Assessment of intestinal microbiota of full-term breast-fed infants from two different geographical locations

Running title: Microbiota in infants from different locations

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Abstract

The intestinal microbiota in the breast-fed infant is considered as ideally healthy. We assessed the microbiota of breast-fed full-term neonates from two different Spanish locations. Statistically significant geographical differences for different bacterial groups were found, underlining the need to consider and define geographical-related effects on microbiota.

Keywords: infant microbiota, breastfed infant

Microbial colonization of the gut provides an essential stimulus for normal intestinal development and maturation of the immune system, contributing to the establishment of a proper intestinal homeostasis and mucosal barrier function [1]. Thus, early establishment of a healthy microbiota provides the first key step in the later well-being of the individual. Gut colonization begins with facultative anaerobes such as enterobacteria and lactobacilli and continues with anaerobic genera such as *Bifidobacterium*, *Bacteroides*, and *Clostridium*. Subsequently, feeding practices affect the population levels of different microbes [2,3]. Breast-milk is the ideal food in early life and it is known to play an important role in the establishment of the intestinal microbiota. The profile of fecal microbiota in the breast-fed infant is considered as ideally healthy. Therefore, promotion of a microbiota resembling that of the healthy breast-fed infant is often taken as a target for improving the functionality of infant formulas by using pro- and prebiotics.

Recent reports have indicated the difficulty of defining the common phylogenetic core of the human healthy intestinal microbiota [4,5]. In infants, the microbiota of the healthy, breast-fed, vaginally delivered, full-term baby is considered the gold standard. However, although the influence of feeding habits, i.e. breast versus formula-fed, and mode of delivery have been extensively studied on the so defined “healthy infant microbiota”, the effect of other factors such as geographical origin have been addressed in a limited number of studies [6].

The aim of this study was to assess the intestinal microbiota of exclusively breast-fed neonates from two different geographical Spanish locations.

Forty healthy full-term exclusively breast-fed infants, born either at the Neonatology Unit of Cabueñes Hospital from Asturias (20 infants; 11 males/9

females) or at the Neonatology Unit of the University Hospital Virgen de la Arrixaca from Murcia (20 infants; 12 males/ 8 females), after an uncomplicated pregnancy were recruited. Infants were vaginally delivered, at a gestational age of 39.2 weeks (95%CI: 38.6-39.7) for Asturian infants and 39.1 (95% CI: 38.5-39.7) weeks for those from Murcia. Birth weights ranged between 3238 and 3586 grams (95% CI) in Asturias and between 3090 and 3411 grams (95% CI) in infants from Murcia. Microbiota of fecal samples collected at 8, 30 and 90 days of life were compared to ascertain the possible effect of geographical origin of samples. Fecal samples were immediately cooled to 4^{o}C after collection, delivered to the laboratory within 4 h, and frozen at –75^{o}C directly on receipt until analysis. None of the mothers or babies received antibiotic therapy during the sampling period. The study was approved by the Regional Committees on Clinical Research from Asturias and Murcia regions. All parents gave written informed consent to participate in the study.

Fecal DNA, as well as DNA from bacterial cultures used for calibration curves, was extracted as previously described [7] by using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). Quantification of the different bacterial populations in feces was performed by quantitative PCR using primers shown in Table 1. All reactions were performed in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR Master Mix (Applied Biosystems). 1 µL (~5ng) of template fecal DNA and 0.2 µM of each primer were used in the 25 µL PCR reaction. Thermal cycling consisted of an initial cycle of 95^{o}C 10 min followed by 40 cycles of 95^{o}C 15 s and 1 min at the appropriate temperature (Table 1). In the negative samples the value of the detection limits obtained for the corresponding primer pair was assigned.

Standard curves were made with pure cultures of appropriate strains (Table 1) which were grown overnight in GAM medium (Nissui Pharmaceutical Co, Tokio, Japan) under anaerobic conditions. Samples were analyzed by duplicate in at least two independent PCR runs.

No differences in background parameters (birth weight, gestational age) were observed between both study groups. A high inter-individual variability was observed on the levels of the different microbial groups. However, despite this variability our results showed statistically significant (U-test; $p<0.05$) higher levels of *Bacteroides* (8.30±1.90 vs. 6.90±1.86 Log no. cells g^{-1}) and *Staphylococcus* (6.64±1.08 vs. 5.62±1.01) at 8 days of age and lower of Enterobacteriaceae (9.17±0.84 vs. 9.90±0.65) at 90 days of life in infants from Murcia than in those from Asturias (Figure 1). Similar levels of Enterococcaceae, Clostridia XIVa and IV groups, *Atopobium*, *Bifidobacterium* and *Lactobacillus* were found between both groups (Figure 1). When all the sampling points were taken together, a trend ($p=0.08$) to lower counts of lactobacilli and higher of *C. leptum* group ($p=0.06$), as well as significantly higher levels of *Bacteriodes* and *Staphylococcus* were observed in Murcian infants as compared to those from Asturias (data not shown). Interestingly, at qualitative level *C. leptum* was more frequently detected in newborns from Asturias than in those from Murcia (64 vs. 25% of infants at 8 days, 77 vs. 55% at 30 days and 92 vs. 65% at 3 months of age; Fisher's exact test, $p<0.05$) whilst no statistically significant differences were obtained in the occurrences of the other microbial groups tested.

In both populations a significant negative correlation was observed between the levels of *Bacteriodes* and those of Enterococcaceae (Pearson's correlation

coefficients, -0.208 and -0.268 for Asturias and Murcia infants, respectively, $p<0.05$ for both cases) whilst *Bacteriodes* levels correlate positively with those of *Bifidobacterium* (Asturias 0.319; Murcia 0.409, $p<0.05$ for both cases). Similarly significant positive correlations ($p<0.05$) were observed in both groups of infants between levels of Enterococcaceae and Enterobacteriaceae (0.473 and 0.276 for Asturias and Murcia, respectively), between *C. leptum* and *C. coccoides* groups (Asturias 0.234; Murcia 0.360) and between bifidobacterial levels and those of lactobacilli (0.368 and 0.258) or *Atopobium* (0.412 and 0.213 for Asturias and Murcia, respectively). Interestingly, a very significant positive correlation between *Bacteriodes* and *Atopobium* was observed in Asturian infants (0.662, $p<0.05$) but not in those from Murcia, whilst the contrary happened between *Atopobium* and *C. coccoides* (0.467, $p<0.05$ in infants from Murcia).

When analysing samples from healthy children and adults, Lay and coworkers [8] did not found any geographical differences on microbiota composition. Contrary to this, when assessing the microbiota of adults and elderly from four European countries, quantitative country-specific differences were reported [9]. Moreover, a recent study [6] indicated differences on the gut microbiota composition of 6-weeks-old babies from five European countries. Interestingly, the study by Fallani and co-workers [6] observed, among others, higher levels of *Bacteroides* in 6-week-old infants from Granada (southern Spain) than in those from other more septentrional European locations. Similarly, in our study exclusively breast-fed, full-term, vaginally delivered infants from Murcia (southern Spain) presented higher levels of *Bacteroides* than those from

Asturias (northern Spain), indicating that this characteristic may be specific from the south of Spain.

In spite of the high inter-individual variability our results evidenced some statistically significant differences between two cohorts of exclusively breast-fed full-term healthy Spanish neonates born in two different locations (~1000 km far from each other), one in the northern Atlantic coast and the other in the south-east Mediterranean coast. All the infants were born at two hospitals of the Public Health System, where facilities and procedures are expected to be similar and therefore the two groups under study are likely to be quite homogeneous. Therefore, it is not surprising that the observed differences were limited to certain microbial populations whilst most of them showed a high similarity between both infant groups. In agreement with this, the correlations among the levels of the different bacteria analysed seem to be very consistent between both infant groups, although some specific correlations were observed to be different between them. This indicates that not only minor differences in composition are present between both infant groups, but also some interactions among the intestinal microorganisms present may differ depending on the geographical origin.

Despite the differences observed in their microbiota, our two individual cohorts represent healthy breast-fed infants. Our study does not allow establishing firm conclusions about the factors explaining these microbiota differences, both locations are similar in terms of number of inhabitants and per capita income, but dietary habits are known to differ between them which is likely to have an influence. Our findings underline the difficulty of defining the healthy intestinal microbiota and suggest that factors related to geographical origin or dietary

background, likely through modulation of breast-milk composition [10], should be also taken into consideration. To this regard, a healthy intestinal microbiota could be defined as the intestinal microbial community that assist the host to maintain a healthy status under certain environmental conditions. This emphasizes that under specific environmental conditions the intestinal microbiota may contribute to health but the same composition under different conditions may lead to disease as suggested by the role that intestinal microbiota seems to play in obesity [11].

Although small, geographical or dietary differences in gut microbiota as those reported here may also have a deep impact on pro/prebiotics research. Interestingly, pioneer studies carried out in Finland demonstrated that atopic diseases can be prevented by administration of probiotics [12]. However, a study carried out in Germany following the same design and using the same probiotic strain found no effect [13]. Unfortunately background microbiotas were not compared. If, similarly to our infants, differences in background microbiota exist, they are likely to modify the effect of probiotics on microbiota composition, which may affect the clinical outcome of probiotic/prebiotic intervention studies. It has been previously indicated that a careful characterization of the intestinal microbiota in the target population should constitute the basis for probiotic and prebiotic use [14]. Our results stress this observation and underline the need to define the microbial core of the healthy infant microbiota taking into account the possible differences due to geographical origin.

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Conflict of interest statement

All authors disclose any conflict of interest.

References

1 Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol 2010;10:159-169.

2 Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511-521.

3 De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massard S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci USA 2010;107:14691-14696.

4 Tap J, Mondot S, Levenez F, Pelletier E, Caron C, Furet JP, et al. Towards the human intestinal microbiota phylogenetic core. Environ Microbiol 2009;11:2574-2584.

5 Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-67.

6 Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, et al. Intestinal microbiota of 6-week-old infants across Europe: Geographic influence beyond delivery mode, breast-feeding and antibiotics. J Pediatric Gastroenterol Nutr 2010;51:77-84.

7 Gueimonde M, Tölkko S, Korpimäki T, Salminen S. New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl Environ Microbiol 2004;70:4165-4169.

8 Lay C, Rigottier-Gois L, Holmstrom K, Rajilic M, Vaughan EE, de Vos WM, et al. Colonic microbiota signatures across five northern european countries. Appl Environ Microbiol 2005;71:4153-4155.

9 Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, et al.. Differences in fecal microbiota in different European study populations in relation to age, gender and country: a cross-sectional study. Appl Environ Microbiol 2006;72:1027-1073.

10 Nasser R, Stephen AM, Goh YK, Clandinin MT. The effect of a controlled manipulation of maternal dietary fat intake on medium and long chain fatty acids in human breast milk in Saskatoon, Canada. Int Breasfeed J 2010;5:3.

11 Ley RE: Obesity and the human microbiome. Curr Opin Gastroenterol 2010;26:5-11.

12 Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 2001;357:1076-1079.

13 Kopp MV, Hennemuth I, Heinzmann A, Urbanek R. Randomized, double-blind, placebo controlled trial of probiotics for primary prevention: no clinical

effects of *Lactobacillus* GG supplementation. Pediatrics 2008;121:e850-e856.

14 Isolauri E, Salminen S. Probiotics: use in allergic disorders. A Nutrition, Allergy, Mucosal immunology and Intestinal microbiota (NAMI) research group report. J Clin Gastroenterol 2008;42:S91-S96.

15 Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl Environ Microbiol 2004;70:7220-7228.

16 Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol 2006;72:4214-4224.

17 Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. Brit J Nutr 2009;101:541-550.

18 Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 2004;97:1166-1177.

19 Matsuda K, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Appl Environ Microbiol 2007;73:32-39. *Erratum in: Appl Environ Microbiol 2007;73:6695.*

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

Microbial target	Strain used for standard curve	Primer Sequence 5'-3'	Tm	Ref
Atopobium group *Atopobium- Collinsella*	*Collinsella intestinalis* DSMZ13280	F: GGGTTGAGAGACCGACC R: CGGRGCTTCTTCTGCAGG	55ºC	[15] [15]
Bacteroides group *Bacteriodes-Prevotella-Porphiromonas*	*Bacteriodes thetaiotaomicron* DSMZ2079	F: GAGAGGAAGGTCCCCCAC R: CGCKACTTGGCTGGTTCAG	60ºC	[16] [17]
Bifidobacterium	*Bifidobacterium longum* NCIMB8809	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	60ªC	[5] [5]
Clostridia XIVa group *C.coccoides-E. rectale*	*Clostridium coccoides* DSMZ935	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	55ºC	[18] [18]
Clostridia IV *C. leptum-F. praustnitzii*	*Clostridium leptum* DSMZ753	F: TTAACACAATAAGTWATCCACCTGG R: ACCTTCCTCCGTTTTGTCAAC	60ºC	[17] [17]
Enterobacteriaceae	*Escherichia coli* LMG2092	F: TGCCGTAACTTCGGGAGAAGGCA R: TCAAGGACCAGTGTTCAGTGTC	60ºC	[19] [19]
Enterococcaceae	*Enterococcus faecalis* IPLAIF3/1	F: CCCATCAGAAGGGGATAACACTT R: ACCGCGGGTCCATCCATC	60ºC	[19] [19]
Lactobacillus Group	*Lactobacillus gasseri* IPLAIF7/5	F: AGCAGTAGGGAATCTTCCA R: CATGGAGTTCCACTGTCCTC	60ºC	[18] This study
Staphylococcus	*Staphylococcus epidermidis* IPLAIF1/6	F: ACGGTCTTGCTGTCACTTATA R: TACACATATGTTCTTCCCTAATAA	60ºC	[19] [19]

Figure 1. Bacterial levels, at the different sampling times, determined by qPCR in feces of breast-fed infants from two Spanish locations (about 1000 km apart from each other); Asturias (black diamonds) or Murcia (open squares). Asterisks indicate statistically significant differences between both groups at the corresponding sampling time ($p<0.05$).

7. Figure(s)

Figure 1