Intestinal Microbiota Development in Preterm Neonates and Effect of Perinatal Antibiotics

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Objectives To assess the establishment of the intestinal microbiota in very low birthweight preterm infants and to evaluate the impact of perinatal factors, such as delivery mode and perinatal antibiotics.

Study design We used 16S ribosomal RNA gene sequence-based microbiota analysis and quantitative polymerase chain reaction to evaluate the establishment of the intestinal microbiota. We also evaluated factors affecting the microbiota, during the first 3 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 and by a higher initial percentage of *Lactobacillaceae* in preterm infants compared with full term infants. Perinatal antibiotics, including intrapartum antimicrobial prophylaxis, affects the gut microbiota, as indicated by increased *Enterobacteriaceae* family organisms in the infants.

Conclusions Prematurity and perinatal antibiotic administration strongly affect the initial establishment of microbiota with potential consequences for later health. *(J Pediatr 2015;166:538-44)*.

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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 is essential for the normal development of the host,[2] with the early neonatal period representing the most important opportunity for microbiota-induced host-homeostasis.[3] However, with the exception of delivery mode and feeding habits, the effects of other factors on the process of microbiota development in the newborn remain poorly understood.

Despite high inter-individual variability, metagenomic and 16S ribosomal RNA (rRNA) gene sequencing studies have recently identified the existence of multiple microbiota enterotypes in humans,[4] and microbiota alterations related with diseases have been observed.[5,6] In spite of the importance of the initial steps of microbiota establishment for the later well-being, information for preterm infants has become available only recently.[7-14] The available data indicate that the microbiota of the preterm differs from that of full-term infants, suggesting potential targets for microbiota modulation.

The fecal microbiota profile of the healthy full-term, vaginally delivered, exclusively breast-fed (FTVDBF) infant has been considered to be the standard for a healthy infant microbiota, and recent studies have tried to define its composition.[15,16] Indeed, the promotion of a microbiota resembling that of the FTVDBF infant has been considered as a target for improving infant formulas.[17] Preterm infants have an immature immune system[18] and a compromised gut mucosa.[19] These represent risks for both vertically transmitted infections and late-onset nosocomial infections. The process of microbiota establishment is also affected in the preterm, with an increase of *Enterobacteriaceae*, a delayed colonization by commensal bacteria and a higher colonization by pathogens such as *Klebsiella* sp, than full-term babies.[7-9,20]

Our aim was to assess the establishment of the intestinal microbiota in very low birthweight (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.

FTVDBF	Full-term, vaginally delivered, exclusively breast-fed
IAP	Intrapartum antimicrobial prophylaxis
NEC	Necrotizing enterocolitis
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
rRNA	Ribosomal RNA
VLBW	Very low birthweight

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Methods

The study was approved by the Regional Ethical Committee of the Servicio de Salud Pública del Principado de Asturias, and informed written consent was obtained from the parents. Thirteen Caucasian FTVDBF infants, (7 males/6 females) born after uncomplicated pregnancy, and 27 Caucasian VLBW preterm infants (12 males/15 females) were recruited at the Neonatology Units of Cabueñes Hospital and Central University Hospital from Asturias (Spain). All full-term infants were vaginally delivered, at gestational ages between 37 and 41 weeks (mean 39.2) and with birth weights between 3020 and 4160 g. The term infants were exclusively breast-fed and were discharged from the hospital by the third day of life. Preterm infants (7 delivered vaginally and 20 by cesarean delivery) were born at gestational ages between 24 and 32 weeks (mean 29.6) with birthweights that ranged between 690 and 1800 g. None of the infants had NEC or culture positive early onset infection. None of the full-term infants received antibiotics, but 3 mothers received intrapartum antimicrobial prophylaxis (IAP) with a single dose of ampicillin. Fourteen of the preterm infants' mothers received IAP. One mother received a single dose of penicillin, and other mother received 1 dosage of ampicillin every 6 hours for 3 days. The other mothers received ampicillin plus erythromycin (between 2 and 24 doses of each antibiotic). Twelve infants received ampicillin plus gentamicin for 5-8 days after birth. The 5 other infants received antibiotics starting at 10-13 days of life (3 received vancomycin plus amikacin, 1 received vancomycin, and 1 received gentamycin plus clindamycin plus teichomycin). Only 5 of the 27 mother/premature infant pairs did not receive antibiotics, either intrapartum or postnatally during the sampling period, and in 9 of the mother and infant pairs received antibiotics. All preterm infants received mixed feeding (infant formula and some breast-milk administration during the study). The preterm infants were discharged from the hospital after an average hospital stay of 50 (range 21-93) days.

Fecal samples were collected at the hospital between 24 and 48 hours of life and at 10, 30, and 90 days of age. The first spontaneous or stimulated stool was collected in a sterile container, immediately frozen at −20°C and sent to the laboratory within a week.

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 −80°C until analysis. DNA was polymerase chain reaction (PCR)-amplified, sequenced with a 316 chip at GenProbio Ltd (www.genprobio.com) by using the Ion Torrent PGM system and the Ion Sequencing 200 kit (Life Technologies, Guilford, California) and analyzed as recently reported[21] using the QIIME software suite (http://qiime.org/). Quality filtering retained only full length reads with quality >25 that were used to construct de novo operational taxonomic units (OTU) using UCLUST software with 97% sequence identity as the 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 multi-experiment viewer software and the Pearson correlation as distance metric.

The raw sequences from the samples have been deposited in the Nacional Center for Biotechnology Information Short Read Archive under the BioProject ID code PRJNA230470.

Quantitative PCR Analysis

Quantification of total fecal bacteria was achieved by quantitative PCR (qPCR) conditions and primers described elsewhere.[22] Quantification of the different bacterial populations was as previously reported.[20]

Statistical Analyses

Results were analyzed using SPSS software (SPSS Inc, Chicago, Illinois). The normality of the data, at each sampling point, was checked using the Kolmogorov-Smirnov test. For normal variables, 1-way ANOVA followed by post-hoc Bonferroni test was used. Some of the bacterial groups had non-normal distribution, and the differences between groups of infants were analyzed using the nonparametric Kruskal-Wallis test or, in the case of pairwise comparisons, the Mann-Whitney U-test.

Results

Establishment of Intestinal Microbiota in VLBW Preterm Neonates Compared with FTVDBF Infants

Ion Torrent sequencing of the PCR products for amplification of the V3-V4 region of the 16S rRNA gene from the 160 fecal samples yielded, after filtering, about $\sim 10^5$ 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-day old preterm newborns had significantly ($P < .05$) lower proportions of the families *Bacteroidaceae*, *Clostridiaceae*, *Micrococcaceae*, *Pasteurellaceae*, and *Porphyromonadaceae* and higher ($P < .05$) *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 had a significant reduction ($P < .05$) in the percentage of *Bacteroidaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae*, *Leuconostocaceae*, *Pasteurellaceae*, *Porphyromonadaceae*, unclassified Actinobacteria, and *Veillonellaceae*, and a higher percentage ($P < .05$) of the families *Enterobacteriaceae*, *Micrococcaceae*, and unclassified Gammaproteobacteria than FTVDBF babies. This distribution remained relatively stable during the rest of the study, with significantly ($P < .05$) higher proportions of *Enterobacteriaceae* and significantly ($P < .05$) lower of *Bacteroidaceae* in preterm infants at 30 and 90 days of age.