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3 **ESTABLISHMENT AND DEVELOPMENT OF LACTIC ACID BACTERIA AND**

4 **BIFIDOBACTERIA MICROBIOTA IN BREAST-MILK AND THE INFANT GUT**

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1 **Abstract**

2 The initial establishment of lactic acid bacteria (LAB) and bifidobacteria in the newborn and

3 the role of breast-milk as a source of these microorganisms are not yet well understood. The

4 establishment of these microorganisms in vaginally delivered breast-fed full-term infants,

5 and the presence of viable *Bifidobacterium* in breast-milk was evaluated. In 1 day-old

6 newborns *Enterococcus* and *Streptococcus* were the microorganisms most frequently

7 isolated, from 10 days of age until 3 months bifidobacteria become the predominant group.

8 In breast-milk, *Streptococcus* was the genus most frequently isolated and *Lactobacillus* and

9 *Bifidobacterium* were also obtained. Breast-milk contains viable lactobacilli and

10 bifidobacteria that might contribute to the initial establishment of the microbiota in the

11 newborn.

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13 **Key words:** Bacterial colonization; microbiota; lactic acid bacteria; bifidobacteria; neonate;
14 breast-milk.

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1. Introduction

Intestinal colonization of the newborn is essential for establishment, maturation and maintenance of the gut mucosal barrier [1]. There is increasing evidence that this initial microbial colonization of the intestine has a strong effect on health and specific aberrancies in this process may predispose to disease later in life [2].

Early colonization begins with facultative anaerobes such as enterobacteria, coliforms and lactobacilli and continues with anaerobic genera such as *Bifidobacterium*, *Bacteroides*, and *Clostridium*. Subsequently, feeding practices affect the concentrations of different microbes [1]. The greatest difference between the microbiota of breast-fed and formula-fed infants lies in numbers and species composition of bifidobacteria. Indeed, the health-promoting effects of breast-milk have been linked partly to different bifidogenic factors and more recently to the presence of lactic acid bacteria (LAB) and bifidobacteria in breast-milk [3-5]. Increasing the LAB and bifidobacteria levels is a target for infant formulas and the most common approach to this end has been to include prebiotic compounds. A different approach is the supplementation with probiotic bacteria, mainly lactobacilli and bifidobacteria. The use of strains isolated from breast-milk would increase the similarity between breast-milk and infant formulas. In this context, the genus *Bifidobacterium* is especially attractive due to its predominant role in the healthy infant microbiota and its positive effects and safety records. Several studies have focused on the infant microbiota. However, there is still limited information on the initial establishment of LAB and bifidobacteria in the newborn and the role of breast-milk as a source of bacteria for infant gut colonization. Increasing our understanding on the initial process of establishment of the LAB and bifidobacterial microbiota will allow the development of strategies to facilitate this colonization process in formula-fed or preterm infants.

The aim of the present work was to assess the establishment and development of the LAB

and bifidobacterial microbiota in vaginally delivered, exclusively breast-fed full-term

infants, as well as in their mothers' milk, during the first 3 months of age. A second aim was

to assess the presence of viable *Bifidobacterium* strains in breast-milk.

2. Materials and Methods

2.1. *Samples.* 20 mother- infant (full-term) pairs were recruited at the Cabueñes Hospital

(Gijon, Spain). Breast-milk and infant faecal samples were taken at 1, 10, 30 and 90 days of

age. Breast-milk samples were obtained by manual expression. All infants (11 males/9

females) were born at the Neonatology Unit of the Hospital after an uncomplicated

pregnancy. Infants were vaginally delivered, at a gestational age of 39.2 weeks (95% CI;

38.6-39.7) and a birth weight of 3403 grams (95% CI; 3238-3568). None of the mothers or

babies received antibiotic therapy during the sampling period. Five mothers received a single

course pre-partum treatment with ampicillin.

The study was approved by the Ethical Committee of the Regional Asturias Public Health

Service (SESPA) and informed written consent was obtained from the mothers.

2.2. *Microbial plate counts.* Fresh faecal samples were immediately placed in an anaerobiosis

jar (Anaerocult A system, Merck, Darmstadt, Germany) at the Hospital and transported to the

lab within 2 hours. At reception samples were introduced and processed in an anaerobic

atmosphere (10% H_2, 10% CO_2 and 80% N_2) in a chamber Mac 500 (Don Whitley Scientific,

West Yorkshire, UK). To determine the levels of LAB and bifidobacteria, samples were

serially diluted in a reducing medium containing BHI broth (Merck) supplemented with 0.5%

glucose, 0.5% yeast extract (Merck), 0.25% L-cysteine (Sigma Chemical Co, St. Louis, MO,

USA), 10 µg/L vitamin K1 (Merck) and 0.02 g/L Hemin (sigma). Dilutions were plated in

MRS medium (Difco, Becton, Dickinson and Company, Le Pont de Claix, France)

1 supplemented with 0.25 % L-cysteine (Sigma) (MRSc) and incubated in anaerobiosis for 48

2 hours. Colonies were then counted and isolated for further identification.

3 2.3. *Identity of isolates by partial sequence analysis of the 16S rRNA gene.* Colonies

4 displaying different morphology were differentially counted and isolated from counting plates

5 for subsequent identification by partial sequence analysis of the 16S rRNA gene. In brief,

6 isolates were grown overnight in MRSc broth at 37ºC in anaerobic cabinet. Then, 1mL of

7 cells was harvested by centrifugation and the DNA extracted using the GenElute™ Bacterial

8 Genomic DNA Kit (Sigma) following manufacturer's instructions. Partial amplification of the

9 16S RNA gene and identification of isolates was carried out as previously described [6].

10 PCR products were purified using the GenElute™ PCR clean-up Kit (Sigma). Automated

11 sequencing of the PCR products was done at Secugen SL (Madrid, Spain) in an automated

12 sequencer ABI Prism (Applied Biosystems, Foster City, CA, USA).

13 2.4. *Genetic typing of* Bifidobacterium *isolates*. DNA extracts from the different isolates

14 identified as *Bifidobacterium* were used for strain typification by randomly amplified

15 polymorphic DNA (RAPD) analysis by using the primer (OPA-2) and conditions previously

16 described [7]

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18 **3. Results**

19 From day 1 to day 10 of life bacterial counts from infant faeces, obtained in MRSc medium,

20 raised from about 8.7 to10 log cfu/g and remained stable during the rest of the study (Figure

21 1). Contrary to this, bacterial levels in breast-milk decreased along the study from 5 log

22 cfu/mL at day 1 to 3.7 log cfu/mL at 90 days (Figure 1).

23 240 colonies were picked up from the counting plates, isolated and identified. In faeces from

24 1 day-old newborns members of the genera *Enterococcus* and *Streptococcus* (31% and 28%

25 of isolates, respectively) were the microorganisms most frequently isolated (Figure 2). Among

1   them *Enterococcus faecalis* and *Streptococcus salivarius*, respectively, were the main species.

2   On the other three sampling points (days 10, 30 and 90) microorganisms belonging to

3   *Bifidobacterium* were the most frequently found (between 42-59% of isolates depending on

4   the sampling point) followed by *Streptococcus*, *Lactobacillus* and *Enterococcus*. These

5   comprise mainly the species *Bifidobacterium longum* followed by *Bifidobacterium breve*,

6   *Bifidobacterium bifidum* and *Bifidobacterium pseudocatenulatum*, *S. salivarius* followed by

7   *Streptococcus vestibularis*, *Lactobacillus gasseri* and *E. faecalis*, respectively. In most of the

8   samples (20% of day 1 samples and about 60% of the samples from the other sampling

9   points) bifidobacteria were the microorganisms present at higher levels in the MRSc plates

10   and ranged from 7.8 to 10.7 log cfu/g depending on the individual and the sampling time.

11   With regard to breast-milk, *Streptococcus*, mainly represented by the species *S. salivarius*,

12   was the LAB genus with higher frequency of isolation, ranging from 36 to 65% of isolates

13   depending on the sampling time. Members of the genus *Staphylococcus* (a genus not

14   belonging to LAB) were also found in the culture medium and they constituted between 29

15   and 50% of the breast-milk isolates. Among them *Staphylococcus epidermidis* was the most

16   frequent species. A 5% of the total breast-milk isolates belonged to *Lactobacillus* genus and

17   another 5% were *Bifidobacterium* or relatives which are anaerobic microorganisms that have

18   not been frequently isolated from breast-milk. Among lactobacilli, *L. gasseri* was the species

19   most frequently found. The bifidobacterial strains isolated from breast-milk samples were *B.*

20   *longum* (3 isolates), *B. breve* (3 isolates) and the *Bifidobacterium*-like microorganism

21   *Parascardovia denticolens* (1 isolate). Their levels ranged between 2.5 and 4.8 log cfu/mL.

22   Bifidobacterial strains showing identical RAPD profiles were found in samples from breast-

23   milk and the corresponding infant faeces as well as in samples from the same infant at

24   different sampling times (data not shown). All the strains isolated from different breast milk-

25   infant pairs showed different RAPD profiles.

4. Discussion

It has been reported that breast-fed infants have less allergies and gastrointestinal infections than formula fed infants [2]. Therefore, the breast-fed infant microbiota may be considered the standard of a healthy gut microbiota and needs to be both qualitatively and quantitatively assessed. LAB and bifidobacteria are often considered as members of a healthy microbiota. LAB account for less than 1% of the total but bifidobacteria may be predominant members of the intestinal microbiota in breast-fed infants [8]. This predominance of bifidobacteria appears to be characteristic of the healthy breast-fed infant gut microbiota and therefore it may have a key role on later health.

Our results show the initial establishment and development of the intestinal LAB and bifidobacterial microbiota in breast-fed babies and the presence of these microorganisms and their evolution in breast-milk. In general, the levels of faecal LAB and bifidobacteria found are in the range of those previously reported for this human population [9,10]. Breast-milk is difficult to sample and microbiological contamination can never be discarded. *S. salivarius* and the non-LAB microorganism *Stap. epidermidis*, which has been reported to be a species characteristic of the breast-fed infant [11], were the microorganisms more frequently isolated. Martin and co-workers [3] isolated LAB from breast milk and showed that the same LAB strains present in breast-milk are also found in faeces of the corresponding infant. In a previous study the presence of bifidobacterial DNA in breast-milk was reported [4]. The question that remained unanswered at that time was whether viable bifidobacteria were present in breast-milk. Recently, similarly to that found in our study Martin *et al.* [5] reported the isolation of bifidobacterial strains from breast-milk samples taken 4-7 days after delivery, demonstrating the presence of alive bifidobacteria in human milk. These authors identified *B. breve* as the most frequently isolated species whilst in our study *B. longum* was equally frequent.

1   We found that bifidobacterial strains showing the same genetic profiles (RAPD analyses)

2   were present in breast-milk and the corresponding infant faeces at different sampling points,

3   suggesting vertical transfer from the mother's milk to the infant. Identical profiles were not

4   found among isolates from different infants indicating that during the first months of life the

5   numerically predominant bifidobacterial populations are individual-specific.

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7 **5. Conclusions**

8   Our results indicate that breast-milk contains viable lactobacilli and bifidobacteria that might

9   contribute to the establishment and development of the microbiota in the newborn. The

10   microorganisms isolated in this study may constitute promising strains for their inclusion in

11   infant formulas.

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1   **Figure 1.** Bacterial counts obtained in MRSc medium for infant faeces (IF) and breast milk

2   (BM) at the different sampling points assessed.

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7   **Figure 2.** Percentages of isolation of different bacterial genera from the plates of MRSc

8   medium in infant faeces (UP) and breast milk (DOWN) at the different sampling points

9   analysed.

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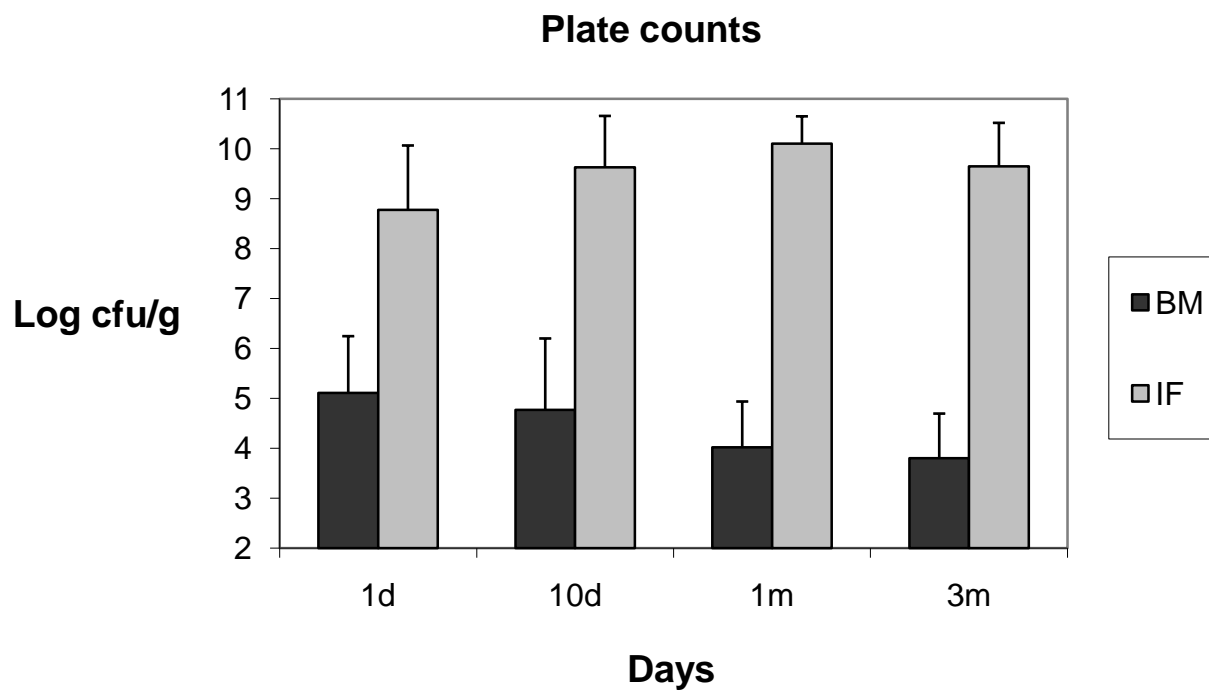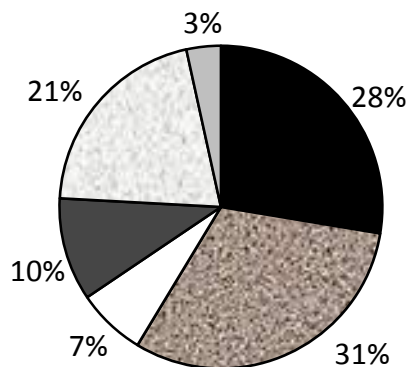15

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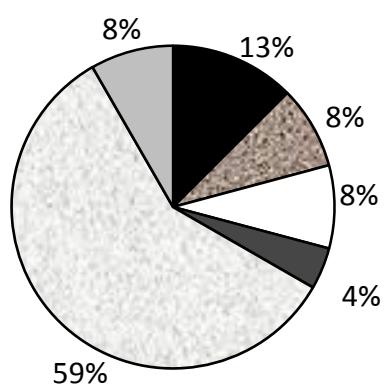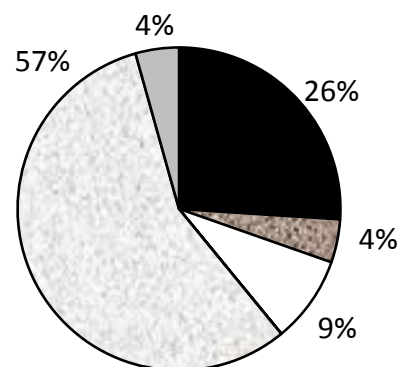
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Figure 1

Figure 1

Plate counts

Figure 2

Figure 2