RESEARCH ARTICLE

Establishment and development of intestinal microbiota in preterm neonates

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Abstract

Microbial colonization of the infant gut is essential for the development of the intestine and the immune system. The profile of intestinal microbiota in the full-term, vaginally delivered, breast-fed infant is considered as ideally healthy. However, in preterm infants this process is challenging, mainly because of organ immaturity, antibiotics use, and hospital stay. To assist in a proper microbiota development in these infants, a detailed knowledge of the colonization process, and the differences from that of full-term breast-fed infants, is needed. We assessed the establishment of the gut microbiota and its metabolic activity in preterm neonates ($n = 21$) during the first 3 months of life and compared it with that of vaginally delivered, exclusively breast-fed full-term infants ($n = 20$) using qualitative and quantitative culture-independent methods. Differences in the gut microbiota composition between both groups were observed. Preterm infants showed higher levels of facultative anaerobic microorganisms and reduced levels of strict anaerobes such as *Bifidobacterium*, *Bacteroides*, and *Atopobium*. Short-chain fatty acids concentrations were lower in preterm infants during the first days of life. Alterations occur in the process of microbiota establishment in preterm infants, indicating the need for intervention strategies to counteract them.

Introduction

Microbial colonization of the digestive tract starts at birth, providing a massive microbial challenge for the maturation of the immune system of the newborn. This colonization of the gut is also essential for a normal development of the intestine, playing a key role in the establishment of intestinal homeostasis and mucosal barrier function (Hooper & Macpherson, 2010). A healthy microbiota has been defined as the intestinal microbial community that assists the host to maintain a healthy status under certain environmental conditions (Peso Echarri *et al.*, 2011). In the case of neonates, the healthy microbiota would be that microbial composition which leads to a reduced risk of infection and provides appropriate signals for gut and immune maturation. In this context, the early establishment of a healthy microbiota would have a profound effect on the future well-being of the individual (Conroy *et al.*, 2009).

Gut colonization of the newborn begins with facultative anaerobes, such as enterobacteria and streptococci and continues with anaerobic genera, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* (Mackie *et al.*, 1999; Fanaro *et al.*, 2003). Several factors may affect this process; among them, the mode of delivery and feeding habits have been extensively studied (Penders *et al.*, 2006; Reid *et al.*, 2011). Breast-milk is known to play an important role in the establishment of the intestinal microbiota, with bifidobacteria constituting the most characteristic microbial group in breast-fed infants. Recent studies have demonstrated a high specialization of the typical infant's bifidobacterial species, such as *Bifidobacterium longum* ssp. *infantis*, for the use of the oligosaccharides present in human milk (Sela & Mills, 2010). Moreover, different

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studies have evidenced a beneficial and protective role of breast-feeding (Ip *et al.*, 2009). These have led to the consideration of the fecal microbiota profile of the healthy full-term, vaginally delivered, exclusively breast-fed (FTVDBF) infant as the standard for a healthy infant microbiota, and recent studies have tried to define its composition (Karlsson *et al.*, 2011; Peso Echarri *et al.*, 2011). Indeed, the promotion of a microbiota resembling that of the FTVDBF infant has often been considered as a target for improving the functionality of infant formulas (Aggett *et al.*, 2003), and microbiota targets for long-term health have been identified in the neonatal gut (Conroy *et al.*, 2009).

Most of the studies on the microbiota establishment process have focused on full-term infants. However, in preterm newborns, this process is more challenging, because of organ immaturity, the frequent use of antibiotics and the stay at the hospital's neonatal unit instead of a home setting. These infants would benefit from intervention strategies directed at favoring the establishment of a healthy microbiota. To develop such strategies, a detailed knowledge of both the microbiota establishment process in preterm neonates and how this process differs from the potentially healthy model, that of FTVDBF babies, is needed.

Traditional plate-counting methods have indicated an altered microbial colonization pattern in the gut of preterm infants (Fanaro *et al.*, 2003; Westerbeek *et al.*, 2006). More recently, qualitative culture-independent studies (mainly PCR-DGGE/TGGE analyses) have been carried out in preterm babies, especially in very-low-birthweight neonates, showing a reduced diversity of the intestinal microbiota, likely acquired from the hospital environment (Schwiertz *et al.*, 2003; Magne *et al.*, 2006; Roudiere *et al.*, 2009; Rouge *et al.*, 2010; Jacquot *et al.*, 2011) and suggesting the gestational age as a critical factor for colonization by *Bifidobacterium* (Butel *et al.*, 2007). Sequencing of amplified 16S rRNA genes has also been carried out (Magne *et al.*, 2006; Wang *et al.*, 2009; Mshvildadze *et al.*, 2010; Morowitz *et al.*, 2011), indicating a high relative abundance of the phylum Proteobacteria, mainly represented by the family *Enterobacteriaceae*, as well as the families *Enterococcaceae*, *Streptococcaceae*, and *Staphylococcaceae*, with a high representation of potentially pathogenic microorganisms such as *Klebsiella*, *Weissella*, or *Clostridium*. During the last year, some metagenomic studies on preterm infant microbiota also became available (Morowitz *et al.*, 2011). In general, a delay in the appearance of commensal bacteria and an increased colonization by potentially pathogenic microorganisms has been reported in these infants. However, the techniques used in such studies were not truly quantitative, and therefore, a quantitative description of the process of microbiota establishment in the preterm infant is still needed.

The aim of the present study was to evaluate the process of establishment of the intestinal microbiota in preterm infants, and compare it to that of FTVDBF healthy neonates, using both qualitative and quantitative culture-independent techniques and by evaluating the metabolic activity of this microbiota. To this end, the levels of 18 microbial groups previously reported to be present in feces of preterm infants (Westerbeek *et al.*, 2006; Wang *et al.*, 2009; Mshvildadze *et al.*, 2010; Rouge *et al.*, 2010), as well as some potentially pathogenic microorganisms, were determined by quantitative PCR (qPCR). Bacterial diversity was assessed using PCR-DGGE. In addition, the concentration of short-chain fatty acids (SCFA) was determined by gas chromatography as a measure of metabolic activity.

Materials and methods

Volunteers and samples

Twenty healthy Caucasian FTVDBF infants (11 males/9 females) born after an uncomplicated pregnancy, and 21 Caucasian preterm infants (9 males/12 females) were recruited at the Neonatology Unit of Cabueñes Hospital in Asturias (Northern Spain). All full-term infants were vaginally delivered, at a gestational age ranging between 38 and 41 weeks (mean 39.3) with birthweights between 3020 and 4160 g, were exclusively breast-fed during the period considered for the study, and were not exposed to antibiotics pre-, peri-, or postnatally. All these infants were discharged from the hospital at the third day of life. The preterm infants (eight delivered vaginally and 13 by cesarean section) were born at a gestational age between 30 and 35 weeks (mean 32.7) and their birthweights ranged between 1190 and 2820 g. Six of the preterm infants' mothers received intrapartum antibiotics (three cases of ampicillin, two cases of ampicillin plus erythromicin, and one case of clindamycin), and six of the infants received antibiotics at birth (all of them received ampicillin plus gentamicin during between 5 and 8 days). Only 10 of the 21 mother/premature infant pairs did not receive antibiotics, either intrapartum or postnatally, during the sampling period. All preterm infants received mixed feeding and were discharged from the hospital after an average of 24 days of hospitalization.

Fecal samples were collected at the hospital at 2 (between 24 and 48 h after birth), 10, 30, and 90 days of age. When infants were not interned at the neonatal unit, the pediatrician scheduled appointments at the hospital for sampling. The first spontaneous or stimulated (after perianal stimulation) deposition was taken in a sterile

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container and immediately frozen at −20 °C. Fecal samples were sent within a week to the laboratory where they were stored at −80 °C until DNA extraction.

The study was approved by the Regional Ethical Committee of Asturias Public Health Service (SESPA), and an informed written consent was obtained from each mother.

DNA extraction

Fecal samples were melted, weighed, diluted 1/10 in sterile PBS solution, and homogenized in a LabBlender 400 stomacher (Seward Medical, London, UK) at full-speed for 4 min. DNA was extracted from the homogenized feces, as well as from bacterial cultures used for standard curves, using the QIAamp DNA stool kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's specifications as previously described (Gueimonde *et al.*, 2004). The extracted DNA was kept frozen at −70 °C until analysis.

Fecal microbiota analyses

Quantitative analysis of fecal microbiota by PCR

Quantification of the different bacterial populations in feces was carried out by qPCR using the primers shown in Table 1. All reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA) in a 7500 Fast Real Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). One microlitre of template fecal DNA (~ 5 ng) and 0.2 μM of each primer were used in the 25-μL PCR. Thermal cycling consisted of an initial cycle of 95 °C 10 min, followed by 40 cycles of 95 °C 15 s, and 1 min at the appropriate primer-pair temperature (Table 1). Specificity of the designed *Weissella* primers was tested against an array of intestinal microorganisms belonging to different genera. In the negative samples, the value of the detection limits obtained for the corresponding primer pair was assigned (ranging between 10^3 and 10^4 cells g^{-1} depending on the bacterial group). Standard curves were made with pure cultures of appropriate strains (Table 1), which were grown overnight in GAM medium (Nissui Pharmaceutical Co, Tokyo, Japan) under anaerobic conditions. Samples were analyzed in duplicates in at least two independent PCR runs.

Qualitative analysis of fecal microbiota by PCR-DGGE

The qualitative profile of the microbiota in feces at the different sampling points was determined by PCR-DGGE in a total of 16 randomly selected infants, eight from each group. Universal primers (F-968 GAACGCGAAGAACCT-TAC and R-1401 CGGTGTGTACAAGACCC) were used to assess microbial diversity, which was defined by the number of amplification bands generated from each sample, and PCR products were separated by DGGE in a DCode system (BioRad Laboratories) using conditions previously described (Salazar *et al.*, 2011). The number of bands in each sample was visually determined. Samples were analyzed in duplicates in at least two independent PCR-DGGE.

Determination of SCFAs in feces

The analysis of SCFA was carried out as follows. Supernatants from 1 mL of the homogenized feces were obtained by centrifugation (10 000 **g**, 30 min, 4 °C) and filtration (0.20 μm). A chromatographic system composed of two 6890N GC (Agilent Technologies Inc., Palo Alto, CA) connected to an FID and an MS 5973N detector (Agilent) was used for quantification and identification of SCFAs as described previously (Salazar *et al.*, 2011).

Statistical analysis

Results were analyzed using the SPSS software (SPSS Inc., Chicago, IL). The normality of the data, at each sampling point, was checked using the Kolmogorov-Smirnov test. Some of the bacterial groups showed non-normal distribution, and therefore, differences in bacterial levels between groups of infants were analyzed using nonparametric tests (Mann–Whitney *U*-test). The occurrence of different microbial groups between preterm and full-term infants was analyzed by the chi-square test.

Results

Microbial population dynamics and development of the newborn's intestinal microbiota

In spite of the high interindividual variability observed, noticeable qualitative and quantitative differences were found in the intestinal microbiota composition between premature and healthy FTVDBF babies.

Microorganisms from families *Enterobacteriaceae* and *Enterococcaceae*, the genera *Bifidobacterium*, the *Bacteroides* and *Lactobacillus* groups, as well as *Weissella* were detected in all infant fecal samples, either from full-term or premature infants, throughout the entire sampling period considered in this work. Against this, microorganisms belonging to the genus *Shigella* and to the species *Staphylococcus aureus* were never detected, while *Akkermansia*

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Table 1. Bacterial groups, standard cultures, primers, and annealing temperatures used for qPCR in this study

Microbial target	Strain used for standard curves	Primer sequence 5′–3′	Tm (°C)	Target gene	Reference
Akkermansia	*Akkermansia muciniphila* CIP107961	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	60	16S	Collado *et al.* (2007)
Atopobium group *Atopobium – Collinsella*	*Collinsella intestinalis* DSMZ13280	F: GGGTTGAGAGACCGACC R: CGGRGCTTCTTCTGCAGG	55	16S	Matsuki *et al.* (2004)
Bacteroides group *Bacteroides – Prevotella – Porphiromonas*	*Bacteroides thetaiotaomicron* DSMZ2079	F: GAGAGGAAGGTCCCCCAC R: CGCKACTTGGCTGGTTCAG	60	16S	Peso Echarri *et al.* (2011)
Bifidobacterium	*Bifidobacterium longum* NCIMB8809	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	60	16S	Gueimonde *et al.* (2004)
Clostridia IV *Clostridium leptum – Faecalibacterium prausnitzii*	*Clostridium leptum* DSMZ753	F: TTAACACAATAAGTWATCCACCTGG R: ACCTTCCTCCGTTTTGTCAAC	60	16S	Ramirez-Farias *et al.* (2009)
Clostridia XIVa *Blautia coccoides – Eubacterium rectale*	*Blautia coccoides* DSMZ935	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	55	16S	Rinttila *et al.* (2004)
Clostridium difficile	*Clostridium difficile* JCM1296	F: TTGAGCGATTTACTTCGGTAAAGA R: CCATCCTGTACTGGCTCACCT	58	16S	Rinttila *et al.* (2004)
Clostridium perfringens	*Clostridium perfringens* IPLA531	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	60	16S	Rinttila *et al.* (2004)
Desulfovibrio	*Desulfovibrio intestinalis* DSMZ11275	F: CCGTAGATATCTGGAGGAACATCA R: ACATCTAGCATCCATCGTTTACAGC	62	16S	Fite *et al.* (2004)
Enterobacteriaceae	*Escherichia coli* LMG2092	F: TGCCGTAACTTCGGGAGAAGGCA R: TCAAGGACCAGTGTTCAGTGTC	60	23S	Matsuda *et al.* (2007)
Enterococcaceae	*Enterococcus faecalis* IPLAIF3/1	F: CCCATCAGAAGGGGATAACACTT R: ACCGCGGGTCCATCCATC	60	16S	Matsuda *et al.* (2007)
Klebsiella pneumoniae	*Klebsiella pneumoniae* subsp. *pneumoniae* CECT143	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC	57	16-23S	Liu *et al.* (2008)
Lactobacillus group *Lactobacillus-Weissella*	*Lactobacillus gasseri* IPLAIF7/5	F: AGCAGTAGGGAATCTTCCA R: CATGGAGTTCCACTGTCCTC	60	16S	Peso Echarri *et al.* (2011)
Shigella	*Shigella sonnei* CECT4887	F: ACCATGCTCGCAGAGAAACT R: TACGCTTCAGTACAGCATGC	60	*ipaH*	Lin *et al.* (2010)
Staphylococcus	*Staphylococcus epidermidis* IPLAIF1/6	F: ACGGTCTTGCTGTCACTTATA R: TACACATATGTTCTTCCCTAATAA	60	16S	Matsuda *et al.* (2007)
Staphylococcus aureus	*Staphylococcus aureus* IPLA SA	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	55	*nuc*	Fang & Hedin (2003)
Streptococcus	*Streptococcus salivarius* IPLABM7/1	F: GTACAGTTGCTTCAGGACGTATC R: ACGTTCGATTTCATCACGTTG	60	*tuf*	Picard *et al.* (2004)
Weissella	*Weissella confusa UI6*	F: CGTGGGAAACCTACCTCTTAGCAG R: GACCATCTCTTAGTGATAGCAGAACCAT	62	16S	This study

was only found in about 5% of the infants' feces (6% of full-term and 5% of preterm infants) at 2 days of age, not being detected at later times. With respect to the other microorganisms analyzed, their presence varied depending on the infant group and sampling point (Table 2). Notably, during the first few days of life, the potentially pathogenic microorganism *Klebsiella pneumoniae* was more frequently detected ($P < 0.05$) in preterm infants than in full-term ones. Moreover, *Clostridium difficile* was only detected (although with a low frequency) in fecal samples from premature babies. The occurrence of *Streptococcus* at 2 days of age was significantly lower ($P < 0.05$) in preterm infants; at 10 days of age, the occurrence of *Clostridium* XIVa group, *Atopobium*, and *Staphylococcus* was also lower in fecal samples from these babies while that of *Desulfovibrio* was significantly higher ($P < 0.05$). At 1 month of life, positive samples for *Clostridium perfringens* and *Atopobium* were significantly higher and lower ($P < 0.05$), respectively, in premature than in full-term infants. All these qualitative differences

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Table 2. Occurrences (%) of the bacterial groups analyzed showing variability between full-term (T) and premature (P) infant groups

		Microorganisms								
Age	Group	Cl. IV	Cl. XIVa	Atop.	Strept.	Staph.	K. pne.	C. perfr.	Desul.	C. diff.
2 days	T	35.3	29.4	5.9	70.6	47.1	47.1	29.4	35.3	0.0
	P	38.1	9.5	9.5	19.0**	19.0	100.0***	47.6	33.3	0.0
10 days	T	68.4	70.0	47.1	78.6	89.5	57.1	57.1	28.6	0.0
	P	76.2	38.1*	14.3*	71.4	52.4**	100.0**	57.1	66.7*	4.8
30 days	T	72.2	78.9	62.5	92.3	66.7	84.6	46.2	23.1	0.0
	P	68.4	57.9	26.3*	89.5	57.9	100.0	89.5**	42.1	21.1
90 days	T	66.7	100.0	81.3	84.6	66.7	84.6	76.9	30.8	0.0
	P	88.2	88.2	58.8	88.2	47.1	100.0	82.4	35.3	11.8

Cl. IV, *Clostridium* cluster IV; Cl. XIVa, *Clostridium* cluster XIVa; Atop, *Atopobium* group; Strept, *Streptococcus*; Staph, *Staphylococcus*; K. pne, *Klebsiella pneumoniae*; C. perfr, *Clostridium perfringens*; Desul, *Desulfovibrio*; C. diff, *Clostridium difficile*.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

in the detection of the different microorganisms analyzed were attenuated over time, ceasing to be significant by the age of 3 months (Table 2).

In general, the quantitative levels of most microbial groups tended to increase over time (Fig. 1). Notable differences in bacterial levels were observed between both groups of infants. Feces from preterm newborns showed higher levels ($P < 0.05$) of *Enterococcaceae* and of *Lactobacillus* group, and more specifically within this group for the genus *Weissella*, during the period of 3 months sampled. The levels of *Enterobacteriaceae*, *K. pneumoniae*, and *Desulfovibrio* were also significantly higher in feces from preterm babies at the first sampling points (no later than 30 days) (Fig. 1a and b). On the other hand, premature infants showed significantly lower levels of *Streptococcus* and *Staphylococcus* in the initial sampling points, and of *Bifidobacterium*, *Bacteroides*, and *Atopobium* during the whole sampling period under study (Fig. 1a).

Although the number of preterm infants may not be large enough for within-group comparisons with appropriate statistical power, we compared the levels of different microorganisms between vaginally (8 of 21 infants) and cesarean delivered preterm neonates without obtaining statistically significant differences at any sampling point for any microbial group. When the levels of microorganisms in feces of preterm infants receiving antibiotics, or in feces of those whose mothers have received intrapartum antibiotics, were compared with those of preterm infants not exposed to antibiotics (10 of 21 infants), the sole statistically significant difference ($P < 0.05$) refers to the lower levels of *Bifidobacterium* found at 10 days of age in the former group, without evidencing any other differences for other microorganisms along the time points considered in this study.

Bacterial diversity determination, as assessed by the number of bands produced by PCR-DGGE analyses, showed lower diversity in fecal microbiota of preterm infants than in the full-term group during the first 3 months of life, the differences being significant ($P < 0.05$) at 2 and 30 days of age (Fig. 2).

Metabolic activity of the intestinal microbiota of newborns

The metabolic activity of the intestinal microbiota was determined by measuring the SCFA concentration in feces. Total SCFA (results not shown), as well as the main SCFA (acetate, propionate, and butyrate) concentration in feces increased over time in both groups of infants (Fig. 3). Despite the high interindividual variation, lower levels of total SCFA, and more specifically of acetate and propionate, were observed in preterm infants ($P < 0.01$ for acetate at 2 days and for propionate at 2 and 10 days of age) (Fig. 3). Nevertheless, when the relative abundance of each of the main SCFA was calculated, no differences ($P > 0.05$) were observed between both groups of infants.

Discussion

Our results showed an altered intestinal colonization by commensal microorganisms, increased occurrence of potential pathogens, as well as high interindividual variability and reduced microbial diversity in preterm infants, thus confirming previous reports (Schwiertz *et al.*, 2003; Magne *et al.*, 2006; Rouge *et al.*, 2010; Jacquot *et al.*, 2011). In addition, our study extends these observations to the quantitative levels of different bacterial groups.

Despite the high interindividual variability in bacterial levels, significant differences were found between preterm and full-term infants for several microbial groups. Delivery mode and antibiotics consumption are two factors that may affect microbiota composition. Although the limited number of infants does not allow for establishing

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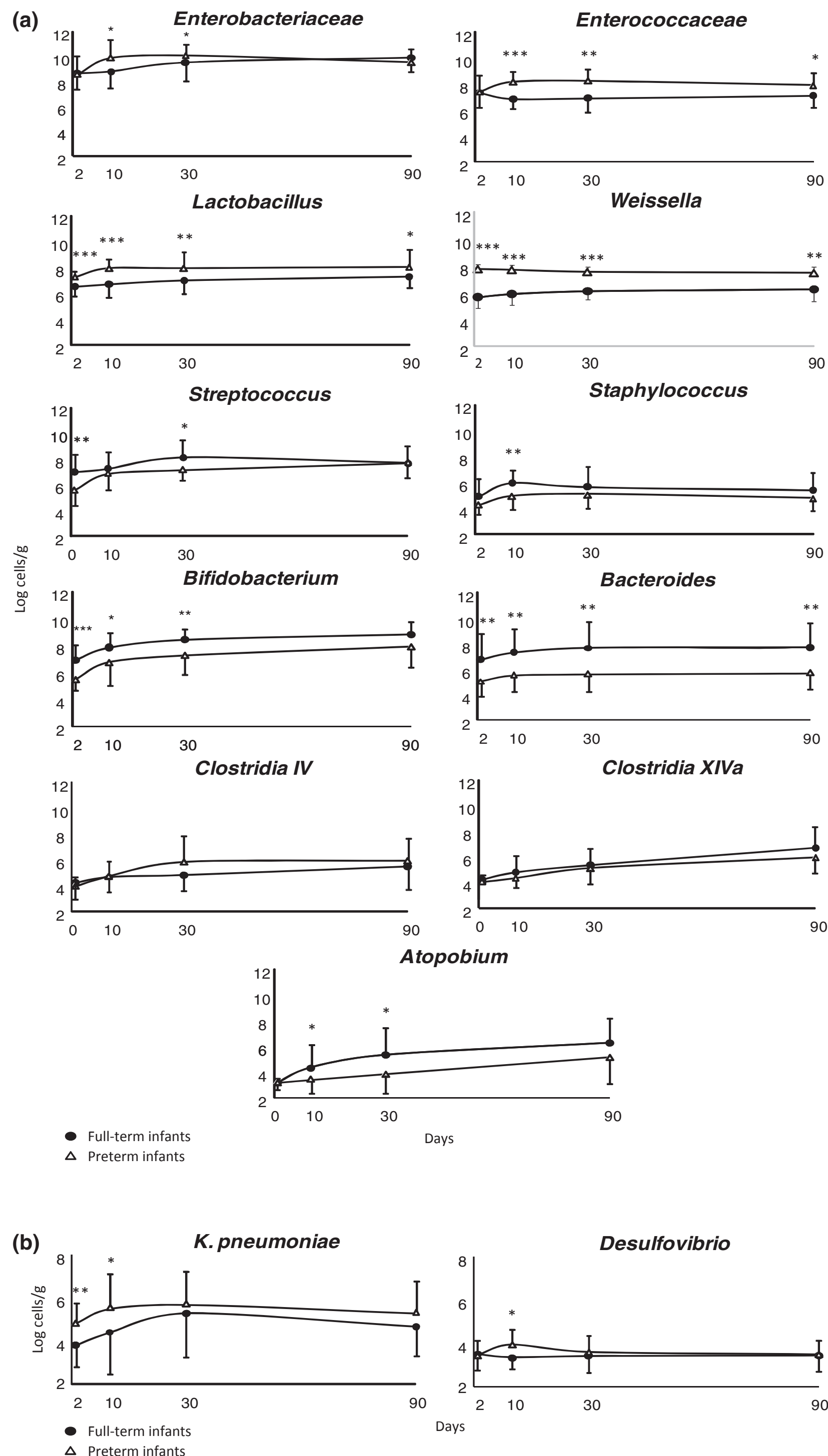

Fig. 1. Fecal levels (mean ± SD) of the different microorganisms analyzed by qPCR. (a) *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillus* group, *Weissella*, *Streptococcus*, *Staphylococcus*, *Bifidobacterium*, *Bacteroides* group, *Clostridium* IV group, *Clostridium* XIVa group, and *Atopobium* group. (b) *Klebsiella pneumoniae* and *Desulfovibrio*. Asterisks indicate statistically significant differences at the corresponding sampling time. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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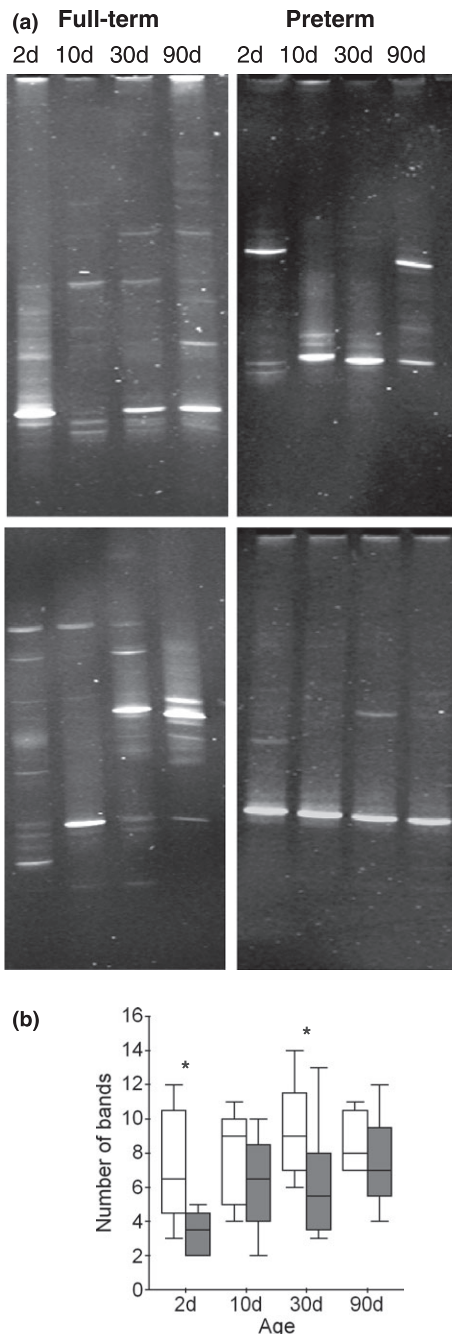

Fig. 2. (a) DGGE gels obtained for two representative infants from each group (full-term and preterm) at the different time points analyzed (2, 10, 30, and 90 days). (b) Microbial fecal diversity as measured by the number of bands obtained by PCR-DGGE analyses at the different time points. White boxes, full-term infants; gray boxes, premature infants. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

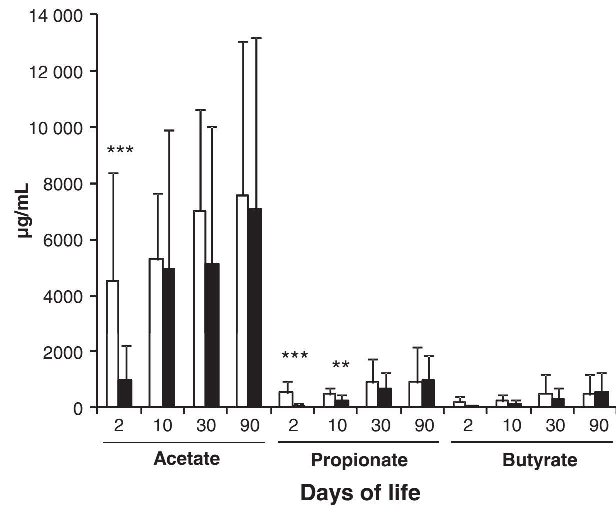

Fig. 3. Concentration (mean ± SD) of the main SCFAs (acetate, propionate, and butyrate) in fecal samples from both full-term (white columns) and premature (black columns) infants at the different sampling points analyzed (2, 10, 30, and 90 days of age). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

firm conclusions, within our preterm group delivery mode did not affect bacterial levels. It is known that the use of antibiotics may influence gut microbiota composition. In our study, antibiotic administration, intrapartum or to the infant, resulted in a reduction of *Bifidobacterium* levels at 10 days of age, whereas this fact does not seem to influence levels of the other microbial groups tested. These results suggest that immaturity itself may affect microbiota composition, and it may account for some of the differences observed. However, the effect of other potential confounding factors, often related to prematurity such as diet and hospital environment, cannot be ruled out.

We observed increased levels of facultative anaerobic microorganisms, such as *Enterobacteriaceae*, *Enterococcaceae*, and *Lactobacillus* group (including *Weissella*) in preterm neonates, together with reduced levels of anaerobes, including *Bifidobacterium*, *Bacteroides*, and *Atopobium*, which seems to indicate an alteration in the establishment of the normal anaerobic gut microbiota. This fact may lead to a delayed maturation of the immune system (Kelly *et al.*, 2007), which may have profound effects on the health of the immature preterm infant because of, among other factors, an increased risk of infection.

Enterobacteriaceae and *Enterococcaceae* were the predominant microbial groups in preterm infants, these babies also showing reduced levels of bifidobacteria. This confirms previous studies carried out using other techniques (Hoy *et al.*, 2000; Magne *et al.*, 2006). Interestingly, in our study, the microorganisms targeted with the *Lactobacillus*-group primers seem to have a predominant role on the preterm infant gut microbiota. The observation of higher levels of these microorganisms in preterm

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infants is striking, as a lower colonization by lactobacilli has been previously reported (Hall *et al.*, 1990). These differences may partially account for the different methodologies used, traditional culture by Hall and co-workers vs. qPCR in our case. However, it is also important to underline that the primers used by us do amplify not only lactobacilli but also related microorganisms from the genus *Weissella*. For this reason, we decided to quantify specifically the levels of microorganism belonging to this genus; we found that *Weissella* appeared to account for most of the observed differences, being present at higher levels in preterm than in FTVDBF neonates.

In addition to the above-mentioned differences, preterm neonates showed an increased occurrence of other potentially pathogenic microorganisms. Occurrence and levels of *K. pneumoniae* in these infants were significantly higher than those found in FTVDBF babies during the first days of life, which may result in an increased risk of infection by this microorganism. In this regard, *K. pneumoniae* has been previously reported to be often present on the preterm infant microbiota (Hoy *et al.*, 2000; Schwiertz *et al.*, 2003). Although the differences in our case did not reach statistical significance, likely due to the low incidence of the microorganism, it is interesting to mention that *C. difficile* was detected exclusively in preterm infants and not in the feces of FTVDBF control babies. Recently, using a qualitative culture-independent technique, it has been shown that colonization by *C. difficile* may be related to the microbiota composition. Infants harboring this microorganism were found to be more frequently colonized by *K. pneumoniae* and less often by *Bifidobacterium longum* and *Staphylococcus epidermidis* (Rousseau *et al.*, 2011). To this respect, our preterm infants showed lower levels of the later two microorganisms and higher levels of the former, which may at least partially explain the appearance of *C. difficile* only in these infants and not in the control FTVDBF group.

As expected from the differences observed in the gut microbiota composition, the concentration of fecal SCFA also showed differences between both infant groups, being higher in full-term infants. The lower levels of fecal SCFA obtained in premature infants may be related to the use of antibiotics (Szylit *et al.*, 1998). Gestational age has also been related to SCFA levels as reported by other authors, and the levels being lower in extremely preterm neonates (Favre *et al.*, 2002). The heterogeneity in the gestational age of our premature infants (between 30 and 35 weeks) may, thus, partially account for the interindividual variation in SCFA levels observed within the group.

It is important to underline that the present work was carried out with preterm infants with gestational age over 30 weeks, which constitute the most frequent group of preterm infants in neonatal units. However, we did not include extreme preterm infants, a group in which differences in the process of establishment of the gut microbiota can be expected to be even higher. On the other hand, in the present work, all the infants were recruited from the same hospital. Recent studies have reported differences on gut microbiota composition among infants from different locations (Fallani *et al.*, 2010; Peso Echarri *et al.*, 2011). These differences may require the application of different intervention strategies depending on the local microbiota; for instance, selection of probiotic strains with properties matching the needs of a specific environment that may require confirmatory studies prior to extrapolation to other locations or units.

A careful characterization of the intestinal microbiota in the target population should constitute the basis for the development of dietary intervention strategies (e.g. probiotics or prebiotics) directed to counteract microbiota aberrancies (Isolauri & Salminen, 2008). Our results stress this observation and identify qualitative and quantitative alterations in the process of establishment of the gut microbiota in preterm infants when compared with the healthy FTVDBF infant microbiota. Further studies are needed to evaluate the potential positive effects, as well as the risks, of favoring the establishment of a gut microbiota resembling that of FTVDBF babies in preterm neonates. However, the microbiota composition of FTVDBF newborns harboring higher levels of nonpathogenic commensal bacteria and lower levels of pathogens may suggest a potential benefit of such microbiota modulation. In this regard, the design of intervention strategies contributing to the establishment of a proper gut microbiota in the preterm newborn, harboring reduced levels of potentially pathogenic microorganisms, can take advantage of this knowledge. Such strategies may contribute to decrease the risk of disease, for example infection, in these highly susceptible infants.

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