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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.

Original Article.

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

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Abstract

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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.

Introduction

The microbial colonization of the intestine of newborns starts with facultative anaerobes and continues with strict anaerobic genera, with several factors, such as feeding habits or gestational age, affecting this process[1]. This initial microbial colonization results essential for the normal development of the host[2], with the early neonatal period representing the most important moment for the microbiota-induced host-homeostasis[3]. 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 humans[4], 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 available[7-14]. 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 formulas[17]. Preterm infants present an
68 immature immune system[18] and a compromised gut mucosa[19]. 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

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

Methods

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

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

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

Microbiota analyses by Ion Torrent PGM sequencing of 16S rRNA gene-based amplicons. DNA was extracted from fecal samples as previously described[20] and kept frozen at -80ºC until analysis. DNA was PCR-amplified, sequenced in a 316 chip at GenProbio Ltd (www.genprobio.com) by using the Ion Torrent PGM system and the Ion Sequencing 200 kit (Life Technologies) and analysed as recently reported[21] using the QIIME software suite. Quality filtering allowed to retain only full length reads with quality >25 that were used to construct *de novo* OTUs using uclust software and 97% sequence identity as threshold. Reference sequences for each OTUs were identified and used for OTUs taxonomic assignment based on a reference dataset from the Ribosomal Database Project. Hierarchical clustering was constructed using the MeV software and the Pearson's correlation as distance metric.

Quantitative PCR Analysis. For quantification of total fecal bacteria the qPCR conditions and primers described elsewhere[22] were used. Quantification of the different bacterial populations assessed was achieved as previously reported[20].

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

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

Results

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

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

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

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

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

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

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

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

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

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

Discussion

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

Delivery mode and antibiotics treatment are two factors that may affect microbiota composition. In our preterm infants group the delivery mode had a limited effect on gut microbiota composition, in contrast to what has been previously reported for full-term infants[24]. The only difference observed suggested a delayed

colonization by *Bacteroides* during the first days of life in cesarean section (CS) delivered newborns, which has been previously reported in full-term infants[25]. The use of antibiotics may disrupt the neonatal gut microbiota having profound consequences for later health[26]. Indeed, recent animal studies demonstrate that antibiotic-mediated disturbance of the intestinal microbiota in the very early life can increase the risk of late-onset sepsis[27]. Here we have assessed the effect of antibiotics administration, either as IAP to the mother or directly to the infant, upon gut microbiota establishment. Our results indicate an effect of IAP administration. This effect was not immediate as it was hardly detected the first days after delivery but becomes apparent later on. At one month of age infants whose mothers received IAP showed an intestinal microbiota different from that of those infants whose mothers did not receive it. Noteworthy, at this sampling time no statistically significant differences were observed among the four preterm infant groups in background variables such as gestational age, birth-weight or length of hospital stay.

Similarly, effects of IAP administration seem to be also present in full-term infants. Although previous studies did not observe any effect of maternal antibiotics consumption during pregnancy upon infant gut microbiota[28], others evidenced an effect of maternal perinatal antibiotics use on the fecal microbiota of full-term infants[29]. Also with full-term infants, other studies have reported that antibiotics administration during the first hours of life caused a reduced level of intestinal *Bifidobacterium* in the immediate days after administration and increased levels of *Enterobacteriaceae* later on[30,31]. These suggesting a lasting effect of early-life antibiotic administration upon gut microbiota composition, which is in good agreement with our observations. Similarly, incomplete recovery of the gut microbiota after antibiotics administration has been demonstrated in adults[32].

Our results indicate that immaturity affects intestinal microbiota composition and it accounts for some of the differences observed between preterm and FTVDBF infants, such as the reduced percentage of *Bacteroidaceae* during the first months of life or the higher percentage of *Lactobacillaceae* during the first days of life. However, in some other of the differences observed, such as the increased *Enterobacteriaceae* levels in preterm infants, the antibiotics exposure seems to have a role. To this regard, IAP seems to exert a critical influence in the early intestinal microbiota. Therefore, it may be time for considering the potential deleterious effects upon gut microbiota composition when deciding on antibiotic use. According to the data from the Spanish Society for Neonatology (www.se-neonatal.es) in Spain about half of the mothers of VLBW infants receive IAP. Overall, in developed countries IAP is used in over 30% of total deliveries[33]. In spite of not being a clearcut decision pre-partum antibiotics are generally recommended in the case of premature rupture of membranes or when vaginal colonization by group B streptococci is detected, however, they are also frequently used in other clinical situations in which a clear benefit has not been demonstrated[34]. This, together with the high number of infants receiving antibiotics for what later on results to be a noninfectious cause has raised some concerns[35]. In addition, recent data suggest that perinatal antibiotics-mediated microbiota disturbance increases the risk for late-onset sepsis and NEC which may constitute a life threatening risk for preterm newborns[36]. Moreover, very often broad-spectrum antibiotics are used which seems to drastically affect the early gut microbiota establishment process.

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

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

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Figure legends

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

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

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

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

Figure 1

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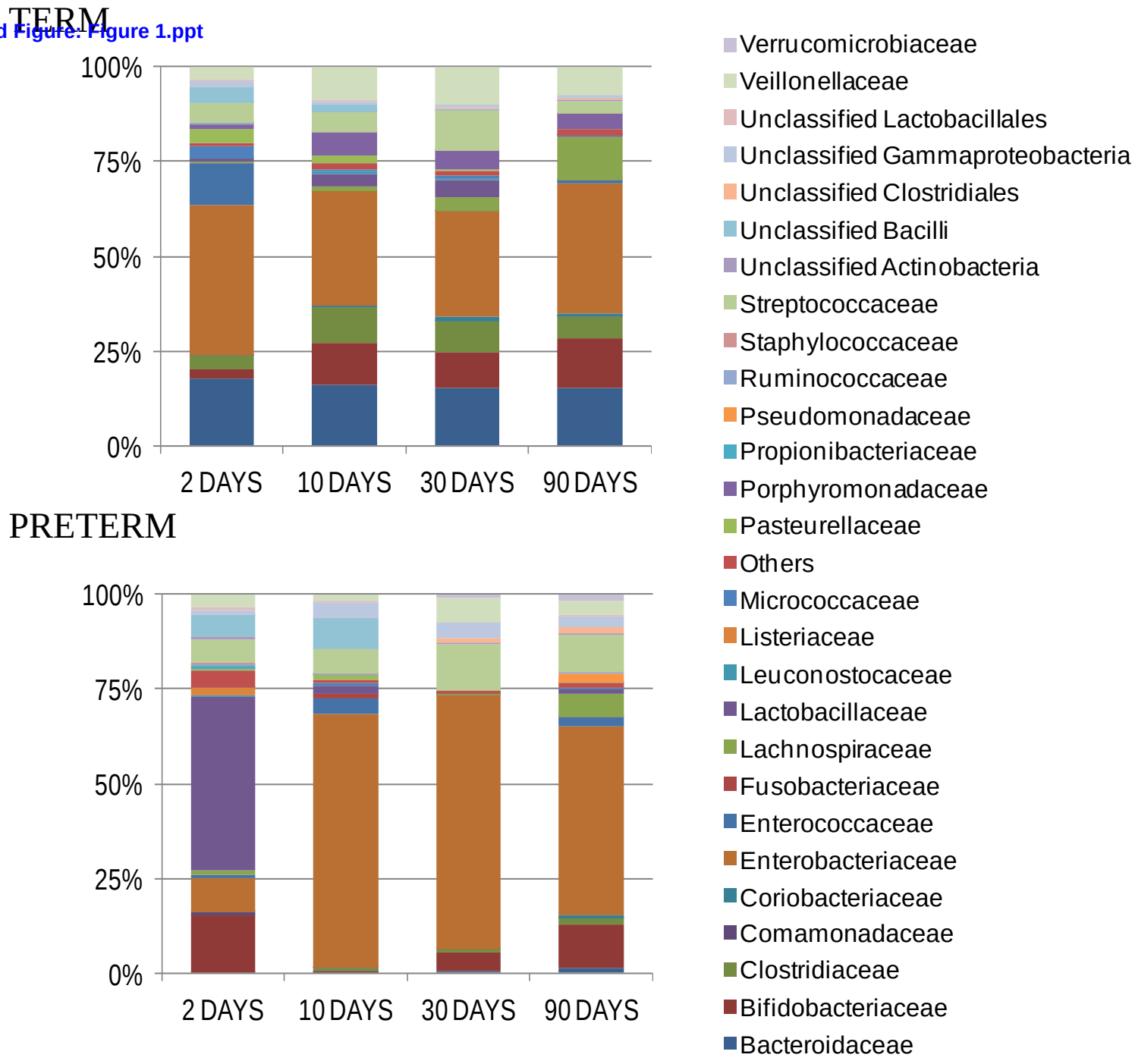

Figure 2 online only

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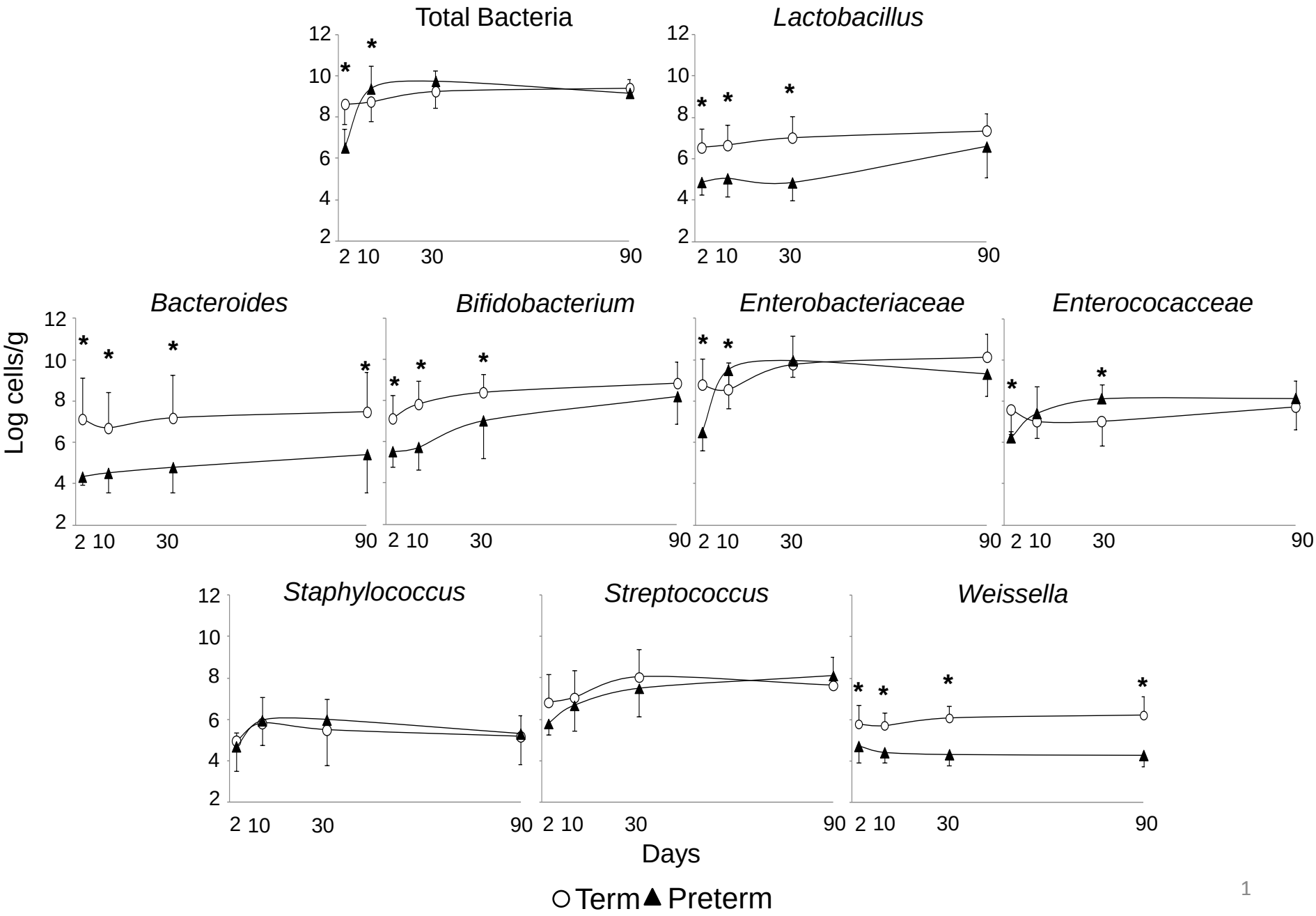

Figure 3

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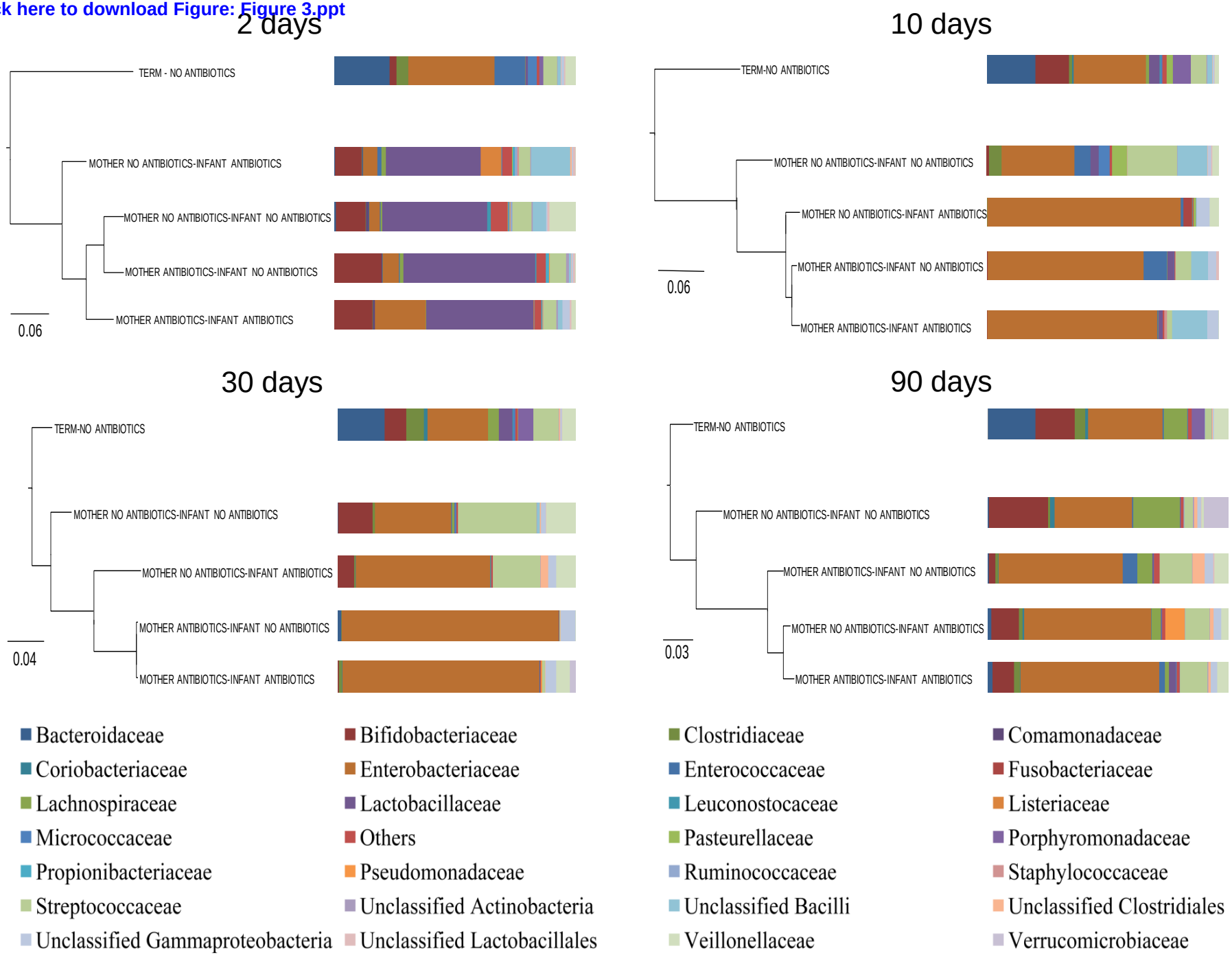

Figure 4 online only

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