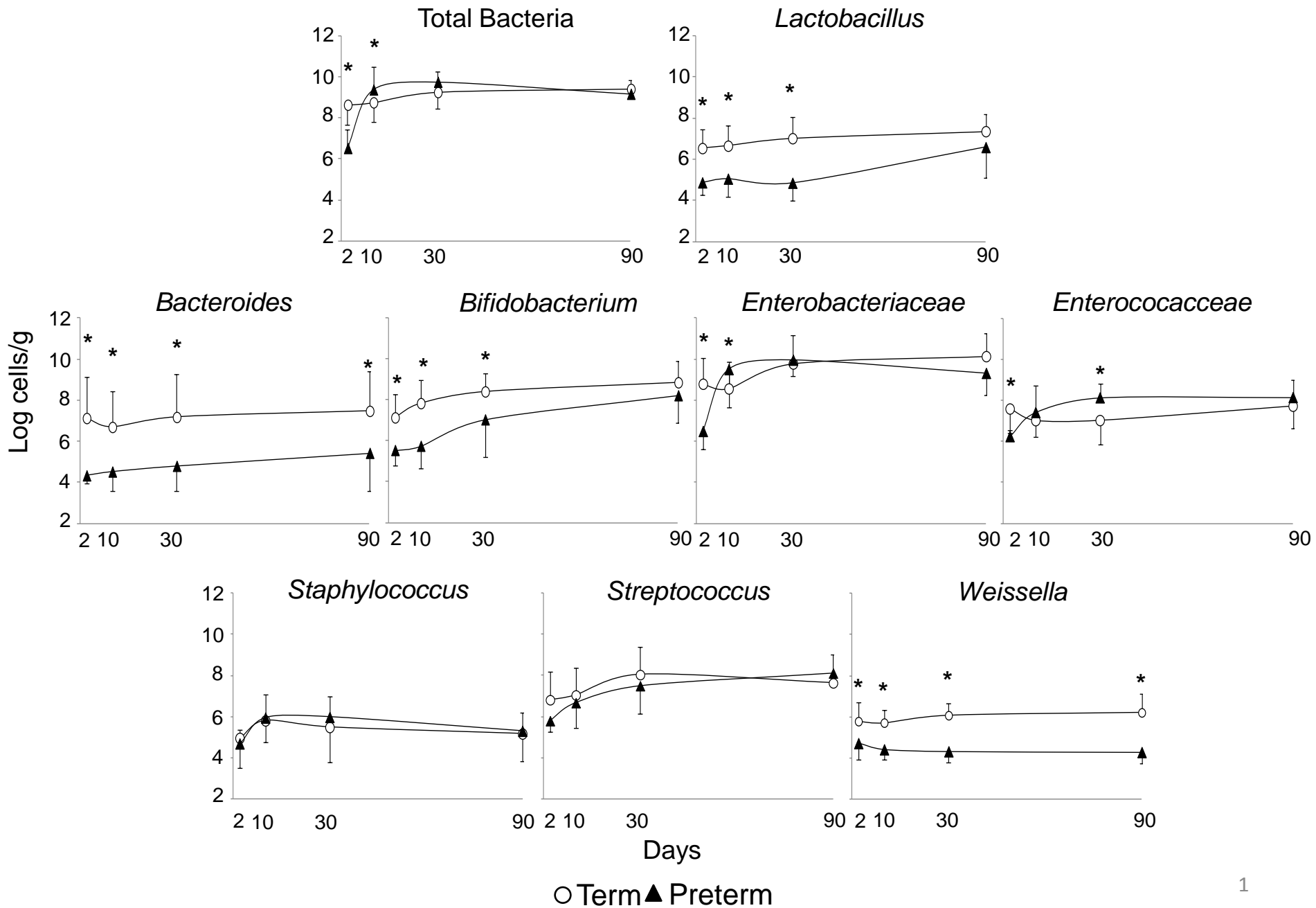
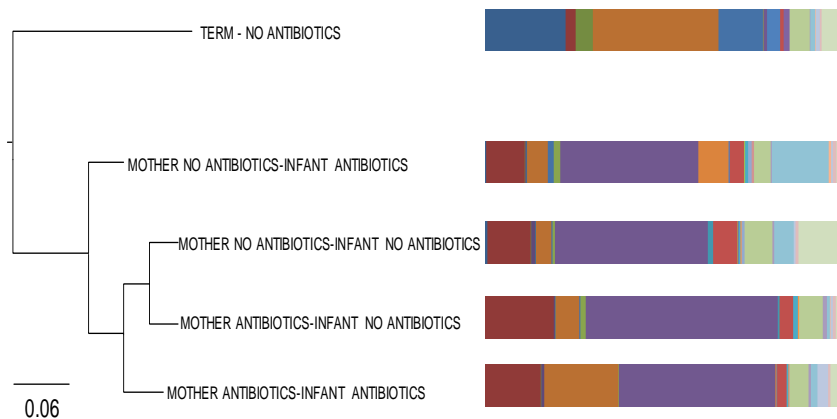
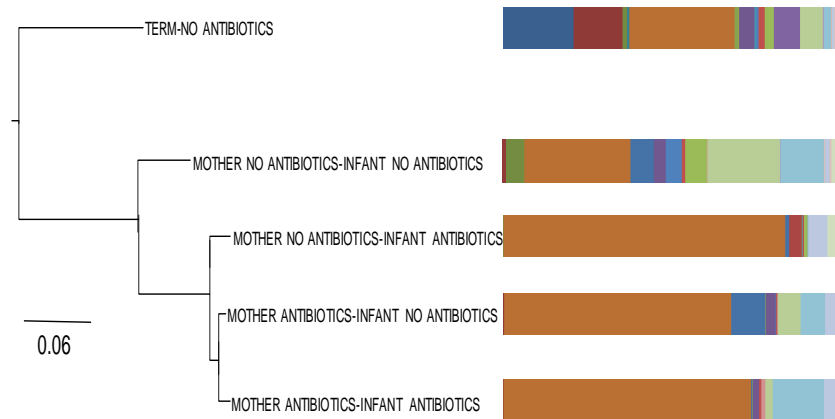
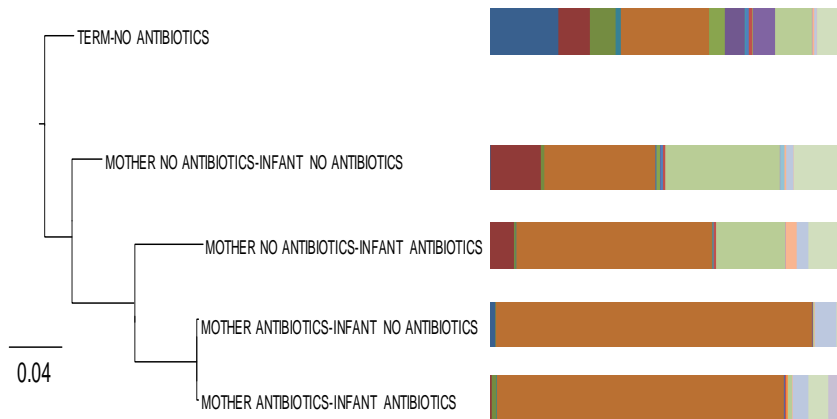
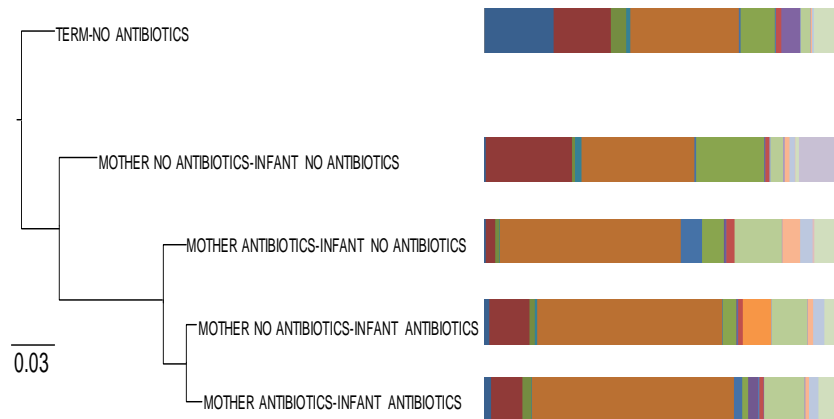


Figure 3[Click here to download Figure: Figure 3.ppt](#)**2 days****10 days****30 days****90 days**

■ Bacteroidaceae

■ Coriobacteriaceae

■ Lachnospiraceae

■ Micrococcaceae

■ Propionibacteriaceae

■ Streptococcaceae

■ Unclassified Gammaproteobacteria

■ Bifidobacteriaceae

■ Enterobacteriaceae

■ Lactobacillaceae

■ Others

■ Pseudomonadaceae

■ Unclassified Actinobacteria

■ Unclassified Lactobacillales

■ Clostridiaceae

■ Enterococcaceae

■ Leuconostocaceae

■ Pasteurellaceae

■ Ruminococcaceae

■ Unclassified Bacilli

■ Veillonellaceae

■ Comamonadaceae

■ Fusobacteriaceae

■ Listeriaceae

■ Porphyromonadaceae

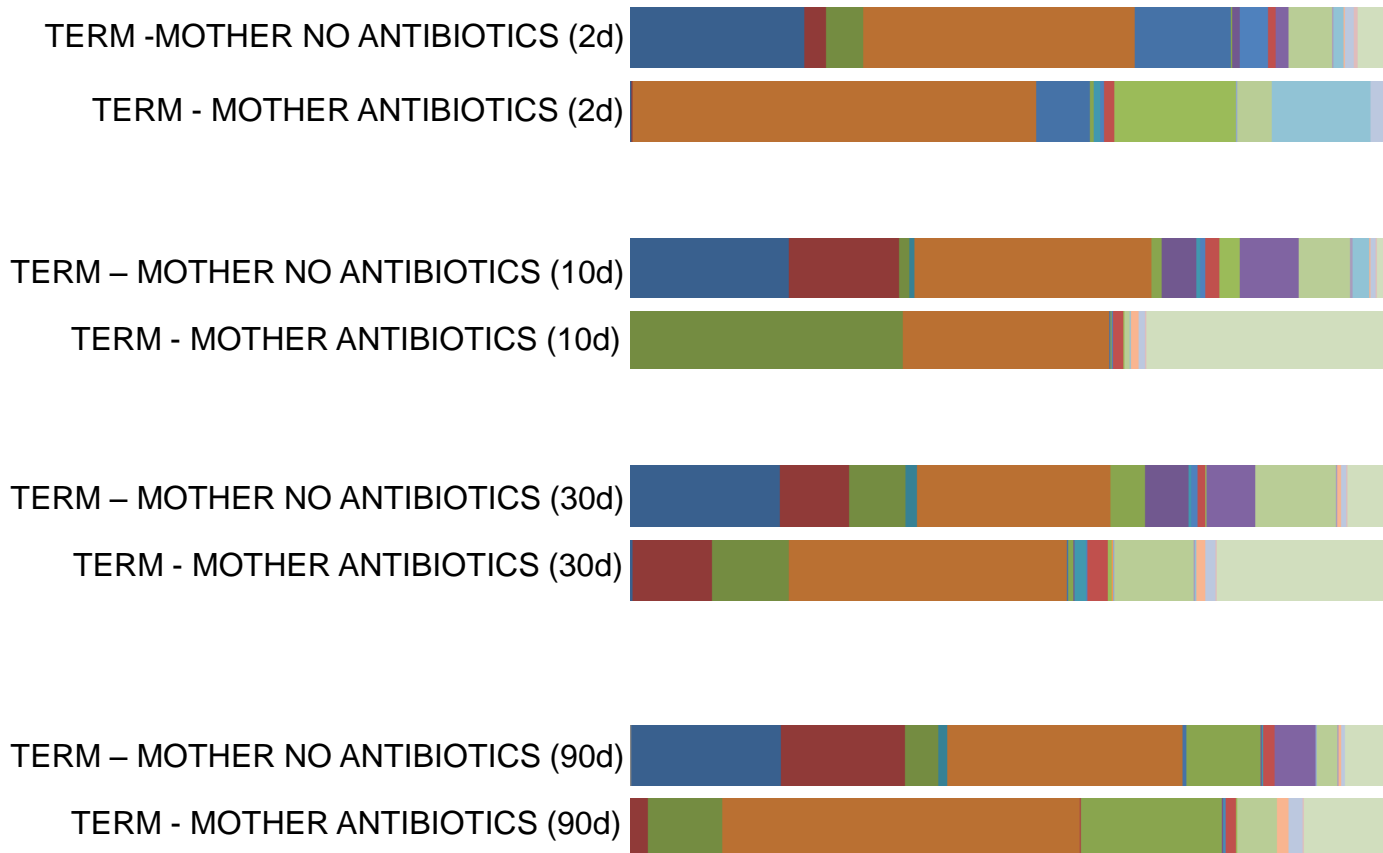
■ Staphylococcaceae

■ Unclassified Clostridiales

■ Verrucomicrobiaceae

Figure 4 online only

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- | | | | |
|------------------------------------|--------------------------------|------------------------|------------------------------|
| ■ Bacteroidaceae | ■ Bifidobacteriaceae | ■ Clostridiaceae | ■ Comamonadaceae |
| ■ Coriobacteriaceae | ■ Enterobacteriaceae | ■ Enterococcaceae | ■ Fusobacteriaceae |
| ■ Lachnospiraceae | ■ Lactobacillaceae | ■ Leuconostocaceae | ■ Listeriaceae |
| ■ Micrococcaceae | ■ Others | ■ Pasteurellaceae | ■ Porphyromonadaceae |
| ■ Propionibacteriaceae | ■ Pseudomonadaceae | ■ Ruminococcaceae | ■ Staphylococcaceae |
| ■ Streptococcaceae | ■ Unclassified Actinobacteria | ■ Unclassified Bacilli | ■ Unclassified Clostridiales |
| ■ Unclassified Gammaproteobacteria | ■ Unclassified Lactobacillales | ■ Veillonellaceae | ■ Verrucomicrobiaceae |