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Sharing of Bacterial Strains Between Breast Milk and Infant Feces

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Abstract

In previous years, it has been shown that human milk is a potential source of bacteria for the infant gut. The results of this work confirm the presence of the same specific bacterial strains of *Bifidobacterium, Lactobacillus*, and *Staphylococcus* in breast milk and infant fecal samples. The identity of bacteria isolated from breast milk and infant feces from 20 mother-infant pairs was investigated at the strain level. DNA from *Staphylococcus*, *Lactobacillus*, and *Bifidobacterium* was detected by qRTi-PCR in nearly all samples analyzed. These samples were cultured on different agar media. One colony representative of each morphology was selected and identified at the species level combining classical tests and molecular techniques (PCR, RAPD, PFGE, and/or MLST genotyping). Breast milk and infant feces from 19 mother-infant pairs shared different *Staphylococcus, Lactobacillus*, and/or *Bifidobacterium* species and strains. Significantly, 2 mother-infant pairs shared 4 bacterial strains although most pairs shared 2. These results confirm that breast milk and infant feces from mother-infant pairs share the same strain(s), indicating that breastfeeding could contribute to the bacterial transfer from the mother to the infant and, therefore, to the infant gut colonization.

Keywords

breast milk, transfer, bacteria, infant gut

Background

Bacterial colonization of the human gut is a complex process that seems to start, at a small scale, during the fetal period.[1–4] Contact with microorganisms belonging to the vaginal, intestinal and mammary microbiota of the mother, and to the surrounding environment of the neonate, leads to a notable intensification of this process after birth.[5–9] As a consequence, factors such as composition of the maternal microbiota, place and way of birth and/or feeding pattern, play key roles in a process that exerts a strong influence on host functions so important as nutrient absorption, formation of host barriers against pathogens, or maturation of the immune system.[10,11]

Breast milk constitutes a continuous source of bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria and lactic acid bacteria.[9,12–14] In addition, human milk contains some components that stimulate the growth of specific bacterial groups in the gut of the breastfed infant.[15] It has been suggested that the origin of bacteria present in breast milk may be the maternal skin in the case of coagulase-negative staphylococci, with *Staphylococcus epidermidis* as the predominant species[16] or the infant mouth for those bacteria that are rarely found as skin residents such as viridans streptococci.[12,16] More recently, another hypothesis has been proposed involving cells of the immune system within the mucosal-associated lymphoid tissue (MALT) system. It has been demonstrated that dendritic cells can penetrate the gut epithelium to directly take up bacteria from the gut lumen,[17] and that antigen-stimulated cells move from the intestinal mucosa to colonize distant mucosal surfaces.[18] In addition, during late pregnancy and lactation, there is a selective colonization of the mammary gland by cells of the immune system (the so-called entero-mammary pathway) and this mechanism has been proposed to explain the

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Table 1. Genus-specific primer pairs used in this study.

Target bacterial group/species	Sequence (5'–3')	Annealing temperature (°C)	References
Bifidobacterium group	g-Bifid-F: CTCCTGGAAACGGGTGG g-Bifid-R: GGTGTTCTTCCCGATATCTACA	50	24, 42
Lactobacillus group	Lab 159: GGAAACAG(A/G)TGCTAATACCG Lab 677: CACCGCTACACATGGAG	61	24, 43
Staphylococcus group	TStaG422: GGCCGTGTTGAACGTGGTCAAATCA TStag765: TIACCATTTCAGTACCTTCTGGTAA	58	24, 44
Staphylococcus epidermidis	J-StGen: TGGCCAAAAGAGACTATTATGA J-StEpi: CCACCAAAGCCTTGACTT	60	14
Universal/non specific	plb16: AGAGTTTGATCCTGGCTCAG mlb16: GGCTGCTGGCACGTAGTTAG	50	25

presence of maternal gut bacteria in breast milk.[19–21] This mechanism could also explain why oral administration of lactobacilli to pregnant women leads to its presence in the feces of breastfed infants born by caesarean section[22] that, therefore, are not exposed to the vaginal environment.

Until now, mother-to-child transmission studies have been focused mainly on potential probiotic bacterial groups, such as lactic acid bacteria and bifidobacteria. However, other dominant bacteria in breast milk, such as staphylococci,[12,14] have received marginal attention for their role in the early colonization of the infant gut despite evidence that they may also be dominant in the gut microbiota of breastfed infants.[14,23] In this context, the aim of this work was to investigate the potential mother-to-child transmission of staphylococci, lactobacilli and bifidobacteria at the strain level. For this purpose, we investigated the genetic background of isolates obtained from both milk and the fecal samples provided by each mother-infant pair.

Methods

Collection of the Samples

Twenty pairs of women and their respective exclusively breastfed infants (aged 7 days to 3 months) participated in the study. All were healthy and without any infant and/or mother perinatal problem. All volunteers gave written informed consent to the protocol, which was approved by the Ethical Committee of Hospital Clínico of Madrid (Spain). Milk samples were collected aseptically in a sterile tube by manual expression using sterile gloves. Previously, nipples and mammary areola had been cleaned with soap and sterile water and soaked in chlorhexidine (Cristalmina; Salvat, Barcelona, Spain). The first drops (~500 µL) were discarded. In addition, a feces sample (~5 g) was collected in individual sterile feces containers from their respective infants. The samples were kept at 4°C until delivery to the laboratory and immediately processed.

DNA Isolation From Breast Milk and Feces

Milk samples (1 mL) were centrifuged at 7,150 × *g* for 20 min and pellets were suspended into 1.4 mL of ASL buffer (QIAgen, Hilden, Germany). Feces (0.2 g) were also suspended in 1.4 mL of ASL buffer (QIAgen). The suspensions were vortexed with 0.1 mm zirconium beads (Biospec, Bartlesville, OK) using a FastPrep instrument (QBioGene, Irvine, CA) at a speed setting of 5.5 m/s for 30 s. Subsequently, total DNA was isolated using the QIAamp DNA Stool Mini Kit (QIAgen) according to the manufacturer's instructions. Purified DNA aliquots were stored at –20ºC.

Quantitative Real-Time PCR (qRTi-PCR) Assays

qRTi-PCR was used to assess the presence of DNA from *Staphylococcus, Bifidobacterium* and *Lactobacillus* in milk and fecal samples, as described by Collado et al.[24] For this purpose, a series of genus-specific primer pairs were used (Table 1). PCR amplification and detection were performed on optical-grade 96-well plates using an iQ5 Cycler Multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Each reaction mixture (25 µL) was composed of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 1 µL of each of the specific primers at a concentration of 0.25 µmol/L, and 1 µL of template DNA. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the non-targeted PCR product. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from pure cultures with a bacterial population ranging from 2 to 9 $\log_{10}$ colony-forming units (CFUs), as determined by plate counts. *S. epidermidis* CECT 231, *Bifidobacterium longum* CECT 4551 and *Lactobacillus delbrueckii* CECT 282, obtained from the Spanish Collection of Type Cultures (CECT), were used to construct standard curves. The bacterial

concentration in each sample was estimated as $\log_{10}$ genome equivalents by the interpolation of the Ct values obtained by the samples into the standard curves. The samples were analyzed in 2 independent qRTi-PCR assays.

Count and Identification of Bacteria in Breast Milk and Feces

Adequate dilutions of the fresh breast milk or fecal samples were spread onto Brain Heart Infusion (BHI; general medium) (Oxoid, Basingstoke, UK), Baird Parker (BP; selective for staphylococci) (BioMerieux, Marcy l'Etoile, France) and, Man, Rogosa and Sharpe (Oxoid, Basingstoke, UK) supplemented with L-cysteine (0.5 g/L) (MRS-Cys; for isolation of lactobacilli and bifidobacteria) agar plates. The plates were incubated for 48 h at 37°C in aerobic conditions, with the exception of MRS-Cys agar plates that were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an anaerobic workstation (MINI-MACS DW Scientific, Shipley, UK).

After incubation and counting, between 5 and 10 isolates from each culture medium were selected, including at least 1 representative of each colony morphology type. They were observed by optical microscopy to determine their morphology and Gram staining. Additionally, they were tested for catalase, oxidase and coagulase activities.

S. epidermidis identification was confirmed by a PCR method based on the amplification of a 249 bp fragment of the *dnaJ* gene from chromosomal DNA with primers J-StGen and J-StEpi (Table 1)[14] in an Icycler thermocycler (Bio-Rad Laboratories, Richmond, CA). A single colony of each isolate was suspended in 20 µL of deionized sterile water; 5 µL of the suspension were used as a template for PCR. For the other bacterial species, identification was confirmed using 16S rRNA primers plb16 and mlb16,[25] and further sequencing the obtained amplicon. Primers plb16 and mlb16 were originally designed for identification of *Lactobacillus acidophilus* members,[25] but several studies have shown that they are also useful for other *Lactobacillus* species and bacterial genera.[14,26] The resulting sequences were used to search sequences deposited in the EMBL database using BLAST algorithm and the identity of the isolates was confirmed on the basis of the highest score (≥99%).

Random Amplification of Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), or Multi Locus Sequence Typing (MLST)

Genetic relatedness among selected bacteria from mother and child was investigated by RAPD to avoid duplication of isolates from the same host sample and to determine if a given genotype was shared by mother and child pairs. RAPD profiles were obtained using primer OPL5 (5'-ACGCAGGCAC-3').[27] This primer was originally designed for lactobacilli but it has been shown to be useful also for typing bifidobacteria and staphylococci.[14] Computer-assisted analysis was performed with InfoQuest FP software (Bio-Rad Laboratories, Inc., Hercules, CA). Cluster analysis of RAPD pattern profiles was performed using the UPGMA method based on the Dice correlation similarity coefficient.

Those isolates with the same RAPD profile were subjected to PFGE. Briefly, chromosomal DNA was obtained from each isolate as previously described[28] and single digestions with *Sma*I, *Not*I, *Apa*I, and *Xba*I (New England Biolabs, Ipswich, MA) were performed depending on the different species isolated. Electrophoresis was carried out in a CHEF DR II apparatus (Bio-Rad, Birmingham, UK) in 1.0% (w/v) SeaKem Gold agarose (FMC, Philadelphia, PA) with 0.5 × TBE buffer (45 mM Tris/HCl, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 15°C. A constant voltage of 200 V was applied to the system. To separate *Sma*I fragments of *S. epidermidis* and *Lactobacillus* species a pulse time was applied from 5 to 15 s for 10 h and then another from 15 to 45 s for 12 h, except for *Lactobacillus fermentum* whose *Not*I fragments were separated by a pulse time from 0.5 to 20 s for 12 h and then another from 30 to 60 s for 8 h. Electrophoretic conditions for separating the *Apa*I fragments in *Staphylococcus hominis* were a pulse time from 0.1 to 30 s for 24 h. Finally, to separate *Xba*I fragments of *Bifidobacterium* isolates a pulse time from 1 to 15 s for 18 h was applied. Low-Range PFG marker and Mid-Range PFG marker I (New England BioLabs) were used as molecular size standards. Agarose gels were stained with ethidium bromide (0.5 µg/mL) and images were digitized with a GelPrinter Plus system (TDI, Madrid, Spain).

In addition, *S. epidermidis* isolates were also analyzed by multi locus sequence typing (MLST) following the recommendations previously published.[29] The method involves the amplification by PCR of 7 housekeeping genes encoding carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), ABC transporter (*gtr*), DNA mismatch repair protein (*mutS*), pyrimidine operon regulatory protein (*pyrR*), triosephosphate isomerase (*tpiA*) and acetyl-CoA acetyltransferase (*yqiL*) from chromosomal DNA. After sequencing, the fragments were trimmed, aligned, and compared with the allele sequences in the *S. epidermidis* MLST database (http://sepidermidis.mlst.net/). The new sequence types (ST) found were submitted to the database and registered under identity numbers 286 to 289.

Antimicrobial Susceptibility Testing

Susceptibility to penicillin, ampicillin, amoxycillin/clavulanate, oxacillin, gentamicin, tobramycin, amikacin, vancomycin,

Table 2. Bacterial counts in the breast milk and the infant feces samples in the different culture media included in this study.

	Bacterial count					
	BHI		BP		MRS-Cys	
Mother/infant pair	Breast milk	Infant feces	Breast milk	Infant feces	Breast milk	Infant feces
1	3.70 ± 0.21	9.66 ± 0.23	3.43 ± 0.18	8.93 ± 0.22	1.95 ± 0.17	9.05 ± 0.19
2	3.91 ± 0.14	9.45 ± 0.30	3.78 ± 0.17	7.63 ± 0.21	3.58 ± 0.15	5.81 ± 0.24
3	3.57 ± 0.19	8.93 ± 0.29	3.51 ± 0.11	7.91 ± 0.31	2.00 ± 0.14	8.49 ± 0.22
4	3.59 ± 0.11	9.22 ± 0.31	3.74 ± 0.17	7.93 ± 0.25	3.53 ± 0.19	8.59 ± 0.27
5	3.95 ± 0.14	8.75 ± 0.33	3.61 ± 0.14	7.78 ± 0.23	3.22 ± 0.21	8.55 ± 0.24
6	3.62 ± 0.17	8.11 ± 0.25	3.50 ± 0.11	7.64 ± 0.24	3.05 ± 0.13	7.57 ± 0.22
7	3.18 ± 0.14	9.40 ± 0.31	3.14 ± 0.14	9.10 ± 0.34	2.38 ± 0.20	9.07 ± 0.21
8	3.17 ± 0.16	7.75 ± 0.21	2.92 ± 0.12	6.05 ± 0.23	2.52 ± 0.12	7.15 ± 0.25
9	3.12 ± 0.14	9.30 ± 0.23	2.54 ± 0.16	9.02 ± 0.31	2.44 ± 0.16	9.00 ± 0.24
10	2.83 ± 0.15	7.87 ± 0.25	2.57 ± 0.16	7.75 ± 0.27	2.70 ± 0.15	7.84 ± 0.27
11	3.23 ± 0.12	7.00 ± 0.23	2.40 ± 0.21	7.00 ± 0.29	2.53 ± 0.16	6.48 ± 0.23
12	3.30 ± 0.11	6.86 ± 0.19	3.22 ± 0.14	6.30 ± 0.23	3.41 ± 0.11	6.77 ± 0.21
13	3.52 ± 0.16	6.98 ± 0.22	3.56 ± 0.17	6.53 ± 0.25	3.11 ± 0.13	6.28 ± 0.29
14	2.68 ± 0.15	6.70 ± 0.24	2.41 ± 0.15	6.58 ± 0.24	2.00 ± 0.14	6.53 ± 0.23
15	3.19 ± 0.11	7.65 ± 0.23	3.09 ± 0.16	7.46 ± 0.27	2.49 ± 0.14	7.52 ± 0.27
16	3.45 ± 0.13	6.72 ± 0.29	2.45 ± 0.14	6.34 ± 0.22	2.59 ± 0.17	6.64 ± 0.24
17	3.27 ± 0.14	8.74 ± 0.24	3.03 ± 0.19	7.06 ± 0.28	2.00 ± 0.16	7.81 ± 0.20
18	3.26 ± 0.11	6.96 ± 0.28	3.52 ± 0.16	6.18 ± 0.26	2.23 ± 0.14	6.53 ± 0.27
19	2.98 ± 0.23	8.83 ± 0.31	2.90 ± 0.11	8.72 ± 0.21	2.61 ± 0.21	9.14 ± 0.29
20	3.22 ± 0.25	7.82 ± 0.26	3.27 ± 0.14	7.72 ± 0.29	2.40 ± 0.10	7.78 ± 0.24

BHI, Brain Heart Infusion. BP, Baird Parker. MRS-Cys, Man, Rogosa and Sharpe supplemented with L-cysteine (0.5 g/L). Values are reported as mean $\log_{10}$ CFU/mL (milk) or $\log_{10}$ CFU/g (feces) ± standard deviation.

teicoplanin, daptomycin, levofloxacin, erythromycin, clindamycin, quinupristin/dalfoprystin, minocycline, linezolid, fosfomycin, co-trimoxazole, rifampicin, mupirocin, and fusidic acid were tested in all *S. epidermidis* isolates by the microdilution method using the semiautomatic system Wider System (Fco. Soria Melguizo, Madrid, Spain).

Results

Detection of Bacterial DNA in Breast Milk and Feces Samples

Breast milk samples from 20 women and fecal samples from their respective breast-fed infants were searched for the presence of DNA from bacteria that are commonly found in human milk. qRTi-PCR revealed the presence of DNA from *Staphylococcus, Bifidobacterium* and *Lactobacillus* in all the samples with the exception of 3 milk samples in which the *Lactobacillus* DNA content could not be detected in the experimental conditions used. Globally, the values ranged from 3.0 to 5.0, 1.5 to 5.5, and 4.0 to 4.8 $\log_{10}$ genome equivalents/mL for the genera *Staphylococcus, Bifidobacterium* and *Lactobacillus*, respectively, in the milk samples. In the fecal samples, the values ranged from 5.0 to 7.0, 5.5 to 9.5, and 4.5 to 8.5 $\log_{10}$ genome equivalents/g for the respective genera.

Bacterial Counts and Identification

In parallel, breast milk and fecal samples were cultured on BHI, BP and MRS-Cys agar plates. Bacterial counts obtained from the milk samples ranged from 2.68 to 3.95, 2.40 to 3.78, and 1.95 to 3.58 $\log_{10}$ CFU/mL in the BHI, BP and MRS-Cys plates, respectively. For the feces samples, counts ranged from 6.70 to 9.66, 6.05 to 9.10, and 6.48 to 9.14 $\log_{10}$ CFU/g in the respective growth media (Table 2).

Identification of the isolates at the species level in breast milk samples revealed the presence of culturable bacteria belonging to the genera *Staphylococcus* (present in all samples), *Lactobacillus* (in 80% of samples), and/or *Bifidobacterium* (in 15% of samples) (Table 3). The bacterial species more frequently found were *S. epidermidis*, *S. hominis, Lactobacillus casei, Lactobacillus gasseri, Lactobacillus gastricus*, *L. fermentum*, *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus salivarius*, *Lactobacillus vaginalis, Bifidobacterium breve*, and *B. longum*, as it is shown in Table 3.

Of interest, for 19 of the mother-infant pairs, the breast milk and feces shared culturable bacterial isolates belonging to 1 or more bacterial species and in the majority of cases (13 mother-infant pairs) 2 bacterial species were found (Table 3). *S. epidermidis* was the bacterial species

Table 3. Bacterial species and strains shared by mother-infant pairs.

	Bacterial species												
Pair	Sepi	Shom	Lcas	Lfer	Lgass	Lgast	Lpla	Lreu	Lsal	Lvag	Bbre	Blon	N	n
1	+/+	+/-							-/+				1	0
2	*/*	*/*											2	2
3	*/*	*/*						-/+				-/+	2	2
4	*/*	*/*		*/*							*/*		4	4
5	*/*		+/-						*/*				2	2
6		*/*		*/*					*/*				3	3
7	*/*						*/*						2	2
8	+/+	+/+				+/-		-/+					2	0
9	+/+	+/+				+/-							2	0
10	+/+	+/+			+/-								2	0
11	*/*			*/*									2	2
12	+/+	+/+			*/*					*/*			4	2
13	*/*				-/+			-/+				*/*	2	2
14	+/-			-/+	*/*				-/+	*/*			2	2
15	*/*	+/-		-/+		+/-		*/*					2	2
16	*/*	*/*	+/-		*/*	+/-						*/*	4	4
17	*/*	*/*				+/-							2	2
18	+/-					+/-		+/-					0	0
19	+/+			+/-			-/+						1	0
20	+/+	+/+		-/+		+/-	-/+						2	0

Sepi, *Staphylococcus epidermidis*. Shom, *Staphylococcus hominis*. Lcas, *Lactobacillus casei*. Lfer, *Lactobacillus fermentum*. Lgas, *Lactobacillus gasseri*. Lgast, *Lactobacillus gastricus*. Lpla, *Lactobacillus plantarum*. Lreu, *Lactobacillus reuteri*. Lsal, *Lactobacillus salivarius*. Lvag, *Lactobacillus vaginalis*. Bbre, *Bifidobacterium breve*. Blon, *Bifidobacterium longum* N, number of bacterial species shared by the mother-infant pair. n, number of bacterial strains shared by the mother-infant pair. +/+, the bacterial species was present in both the breast milk and infant feces samples. +/-, the bacterial species was present in the breast milk sample but it was absent from the infant feces sample. -/+, the bacterial species was absent from the breast milk sample but it was detected in the infant feces sample. */*, the same bacterial strain was isolated from both the breast milk and the infant feces samples.

shared by most mother-infant pairs (17 pairs), followed by *S. hominis* (detected in 11 mother-infant pairs). Both staphylococcal species were found simultaneously in the breast milk and infant feces of 50% of the pairs. Among *Lactobacillus* strains, *L. fermentum* and *L. gasseri* were the most frequenty shared and were found in 3 different mother-infant pairs each, followed by *L. salivarius* and *L. vaginalis*. On the other hand, *L. gastricus and L. casei* were found in 7 and 2 breast milk samples, respectively, but were not detected in any fecal sample (Table 3). Finally, isolates belonging to *Bifidobacterium* were present in only 3 breast milk samples, but the same species could also be found in the corresponding infant feces sample (Table 3).

Other species of lactic acid bacteria (*Lactococcus lactis*, *Leuconostoc garlicum*, *Pediococcus pentosaceous*, *Lactobacillus oris*, *Lactobacillus mucosae*, *Lactobacillus curvatus*, *L. delbrueckii*, *Lactobacillus graminis*, *Weissella* spp.) were also identified in this study in breast milk samples and/or fecal samples but they could only be isolated from 1 or 2 samples (results not shown) and, therefore, they were excluded from the genotyping assays.

Genotyping of the Staphylococci, Lactobacilli, and Bifidobacteria Isolated From Milk and Feces

When the same staphylococci, lactobacilli or bifidobacteria species was found in the breast milk sample and in the respective infant feces sample, all isolates from that bacterial species in that mother-infant pair were initially typified by the RAPD technique. The analysis of the profiles revealed the existence of some RAPD profiles that were shared within some mother-infant pairs (Figure 1).

Later, PFGE typing of the different RAPD genotypes confirmed that the same specific bacterial strain was present in, at least, 11 mother-infant pairs for staphylococci (*S. epidermidis*, *S. hominis*), 9 for lactobacilli (*L. gasseri*, *L. fermentum*, *L. salivarius*, *L. vaginalis*, *L. plantarum*), and 3 for bifidobacteria (*B. breve*, *B. longum*) (Figure 2; Table 3). Of all the mother-infant pairs, numbers 4 and 16 shared the highest number of strains (n = 4; *S. epidermidis*, *S. hominis*, *L. fermentum*, and *B. breve* for pair number 4 and *S. epidermidis*, *S. hominis*, *L. gasseri*, and *B. longum* for pair number 16).

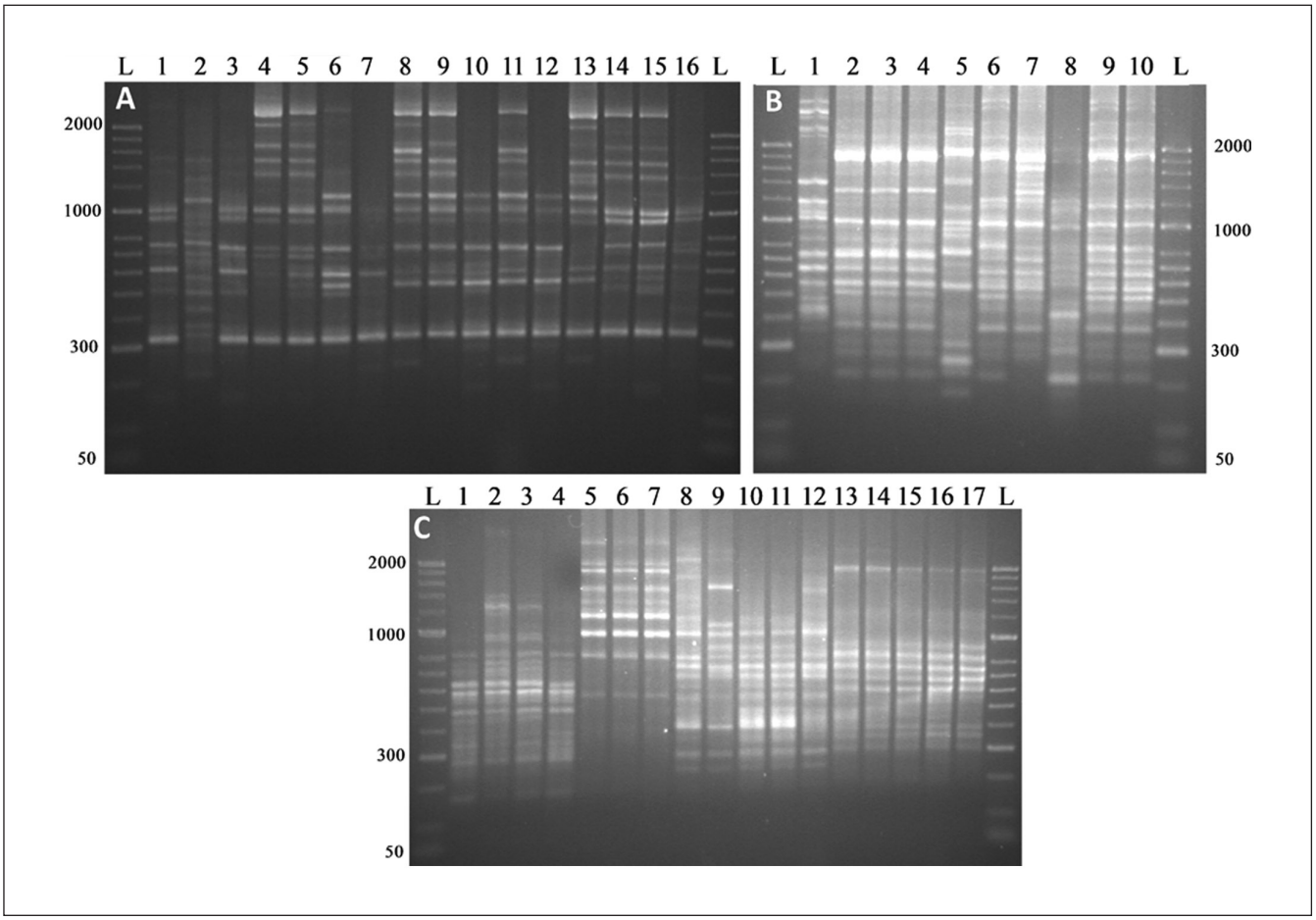

Figure 1. RAPD patterns of isolates from milk samples (M) and infant faeces samples (F). (A) *Staphylococcus epidermidis* isolates. Lane 1: isolate from sample F2; lanes 2–3: isolates from sample M2; lane 4: isolate from sample F4; lanes 5–7: isolates from sample M4; lanes 8–9: isolates from sample F13; lanes 10–12: isolates from sample M13; lanes 13–14: isolates from sample F15; lanes 15–16: isolates from sample M15; (B) *Bifidobacterium longum* isolates. Lanes 1–2: isolates from sample F4; lane 3–4: isolates from sample M4; lanes 5–7: isolates from sample F16; lanes 8–10: isolates from sample M16. (C) *Lactobacillus* isolates. Lanes 1-2: *Lactobacillus salivarius* isolates from sample F5; lanes 3–4: *Lactobacillus salivarius* isolates from sample M5; lane 5: Lactobacillus salivarius isolate from sample F6; lane 6–7: *Lactobacillus salivarius* isolates from sample M6; lanes 8-10: *Lactobacillus plantarum* isolates from F7; lanes 11–12: *Lactobacillus plantarum* isolates from M7; lanes 13–14: *Lactobacillus vaginalis* isolates from F12; lanes 15–17: *Lactobacillus vaginalis* isolates from M12.

S. epidermidis MLST results further confirmed in 4 mother-infant pairs that strains isolated from both milk and feces have identical allele composition. Allelic sequences detected in the 4 pairs of strains had not been previously described, and the new STs found were registered in the MLST web site as ST286 (8,30,23,9,6,9); ST287 (8,30,1,5,7,6,36) ST288 (1,1,1,23,9,1,1), and ST289 (3,3,13,1,2,4,4).

Antibiotic Susceptibility

The antibiotic resistance profile of 8 *S. epidermidis* isolates from the same 4 mother-infant pairs showed that those isolates obtained from the same mother-child pair had identical minimal inhibitory concentration values for all studied antimicrobials. Resistance was only observed for penicillin and ampicillin, and, additionally, to oxacillin in 1 pair and to fosfomycin in another pair. These results further confirm the identity of those *S. epidermidis* isolates from breast milk and infant feces in 4 mother-infant pairs.

Discussion

This study showed that some mother-infant pairs shared bacterial strains belonging to different bacterial genera, which suggests that breast milk may be included among the potencial sources of bacteria to the infant gut. The commensal and potentially probiotic bacteria, including staphylococci, streptococci, bifidobacteria and lactic acid bacteria present in the breast milk may contribute to the maturation of the gut barrier and the gut-associated lymphoid tissue.[9,12–14]

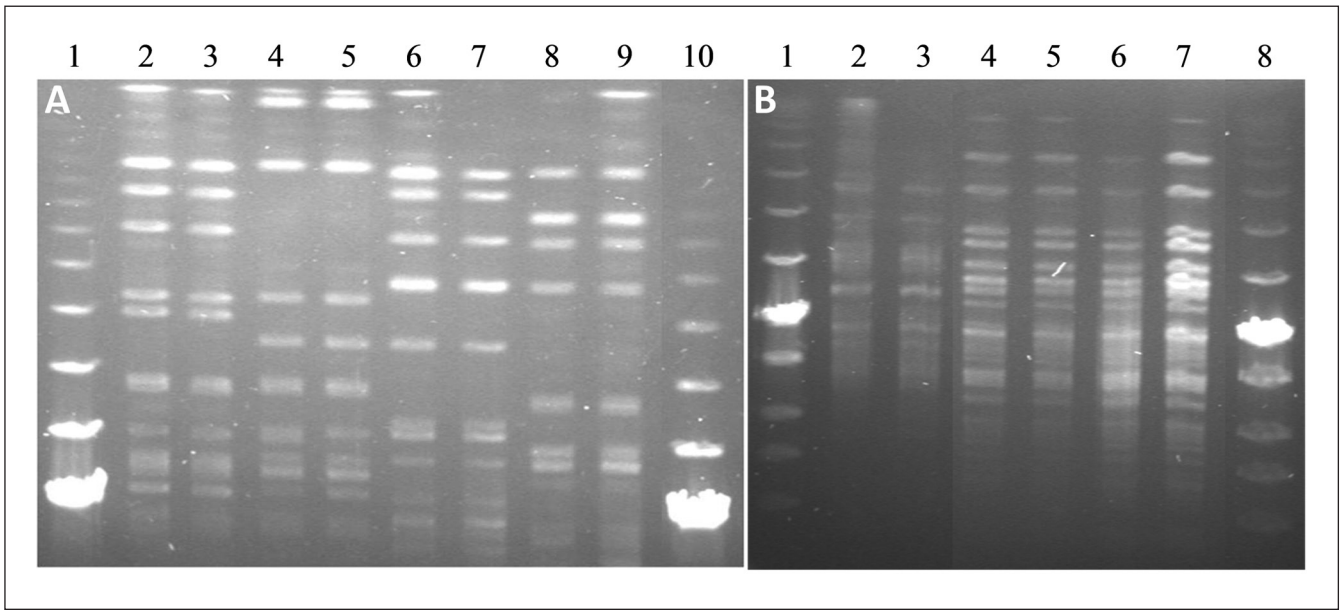

Figure 2. PFGE patterns of isolates from milk samples (M) and infant faeces samples (F). (A) *Sma*I digested genomic DNA of *Staphylococcus epidermidis*. Lanes 1, 10: MidRange II PFGE Marker (New England BioLabs); lane 2: isolate from sample M10; lane 3: isolate from sample F10; lane 4: isolate from sample M12; lane 5: isolate from sample F12; lane 6: isolate from sample M17; lane 7: isolate from sample F17; lane 8: isolate from sample M31; lane 9: isolate from sample F31; (B) *Xba*I digested genomic DNA of Bifidobacteria isolates. Lanes: 1, 8: LowRange PFGE marker (New England BioLabs) lane 2: *Bifidobacterium breve* isolate from sample M12; lane 3: *Bifidobacterium breve* isolate from sample F12; lanes 4-5: *Bifidobacterium longum* isolates from sample M64; lanes 6-7: *Bifidobacterium longum* isolates from sample F64.

In fact, the bacterial composition of the fecal microbiota of the breast-fed infant usually reflects that of breast milk.[12,14]

In the past, different culture-based studies suggested the existence of a mother-to-infant transfer of certain bacteria through breast milk and the recent application of culture-independent molecular techniques, and particularly those based on 16S rRNA genes, has confirmed the influence of this biological fluid on the bacterial colonization of the neonatal gut.

Staphylococcus was the genus isolated with the highest frequency. In fact, comparison of the PFGE profiles revealed that at least 8 to16 mother-infant pairs shared *S. epidermidis* strains and these results were confirmed by using the MLST technique. Similarly, sharing of RAPD profiles were observed among *S. epidermidis* isolates obtained in 12 of 16 mother-infant pairs in a previous study.[14] Several studies showed that *S. epidermidis* was the predominant species in milk and feces of breast-fed infants while it was less prevalent in those of formula-fed infants.[12,14,23,30,31] More recently, it has been shown that coagulase-negative staphylococci colonized 100% of breast-fed Western infants from day 3 onward.[32] Some authors suggest that, in fact, staphylococcal colonization of the infant gut has increased from the 1970s to the present.[32,33]

In relation to lactobacilli, 9 mother-infant pairs shared at least one strain (*L. gasseri*, *L. fermentum*, *L. salivarius*, *L. vaginalis*, *L. plantarum*, and/or *L. reuteri*) as revealed by PFGE profiling. Martín et al.[13] isolated lactic acid bacteria from milk of 8 healthy mothers, and feces of their respective breast-fed infants, and found that some *L. gasseri* isolates obtained from milk and feces of a mother-infant couple displayed identical RAPD profiles. None of the lactic acid bacteria isolated from breast skin or vaginal swabs shared RAPD profiles with those isolated from breast milk or mammary areola, which suggested an endogenous origin.[13] Recently, it has been shown that oral administration of lactobacilli strains isolated from human milk to women led to their transfer from the maternal gut to the mammary gland, since such strains were isolated from their milk some days later.[34,35] The application of molecular techniques, such as PCR-DGGE and library cloning, have also indicated that maternal milk and feces often share DNA from certain species belonging to the *Lactobacillus* group.[8] Therefore, it is not strange that *Lactobacillus* counts are significantly higher in breastfed than formula-fed or weaned infants.[36,37] Breast milk lactobacilli may easily colonize the neonatal gut since gastric conditions (pH, emptying time) in infants are less restrictive than those found in the adult stomach.

In contrast to staphylococci, sharing of bifidobacteria was observed in only 3 mother-infant pairs; this fact is probably due to the fastidious growth requirements of bifidobacterial species. Recently, bifidobacteria were isolated from 12 breast milk and 20 infant feces samples; interestingly, those species

present in the milk samples (*B. breve*, *Bifidobacterium adolescentis* and *Bifidobacterium bifidum*) were also isolated from feces of the respective infants.[9] Molecular techniques used in different works have revealed that the bifidobacterial diversity in breast milk and infant feces was specific of each mother-infant pair.[9,17] Bifidobacteria were first isolated a century ago from infant feces[38] and, since then, they have been associated with a healthy infant gut because of their predominance in breast-fed infants in comparison to formula-fed ones.[39,40]

The fact that, globally, lactobacilli and bifidobacteria were isolated from a higher number of infant feces samples (in comparison with the corresponding breast milk samples) may be the consequence of their rather different concentration (>10^7 CFU/g in feces versus <10^3 CFU/mL in breast milk), together with their fastidious growth requirements, as suggested previously.[9] The main exception were the isolates belonging to the *L. gastricus* species, which could only be isolated from milk. This fact may reflect a lower rate of growth of this particular species in the infant gut although work is in progress to elucidate the potential role of such species in the neonatal gut.

The elucidation of the origin of the bacteria present in breast milk will be an attractive research target in the future. Traditionally, it was considered that they are acquired by skin or oral contamination. Obviously, sampling of breast milk for microbiological analysis must take into account that skin contamination is almost unavoidable and that doubts on the original location (internal mammary gland or skin) of the isolated bacteria may arise; however, the latter source seems very unlikely at least in the case of bifidobacteria since they belong to a strictly anaerobic genus.[41]

In conclusion, the results of this work show the presence of the same specific bacterial strains of *Bifidobacterium, Lactobacillus*, and *Staphylococcus* in breast milk and infant fecal samples and suggest that breast milk may be an additional source of bacteria to the infant gut.

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