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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 due to organ immaturity, antibiotics use and hospitals stay. To assist in a proper microbiota development in these infants a detailed knowledge of the colonization process, and of 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 three months of life and compared it with that of vaginally-delivered, exclusively breast-fed full-term infants (n=20) by using qualitative and quantitative culture-independent methods. Clear differences in the gut microbiota composition between both groups were observed. Preterm infants showed higher levels of facultative microorganisms and reduced levels of anaerobes such as *Bifidobacterium*, *Bacteroides* and *Atopobium*. Short chain fatty acids concentrations were lower in preterm infants during the first days of life. Profound alterations occur in the process of microbiota establishment in preterm infants, indicating the need for intervention strategies to counteract them.

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Introduction

Microbial colonization of the digestive tract starts immediately after birth, providing a massive microbial challenge for 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). Therefore, the early establishment of a healthy microbiota may have a profound effect on the later well-being of the individual (Conroy et al., 2009).

Gut colonization of the newborn begins with facultative anaerobes such as enterobacteria and lactobacilli, and continues with anaerobic genera, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium*. Several factors may affect this process; among them, 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, and a protective effect of breast-feeding has been evidenced (Ip et al., 2009). These have led to consider the fecal microbiota profile of the healthy full-term, vaginally-delivered, exclusively breast-fed (FTVDBF) infant as the standard for a healthy infant microbiota. 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).

Most of the studies on the microbiota establishment process have focused on full-term infants. However, there is a group of newborns in which the establishment of a healthy microbiota is more challenging, due to organ immaturity, the frequent use of antibiotics and the stay at the Hospital's Neonatal Unit instead of home setting- these are the preterm neonates. These

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infants would benefit from intervention strategies directed at favoring the establishment of a healthy microbiota. In order to develop such strategies a detailed knowledge of both the microbiota establishment process in preterm neonates, and how this process differs from the 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 birth weight 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 bifidobacteria (Butel et al., 2007). Cloning and sequencing of amplified 16S rRNA genes have also been carried out (Magne et al., 2006; Wang et al., 2009) indicating a high relative abundance of the phylum proteobacteria. During the last year some metagenomic studies on preterm infant microbiota also became available (Mshvildadze et al., 2010; Morowitz et al., 2011). In general, a delayed colonization by commensal bacteria and increased colonization by pathogens has been suggested. However, the techniques used are not truly quantitative, and therefore, a detailed quantitative description of the preterm infant microbiota establishment process is still lacking.

The aim of this study was to evaluate the process of establishment of the intestinal microbiota in preterm infants as compared with that of FTVDBF

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healthy neonates, by using both qualitative and quantitative culture-independent techniques and by evaluating the metabolic activity of this microbiota.

Materials and methods

Volunteers and samples

Twenty healthy FTVDBF infants, (11 males/9 females) born after an uncomplicated pregnancy, and twenty-one 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 birth weights between 3020 and 4160 grams, and were exclusively breast-fed during the study period. The preterm infants (8 delivered vaginally and 13 by caesarean section) were born at a gestational age between 30 and 36 weeks (mean 32.7) and birth weights ranged between 1190 and 2820 grams. Six of the preterm infants' mothers received intrapartum antibiotics, and 6 of the infants received antibiotics at birth. Only 10 out of the 21 mother/premature infant pairs did not receive antibiotics, either intrapartum or postnatally, during the sampling period. None of the preterm infants were exclusively breastfed during the sampling period, all of them being in mixed feeding.

Fecal samples were collected at 2 (between 24 and 48 hours after birth), 10, 30 and 90 days of age, and immediately frozen until their analysis.

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.

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3 123 *DNA extraction*

124 Fecal samples were melted, weighed, diluted1/10 in sterile PBS solution,
125 and homogenized in a LabBlender 400 stomacher (Seward Medical, London,
126 UK) at full-speed for 4 min. DNA was extracted from the homogenised faeces,
127 as well as from bacterial cultures used for standard curves, using the QIAamp
128 DNA stool kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's
129 specifications as previously described (Gueimonde et al., 2004). Extracted DNA
130 was kept frozen at -70ºC until further analyses.

131 *Fecal microbiota analyses*

132 Quantitative analysis of fecal microbiota by quantitative PCR. Quantification of
133 the different bacterial populations in faeces was performed by quantitative PCR
134 (qPCR) using the primers shown in Table 1. All reactions were performed on
135 MicroAmp optical plates sealed with MicroAmp optical caps (Applied
136 Biosystems, Foster City,CA) in a 7500 Fast Real Time PCR System (Applied
137 Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). 1 µl
138 of template fecal DNA and 0.2 µM of each primer were used in the 25 µl PCR
139 reaction. Thermal cycling consisted of an initial cycle of 95°C 10 min, followed
140 by 40 cycles of 95°C 15 s, and 1 min at the appropriate primer-pair temperature
141 (Table 1). In the negative samples the value of the detection limits obtained for
142 the corresponding primer pair was assigned. Standard curves were made with
143 pure cultures of appropriate strains (Table 1) which were grown overnight in
144 GAM medium (Nissui Pharmaceutical Co, Tokio, Japan) under anaerobic
145 conditions. Samples were analyzed by duplicate in at least two independent
146 PCR runs.

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3 147 Qualitative analysis of fecal microbiota by PCR-DGGE. Qualitative composition
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6 148 of the microbiota in feces at the different sampling points was determined by
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8 149 PCR-DGGE in a total of sixteen randomly selected infants, eight from each
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10 150 group. Universal primers were used to assess microbial diversity, which was
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13 151 defined by the number of amplification bands generated from each sample.
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15 152 PCR reaction products were separated by DGGE in a DCode system (BioRad
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17 153 Laboratories) using conditions previously described (17). The number of bands
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20 154 in each sample was visually determined.
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23 155 *Determination of Short Chain Fatty Acids in feces*
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26 156 The analysis of Short Chain Fatty Acids (SCFA) was carried out as
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28 157 follows. Supernatants from 1 mL of the homogenized feces were obtained by
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30 158 centrifugation (10,000 *g*, 30 min, 4ºC) and filtration (0.20 µm). A
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33 159 chromatographic system composed of two 6890N GC (Agilent Technologies
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35 160 Inc., Pal Alto, CA, USA) connected to a FID and a MS 5973N detector (Agilent)
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37 161 were used for quantification and identification of SCFAs as described previously
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40 162 (Salazar et al., 2011).
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43 163 *Statistical analysis*
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46 164 Results were analyzed using the SPSS software (SPSS Inc. Chicago,
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48 165 USA). The normality of the data, at each sampling point, was checked using the
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50 166 KS test. Some of the bacterial groups showed non-normal distribution, and
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53 167 therefore, differences in bacterial levels between groups of infants were
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55 168 analyzed using non-parametric tests (Mann-Whitney U-test). The occurrence of
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57 169 different microbial groups between preterm and full-term infants was analyzed
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60 170 by chi-square test.

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171 **Results**

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

174 Noticeable qualitative and quantitative differences were found in the
175 intestinal microbiota composition between premature and healthy FTVDBF
176 babies.

177 Microorganisms from families *Enterobacteriaceae* and *Enterococcaceae*,
178 the genera *Bifidobacterium*, the *Bacteroides* and *Lactobacillus* groups, as well
179 as *Weissella* were detected in all infant fecal samples, either from full-term or
180 premature infants, throughout the entire sampling period considered in this
181 work. Against this, microorganisms belonging to the genus *Shigella* and to the
182 species *Staphylococcus aureus* were never detected, whilst *Akkermansia* was
183 only found in about 5% of the infants' feces (6% of full-term and 5% of preterm
184 infants) at 2 days of age, not being detected at later times. With respect to the
185 other microorganisms analyzed, their presence varied depending on the infant
186 group and sampling point (Table 2). Notably, during the first few days of life the
187 pathogenic microorganism *Klebsiella pneumoniae* was found significantly more
188 frequently ($p<0.05$) in preterm infants than in full-term ones, tending to continue
189 being higher also at later sampling points ($p=0.08$ and $p=0.09$ at 30 and 90
190 days, respectively). Moreover, *Clostridium difficile* was only detected (although
191 with a low occurrence rate) in fecal samples from premature babies. The
192 occurrence of *Streptococcus* at 2 days of age was significantly lower ($p<0.05$),
193 and that of *Staphylococcus* tended to be lower ($p=0.065$), in preterm infants; at
194 10 days of age the presence of *Clostridium* XIVa group, *Atopobium* and
195 *Staphylococcus* was also lower in fecal samples from these babies whilst the

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3 196 occurrence of *Desulfovibrio* was significantly higher ($p<0.05$). At one month of
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5 197 life, positive samples for *Clostridium perfringens* and *Atopobium* were
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8 198 significantly higher and lower ($p<0.05$), respectively, in premature than in full-
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10 199 term infants. All these qualitative differences in the presence of the different
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12 200 microorganisms analyzed, were attenuated over time, ceasing to be significant
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14 201 by the age of three months (Table 2).
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18 202 In general, levels of most microbial groups tended to increase over time
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20 203 (Figure 1). Notable differences in bacterial levels were observed between both
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22 204 groups of infants. Feces from preterm newborns showed higher levels ($p<0.05$)
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24 205 of *Lactobacillus* group, and specifically *Weissella*, as well as *Enterococcaceae*
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26 206 during the period of three months sampled, whereas the populations of
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28 207 *Enterobacteriaceae*, *K. pneumoniae* and *Desulfovibrio* were significantly higher
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30 208 in feces from these babies only at the first sampling points (no later than 30
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32 209 days) (Figure 1B). On the other hand, premature infants showed significantly
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34 210 lower levels of *Streptococcus* and *Staphylococcus* in the initial sampling points,
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36 211 and of *Bifidobacterium, Bacteroides* and *Atopobium* during the whole sampling
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38 212 period under study (Figure 1B).
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42 213 In accordance with the results commented above, when the percentages
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44 214 of each bacterial group were calculated, *Enterobacteriacceae* ranged between
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46 215 45 to 63% of total microorganisms in FTVDBF infants, whereas they
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48 216 represented 60 to 83% in the preterm group (Supplementary Figure 1). Among
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50 217 the non-*Enterobacteriaceae* microorganisms, *Bacteroides*, *Enterococcaceae*
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52 218 and *Streptococcus*, followed by *Bifidobacterium* were predominant at 2 days of
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54 219 age in feces from FTVDBF babies. In the preterm group, however, the
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56 220 predominant microorganisms at this time were *Enterococcaceae* and members
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221 of *Lactobacillus* group, followed by *Streptococcus*. Between 10 days and 3
222 months of age *Bifidobacterium*, *Bacteroides* and *Streptococcus* predominate in
223 full-term infants, in contrast with the dominance of *Enterococcaceae* and
224 *Lactobacillus* group, followed by bifidobacteria, observed in premature babies
225 (Supplementary Figure 1).

226 Although the number of preterm infants may not be large enough for
227 within-group comparisons with appropriate statistical power, we compared the
228 levels of different microorganisms between vaginally (8 out of 21 infants) and
229 caesarean delivered preterm neonates without obtaining statistically significant
230 differences at any sampling point for any microbial group. When preterm infants
231 receiving antibiotics, or whose mothers receive intrapartum antibiotics, were
232 compared with those not exposed to antibiotics (10 out of 21 infants) the sole
233 statistically significant difference ($p<0.05$) regarded lower levels of bifidobacteria
234 at 10 days of age in the former group, without observing any other differences
235 for other microorganisms or time points (data not shown).

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

240 *Metabolic activity of the intestinal microbiota of newborns*

241 The metabolic activity of the intestinal microbiota was determined by
242 measuring the SCFA concentration in feces. Total SCFA (results not shown), as
243 well as the main SCFA (acetate, propionate and butyrate) concentration in
244 feces increased over time in both groups of infants (Figure 3). Despite the high

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inter-individual variation, lower levels of total SCFA (data not shown), as well as specifically acetate and propionate, were observed in preterm infants (significant differences, $p<0.05$, at 2 days of age for acetate and propionate and at 10 days for propionate) (Figure 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, with a slight increase (about 6%) in the relative abundance of acetate, and a concomitant decrease in that of propionate and butyrate between 2 and 10 days of age, remaining stable afterwards (data not shown).

Discussion

Our results showed a clear delay in the intestinal colonization by commensal microorganisms, increased occurrence of pathogens, as well as high inter-individual variability and reduced microbial diversity in preterm infants, this confirming previous reports (Schwiertz et al., 2003; Magne et al., 2006; Rouge et al. 2010; Jacquot et al., 2011). In addition, our study extend these observations to the quantitative levels of different bacterial groups.

Despite the high inter-individual variability in bacterial levels, significant differences were found between preterm and full-term infants for several microbial groups, evidencing a deeply altered gut microbial colonization pattern in the former group of babies. Delivery mode and antibiotics consumption are two factors that may affect microbiota composition. Although the limited number of infants do not allow establishing firm conclusions, within our preterm group delivery mode do not seem to have affected bacterial levels. The use of

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antibiotics, intrapartum or administered to the infant, showed limited effects, producing a reduction on *Bifidobacterium* levels at 10 days of age. These results suggest that immaturity itself may explain most of the differences observed in this study.

We observed increased levels of facultative microorganisms, such as *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillus* group (including lactobacilli and *Weissella*), together with reduced levels of anaerobes, including *Bifidobacterium*, *Bacteroides* and *Atopobium*, which seems to indicate a deficiency, or delay, in the establishment of the normal anaerobic gut microbiota in preterm neonates. 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 due to, among other factors, an increased risk of infection.

Enterobacteriaceae and *Enterococcaceae* were the predominant microbial groups in preterm infants, which showed also 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 *Lactobacillus* group seem to have a predominant role on the preterm infant gut microbiota. The observation of higher levels of these microorganisms in the preterm infants is striking, as a lower colonization by lactobacilli in preterm neonates 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. quantitative PCR 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 genera *Weissella*. For these reason we decided to

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quantify specifically the levels of this microorganisms and found that *Weissella* appears 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 potential pathogens. 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 often be present on the preterm infant microbiota (Hoy et al., 2000; Schwiertz et al., 2003). Although the differences did not reach statistical significance, likely due to the low incidence of the microorganism, it was interesting to observe that *C. difficile* was detected exclusively in preterm infants. 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. pneumonia* 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 large 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 by

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319 other authors, the levels being lower in extremely preterm neonates (Favre et
320 al., 2002). The heterogeneity in the gestational age of our premature infants
321 (between 30 and 36 weeks) may, thus, partly account for the inter-individual
322 variation in SCFA levels observed within the group.

323 It is important to underline that the present work was carried out with
324 preterm infants with gestational age over 30 weeks, which constitute the most
325 frequent group of preterm infants in neonatal units. However, we did not include
326 extreme-preterm-infants, a group in which differences in the process of
327 establishment of the gut microbiota can be expected to be even higher.

328 A careful characterization of the intestinal microbiota in the target
329 population should constitute the basis for the development of dietary
330 intervention strategies (e.g. probiotics or prebiotics) directed to counteract
331 microbiota aberrancies (Isolauri & Salminen, 2008). Our results stress this
332 observation and identify several qualitative and quantitative alterations in the
333 process of establishment of the gut microbiota in preterm infants when
334 compared with the golden standard for infant gut microbiota, the healthy
335 FTVDBF infant. The design of intervention strategies to facilitate the
336 establishment of a proper gut microbiota in the preterm newborn should take
337 advantage of this knowledge. Such strategies may greatly contribute to
338 decrease the risk of disease, e.g. infection, in these highly susceptible infants.

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

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

AGE	GROUP	MICROORGANISMS								
		Cl. IV	**Cl. XIVa**	**Atop.**	**Strept.**	**Staph.**	**K. pne.**	**C. perfr.**	**Desul.**	**C. diff.**
2 d	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 d	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 d	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 d	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. perf**, *Clostridium perfringens;* **Desul**, *Desulfovibrio;* **C. diff**, *Clostridium difficile. * p<0.05; ** p<0.01; *** p<0.001*

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Figure 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. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 2. 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; grey boxes, premature infants. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3. Concentration (mean ± sd) of the main short chain fatty acids (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). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Supplementary Figure 1. Percentages of the different fecal microbial groups, in both premature and full-term infants, at the different time points analyzed.

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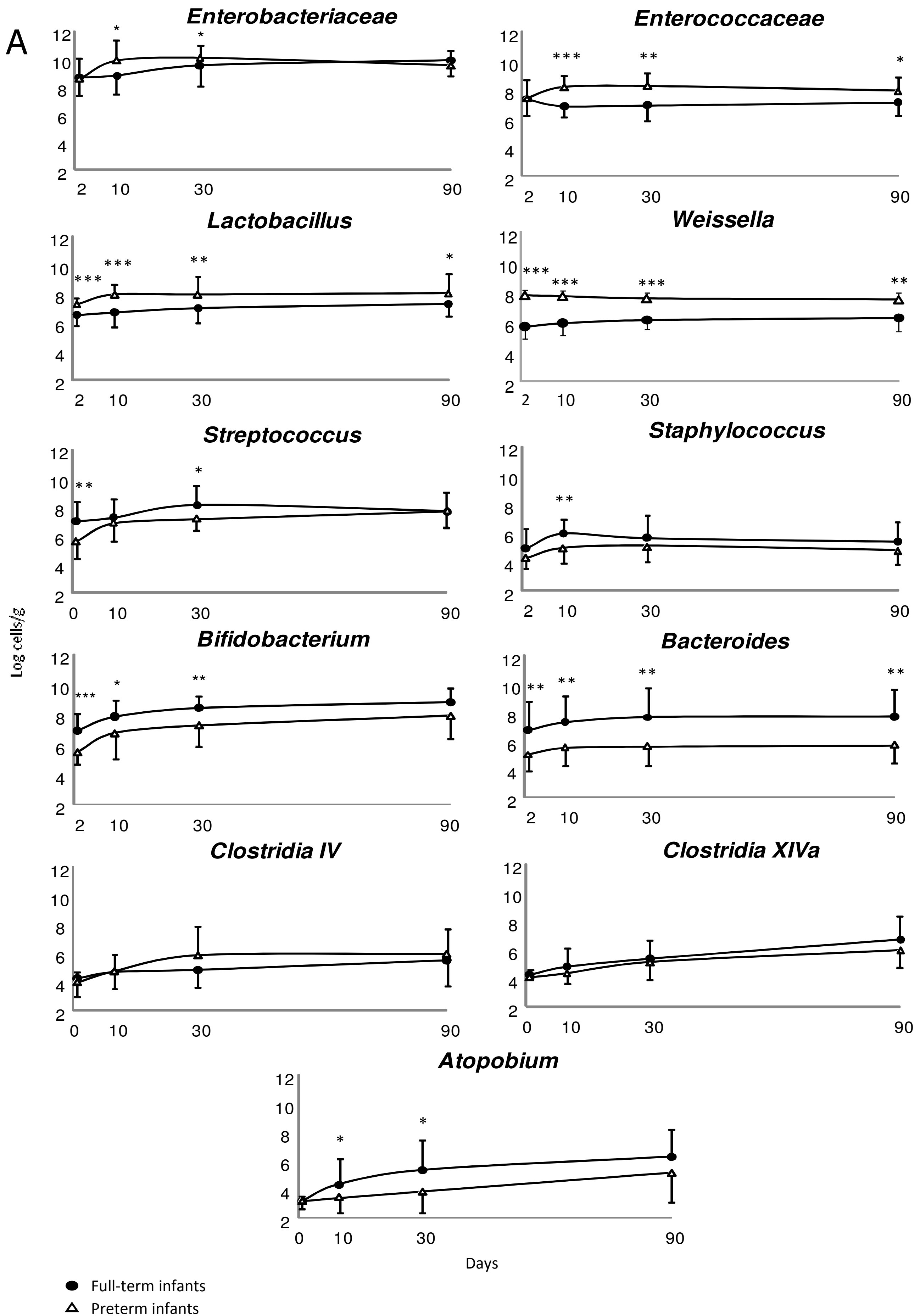
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Clostridia IV
Clostridia XIVa
Atopobium
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Preterm infants

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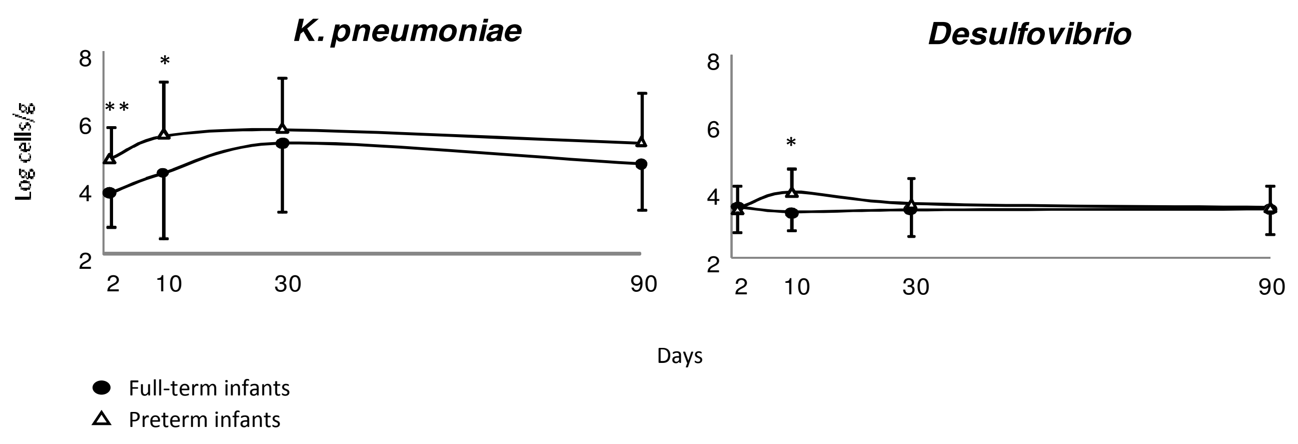

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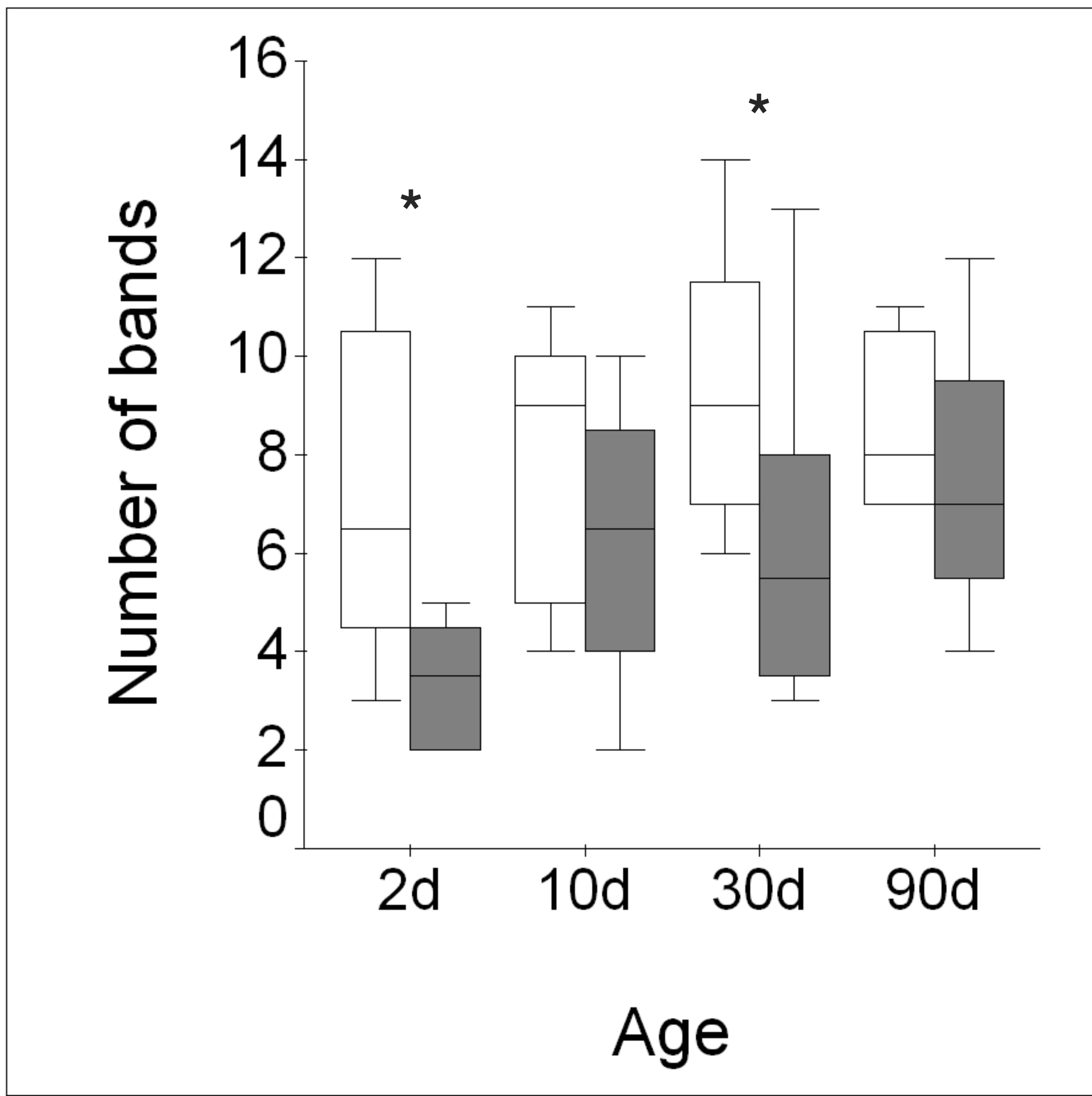

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