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THE MICROBIOTA OF HUMAN MILK IN HEALTHY WOMEN

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Abstract

Recent work has shown that human colostrum and milk, which had been traditionally considered sterile, provides a continuous supply of commensal and potential probiotic bacteria to the infant gut. More than 200 different bacterial species, including staphylococci, lactic acid bacteria and bifidobacteria, have been isolated from human milk samples so far, although the cultivable bacterial diversity found in individual samples is much lower (2 to 8 different species per women). Interestingly, the same bacterial strains have been found in both breast milk and infant feces of different mother-infant pairs, confirming the role of human milk on the bacterial colonization of the infant gut. These commensal bacteria could protect the infant gut and direct, at least partly, the maturation of the immune system, among other functions. Different studies suggest that some bacteria present in the maternal gut could reach the mammary gland during late pregnancy and lactation through a mechanism involving gut dendritic cells and macrophages. Thus, modulation of maternal gut microbiota during pregnancy and lactation could have a direct effect on infant health.

Key words: Human milk, breastfeeding, bacteria, microbiota, dendritic cells, mastitis, probiotics.

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BACTERIAL DIVERSITY OF HUMAN MILK

Human milk constitutes the best feeding during the first months of life since it usually satisfies all the nutritional requirements of the rapidly-growing infant; additionally, it educates the infant immune system and confers a certain degree of protection against pathogens (75). Therefore, it is not strange that breastfeeding of preterm newborns is associated to a significantly lower incidence of necrotizing enterocolitis and septicaemia, a faster tolerance to enteral nutrition and a lower dependence on parenteral nutrition (55,100,102). These effects result from the synergistic action of many bioactive molecules, present in colostrum and milk, including inmunoglobulins, inmunocompetent cells, fatty acids, polyamines, oligosaccharides, lysozyme, lactoferrin, and other glycoproteins, and antimicrobial peptides (77).

In the last decade, several studies have revealed that colostrum and breast milk of healthy women harbour commensal and potentially beneficial bacteria (9,38,45,46,63,65,66,69). This is a relevant finding since human milk was traditionally considered to be sterile despite the lack of scientific evidences supporting such assumption. Obviously, milk bacteria may reach and colonize the infant gut and, therefore, this biological fluid may constitute one the main sources of bacteria to the breastfed infant gut. However, the exact roles of the different bacteria provided by breast milk in infant gut colonization have not been elucidated yet. Bacterial colonization of the human gut is a complex process that seems to starts, at a small scale, during the fetal period (8, 23,43,44). 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 (57,67,68). As a consequence, factors such as composition of the maternal microbiota, mode of delivery 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 (40,72).

Culture-based descriptions of the bacterial diversity of breast have showed the common isolation of staphylococci, streptococci, peptostreptococci, micrococci, corynebacteria, enterococci, lactococci, lactobacilli, propionibacteria, bifidobacteria and other closely related Gram-positive bacteria (30,38,63,69,112), with a occasional presence of Escherichia coli (88). Among them, coagulase-negative staphylococci (such as Staphylococcus epidermidis) and viridans streptococci (such as Streptococcus mitis or Streptococcus salivarius) seem to be the dominant cultivable bacteria found in this biological fluid. The fact that the bacteria cited above are easily isolated from fresh milk of healthy women from distant countries indicates that their presence in this substrate is a common event. Therefore, they should be considered as components of the natural microbiota of the human milk rather than as mere contaminant bacteria.

Up to the present, our research group has isolated more than 200 different bacterial species, belonging to approximately 50 different genera, from human milk. In addition, this has been the source of new bacterial species, such as Streptococcus lactarius (70). However, the number of cultivable bacterial species found simultaneously in the milk of a healthy woman seems to be relatively low and ranges from 2 to 18 different species (3,71). It must be highlighted that some microorganisms (enterobacteria, yeasts) may be present in milk as a result of a non-hygienic sample

collection, including the use of contaminated milk pumps (15,16,62).

The application of culture-independent molecular techniques, and particularly those based on 16S rRNA genes, allowed a complementary assessment of the biodiversity of the human milk microbiota. Typically, this approach involves extraction of the DNA from the biological samples, PCR amplification of 16S rRNA gene fragments with universal or group-specific bacterial primers, analysis of PCR products by fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and/or, the construction of clone libraries to assess the variety of 16S rRNA gene sequences present. The use of culture-independent techniques confirmed the presence of DNA belonging not only to staphylococci, lactic acid bacteria and bifidobacteria but, also, to other bacterial groups, such as some Gram-negative bacteria (34,67,68,69,88).

In addition, the application of the -omics approach (genomics, metagenomics, transcriptomics, proteomics, metabolomics) to the study of the human mammary microbiota is already in progress and there is no doubt that the results provided by such techniques will open new perspectives to understand the initiation and development of the infant gut microbiota. Recently, the first microbiome study focused on human milk was published (41). The authors used microbial identification techniques based on pyrosequencing of the V1-V2 region of the bacterial 16S rRNA gene to characterize the bacterial communities present in milk samples collected from 16 women self-described as healthy at three time-points over four weeks. Results indicated that milk bacterial communities were generally complex and, although a few genera (Streptococcus, Staphylococcus, Serratia) represented greater than 5% of the relative community abundance, eight other ge era represented ≥ 1% of the communities observed across samples. Additionally, assignment of sequences into operational taxonomic units (OTUs) using a 3% similarity cut off identified 100–600 OTUs present in the samples from each subject. These results suggest that human milk may contain a higher bacterial diversity than previously reported. Among the hundreds of operational taxonomic units (OTUs) detected in the milk of every woman, only 9 (Streptococcus, Staphylococcus, Serratia, Pseudomonas, Corynebacteria, Ralstonia, Propionibacterium, Sphingomonas and Bradyrhizobiaceae) were present in every sample from every woman. These 9 "core" OTUs represented approximately half of the microbial community observed, although their relative abundance varied greatly between subjects. The remaining half of the community was not conserved across women. On the other hand, milk bacterial community was generally stable over time within an individual.

In this microbiome study, species belonging to the Lactobacillus group (which includes the genera Lactobacillus, Pediococcus, Leuconostoc and Weissella) and to the Genus Bifidobacterium, were very underrepresented despite they are commonly isolated from human milk This is not strange since the study of bacterial diversity using molecular methods faces important bias in relation to differential DNA isolation and amplification from biological samples, which tend to minimize the presence of DNA from such bacterial groups. Previously, Roger et al. (98) reported that Bifidobacterium component (relative abundance and diversity) of the infant faecal microbiota has been under-represented in recent metagenomic studies investigating the human microbiome (50,84,109). A comparison of compositional and functional data from a 2.5-year case study of the fecal microbiome of a breastfed infant highlighted inconsistencies between the 16S rRNA gene data and those generated from shotgun sequencing, and indicated that a "16S rRNA gene primer" bias was the most likely explanation for the discrepancy (50). This could also explain the relative lack of abundance of bifidobacteria (and/or Actinobacteria) in metagenomic studies of the infant gut microbiota (84).

The results of a second analysis of the breast milk microbiome involving 18 mothers indicated that milk bacteria are not contaminants and suggested that this site-specific microbiome is influenced by several factors that significantly skew its composition (17). More recently, our research group analyzed the metagenome of human milk samples obtained from healthy, mastitis-suffering or obese women, and observed that the bacterial community of breast milk may differ depending on the individual and on the health status of the lactating women (unpublished results)."

BREAST MILK: A SOURCE OF BACTERIA TO THE INFANT GUT

Some studies have already revealed the strong influence that this biological fluid exerts on the bacterial colonization of the neonatal gut. In a pioneer work, samples of fresh breast milk, vaginal swabs and infant feces collected from five mother-infant pairs whose neonates were born by vaginal delivery and from other five that had their babies by programmed caesarean section were submitted to PCR amplification using Lactobacillus group-specific primers (68). Subsequently, the amplicons were analyzed by DGGE and, parallel, clone libraries were constructed to describe the Lactobacillus-group microbial diversity. Interestingly, none of the species detected among the vaginal samples were found in breast milk-derived libraries and only a few were detected in 16S rRNA gene libraries from infant feces. In contrast, the profiles of Lactobacillus sequences retrieved from infant feces were more similar to those retrieved from breast milk of the respective mothers. Similarly, a molecular epidemiological study on the transmission of vaginal Lactobacillus species from mother to the newborn infant showed, that only less than one-fourth of the infants acquired maternal vaginal lactobacilli at birth, and that 1 month later, they had been replaced by lactobacilli associated with human milk (73).

However, sharing of a similar species pattern between samples is not a proof of bacterial transmission and studies at the strain level were required to determine if, actually, breast milk was a source of bacteria to the infant gut. In this context, some recent studies have revealed the presence of the same bacterial strains in both breast milk and infant feces of different mother-infant pairs (3,59,63,71). The bacterial strains shared by the mother-infant pairs included a variety of species belonging to the genera Staphylococcus, Enterococcus, Pediococcus, Lactobacillus, and Bifidobacterium. In fact, human milk may constitute one the main sources of bacteria to the infant gut and, therefore, it is not strange that composition of the infant gut microbiota reflects that of breast milk (38). It has been estimated that a baby consuming approximately 800 ml/day

Table 1. Composition of human milk microbiota. Main bacterial species isolated or detected by independent-culture techniques in breast milk from healthy women.

Staphylococcus	***Streptococcus***	***Lactobacillus***	***Bifidobacterium***	**Other LAB**	**Other Gram + bacteria**	**Gram - bacteria**
S. aureus	*S. bovis*	*L. fermentum*	*B. adolestentis*	*Enterococcus faecalis*	*Actinomyces odontolyticus*	*Acinetobacter johnsonii*
S. epidermidis	*S. mitis*	*L. gasseri*	*B. bifidum*	*Enterococcus faecium*	*Arthrobacter cumminsii*	*Bacteroides* sp.
S. hominis	*S. oralis*	*L. gastricus*	*B. breve*	*Lactococcus lactis*	*Bacillus vietnamiensis*	*Burkholderia multivorans*
S. xylosus	*S. parasanguis*	*L. plantarum*	*B. catenolatum*	*Leuconostoc citreum*	*Bacillus pumilus*	*Citrobacter freundii*
S. haemolyticus	*S. salivarius*	*L. lactarius*	*B. infantis*	*Leuconostoc fallax*	*Corynebacterium aurimucosum*	*Escherichia coli*
S. lugdunensis	*S. infantis*	*L. reuteri*	*B. longum*	*Leuconostoc mesenteroides*	*Corynebacterium coyleae*	*Klebsiella milletis*
	S. peroris	*L. rhamnosus*		*Pediococcus pentosaceous*	*Corynebacterium pseudogenitalium*	*Klebsiella oxytoca*
		L. salivarius		*Weissella cibaria*	*Gemella haemolysans*	*Klebsiella pneumoniae*
		L. vaginalis		*Weissella confusa*	*Kocuria kristinae*	*Kluyvera cryocrescens*
					Kocuria rhizophila	*Pseudomonas aeruginosa*
					Micrococcus luteus	*Pseudomonas pseudoalcaligenes*
					Paenibacillus amylolyticus	*Pseudomonas synxanthia*
					Propionibacterium acnes	*Serratia proteamaculans*
					Propionibacterium granulosum	
					Rothia mucilaginosa	

of milk will ingest between 1×10^5 and 1×10^7 bacteria daily. A high proportion of such bacteria would reach the infant gut in a viable state since, in comparison to adults, the time of transit through the stomach is shorter in breastfed infants, while their stomach pH is higher.

Exposure of the breastfed infant to milk bacteria may be one factor contributing to the differential fecal microbiota between breastfed and formula-fed infants (12,27,36,57,114). Such differences involve all the main bacterial groups commonly found in breast milk. Coagulase-negative staphylococci were, at least quantitatively, the first bacterial group found in a recent work describing the microbiome of human milk (41). Previously, culture-based methods, had revealed that S. epidermidis is the most prevalent species both in human milk and in feces of breastfed infants while it is practically absent in those of formula fed-infants (45). Therefore, this species can be considered as a differential trait of the fecal microbiota of breastfed infants (45). Studies carried 20 years ago already described that staphylococci were common in feces of breastfed infants (6,56,101). More recently, it has been shown that coagulase-negative staphylococci colonized 100% of breastfed Western infants from day 3 onwards (1). Such staphylococci colonized vaginally- and cesarean section-delivered infants equally early.

In relation to lactic acid bacteria, some species belonging to the Lactobacillus group (which includes the genera Lactobacillus, Pediococcus, Leuconostoc and Weissella) are commonly isolated from milk; however, their prevalence may be somehow underrated independently of the use of culture-dependent or culture-independent techniques: their isolation is often more difficult than that of other bacteria while DNA isolation methods and currently used primers often lead to the preferential amplification of DNA sequences belonging to other bacterial groups. Anyway, a molecular survey on the Lactobacillus species present in the gut microbiota of Swedish infants showed that they were significantly more often isolated from the feces of infants receiving breast milk than from weaned infants (2). Similarly, it has been observed that at 6 months, Lactobacillus counts are significantly higher in breastfed than in formula-fed infants (97). Lactococci, enterococci and viridans streptococci are also frequently isolated from breast milk and form feces of breastfed infants (1,3,9,38,45,46,95). Interestingly, streptococci was the second bacterial group found in the microbiome study cited above (41).

Bifidobacteria were first isolated a century ago from infant feces and were quickly associated with a healthy infant gut because of their predominance in breastfed infants in comparison to formula-fed ones (106,107). Since then, it was widely accepted that bifidobacteria represent one of the most important bacterial groups in the infant gut and that a delayed bifidobacterial colonization or decreased bifidobacterial numbers may increase the susceptibility to a variety of gastrointestinal or allergic conditions (83,103). Presence of bifidobacterial DNA (34,69,88) or live bifidobacteria (59,69) in breast milk has been reported. Recent studies have confirmed that breastfed infants harboured a more complex Bifidobacterium diversity than formula-fed infants (51,98,109).

Finally, Escherichia coli, Klebsiella spp. and other enterobacteria can be occasionally found in breast milk of healthy women (67,88). Molecular studies have shown that E. coli is one of the first colonizers of the infant gut, where it can coexist with Gram-positive bacteria (27). Previously, evidence for mother-to-infant transmission of Enterobacteriaceae was obtained in four out of five cases (105). The species E. coli comprises non-pathogenic as well as pathogenic bacteria; commensal strains generally represent normal and ecologically important inhabitants of the human mucosal surfaces (5). In fact, E. coli strain Nissle 1917 (O6:K5:H1) forms the basis of an infant probiotic preparation and different studies have shown that its oral application to full-term and premature infants reduces the number and incidence of infections, significantly stimulates specific humoral, and cellular responses and simultaneously induces non-specific natural immunity (21,33,54). Although infant nutrition has experimented notable advances in the last decades, there are still big qualitative and quantitative differences between human milk and any infant formula. From the microbiological point of view, breast milk contains a spectrum of different bacterial genera, species and strains at a rather moderate level (~103 cfu/ml), together with a wealth of bacterial DNA signatures. In contrast, some infant formulas have incorporated just one or two strains, belonging to the genera Bifidobacterium or Lactobacillus, at a relatively high concentration. Since the different bacteria found human milk may also play different functions in the infant gut (see next section), this aspect should be had in account in future studies comparing the gut colonization patterns of breastfed infants and that of infant fed with infant formula containing bacteria.

Presence of bacteria in human milk may have some practical connotations for Human Milk Banks. Although criteria to define a bacterial contamination vary considerably between Banks (from 103 to 105 cfu/ml), such counts are usually met in fresh milk obtained from healthy women (3,38,62,63,88). Despite the results of a variety of culture-based studies that indicate that enterococci, staphylococci or streptococci are normal inhabitants of human milk, the mere presence of these bacteria would be enough to consider the milk unfit for consumption. Fortunately, cultures are regarded as unnecessary when the extracted milk is used to feed the mother's own baby. Therefore, it is not strange that a bacteriological screening of expressed breast milk in Chinese women (78) showed that between 63 and 86% of the samples would be considered contaminated following the criteria of different Milk Banking Associations (74). However, and to the surprise of the medical staff, this contaminated milk was the one that provided the highest benefits to neonates and infants in a Chinese orphanage, including the lowest rates of necrotizing enterocolitis (78). If the biological importance of milk bacteria is further confirmed, it should lead to a change in the management of Milk Banks, since current bacteriological criteria can have a negative impact on, at least, many term and preterm neonates. In the future, pasteurization of donors' milk could also be reconsidered, particularly when routine screening of donors or donor milk for cytomegalovirus and other viruses becomes available.

FUNCTIONS OF HUMAN MILK BACTERIA IN THE INFANT GUT

In the last years, some studies have shown that bacteria found human milk may play several functions in the infant gut, including protection against pathogens, immunomod-

ulation, and contribution to the host metabolism (Fig. 1). Although most research efforts have been focused on lactobacilli and bifidobacteria, due to their obvious commercial connotations, other bacteria present in human milk, including staphylococci or streptococci seem to have, at least, the same biological relevance. Independently of the species, the safety of specific bacterial strains for human applications must be carefully assessed on a strain-by-strain manner, including in vitro, in vivo and human assays (26,31,53,60).

First of all, they can contribute to the reduction of the incidence and severity of infections in the breastfed infant by different mechanisms, including competitive exclusion (79), production of antimicrobial compounds (65,66,79), and improvement of the intestinal barrier function (79). Recently, the administration of a human milk Lactobacillus strain to infants during 6 months led to 46%, 27%, and 30% reductions in the incidence rates of gastrointestinal infections, upper respiratory tract infections, and total number of infections, respectively (61). Commensal coagulase-negative staphylococci and viridans streptococci provided by breast milk can be particularly useful to reduce the acquisition of undesired pathogens by infants exposed to hospital environments. In fact, some S. epi-

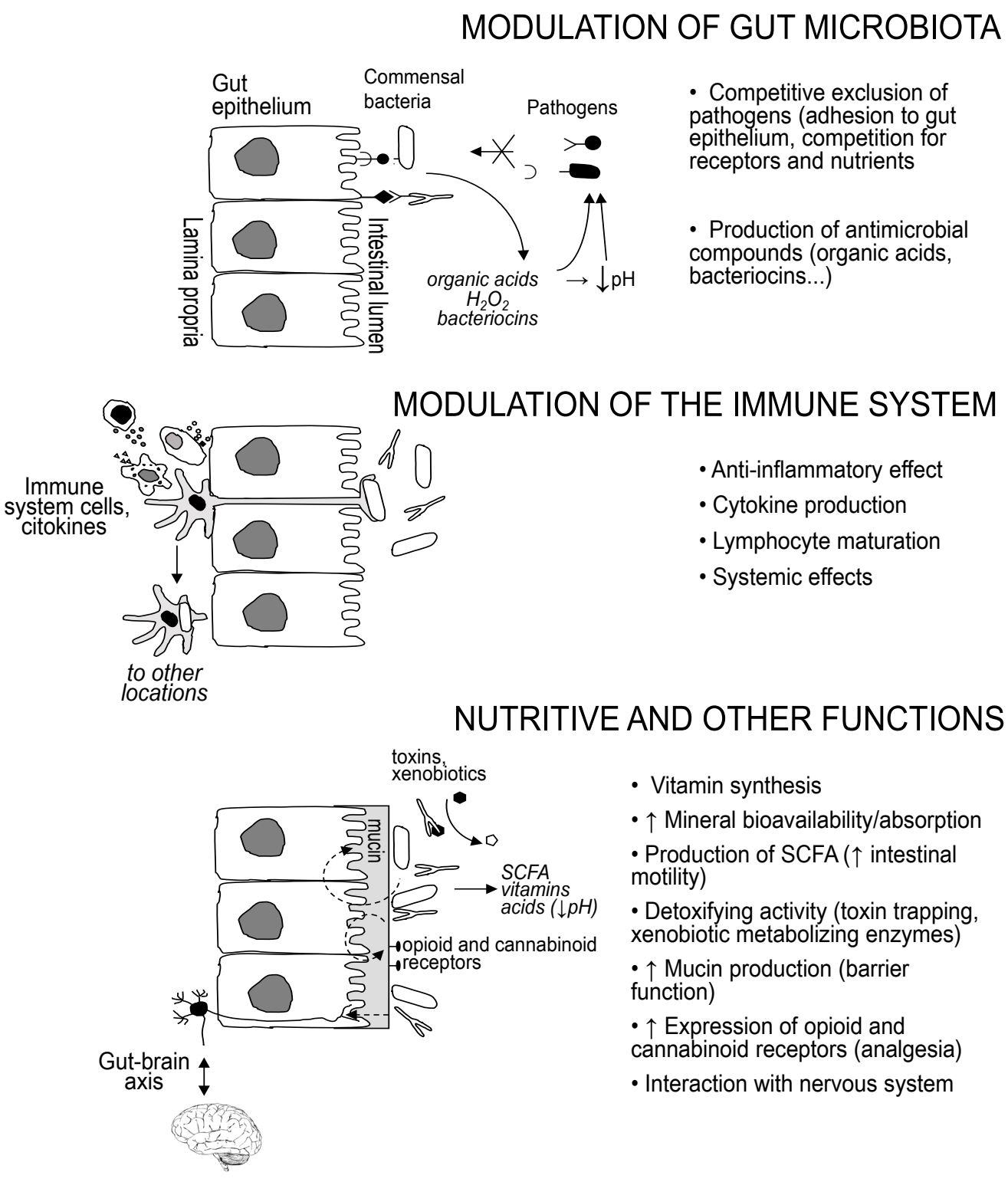

Figure 1. Potential functions of human milk microbiota in the infant gut.

dermidis and viridans streptococcal strains that inhibit in vivo colonization by methicillin-resistant Staphylococcus aureus have been postulated as a future strategy to eradicate such pathogen from the human mucosal surfaces, including those of high-risk newborns exposed to hospital environments (42,86,108). Therefore, their application as probiotics in neonatal units could be considered in the future if research in progress (including complete genome sequencing and analysis) confirm the efficacy and safety of selected strains. In fact, exclusion of pathogenic staphylococci and streptococci that use to be highly prevalent in hospital environments by those found in human milk may be one of the reasons explaining the success of the kangaroo-mother method in the management of preterm neonates (unpublished results).

Milk bacteria may also contribute to the correct maturation of the infant immune system since at least some strains have the ability to modulate natural and acquired immune responses in vitro, in animal models and in humans (24,80,81,89). Interestingly, this immunomodulatory function is flexible and depends on the conditions found in the gut environment. In this context, L. salivarius CECT5713 and L. fermentum CECT5716 enhanced macrophage production of Th1 cytokines, such as IL-2 and IL-12 and the inflammatory mediator TNF-α, in the absence of an inflammatory stimulus. However, both strains led to a reduction of Th1 cytokines when cells were incubated in the presence of lipopolysaccharide (24). An independent study confirmed that both lactobacilli strains display a broad array of effects on both innate and acquired immunity, acting as potent activators of NK cells and moderate activators of CD4+ and CD8+ T cells and regulatory T cells (89). The authors compared these strains with others belonging to the same species but isolated from sources different to breast milk and found some milk strain-specific effects, such as a higher induction of IL-10 and IL-1 production. It is also interesting to note that the presence of viridans streptococci, one of the dominant bacterial groups in human milk, seems to be a feature of the healthy infant gut in contrast to that of the atopy-suffering infants (49).

Finally, human milk bacteria have a remarkable potential to play metabolic roles in the infant, and aspect that is being actively investigated at present. While the glycobiome of some human milk strains may help to create a specific "healthy" microbiota in the infant gut (115), these microorganisms might also contribute to infant digestion through the breakdown of sugars and proteins. Previously, it has been shown that human milk lactobacilli strains are metabolically active in the infant gut and increase the production of functional metabolites such as butyrate. As a result, they improve the intestinal habit, with an increase in faecal moisture, and in stool frequency and volume (31,60).

ORIGIN OF THE BACTERIA ISOLATED FROM BREAST MILK

The origin of the bacteria present in breast milk has been a controversial issue in the last years. In the past, it was suggested that, if milk harbored bacteria, it was just due to contamination with bacteria from the mother's skin or the oral cavity of the infant. Infrared photography (91) has shown that a certain degree of retrograde flow back into the mammary ducts can occur during suckling. Such back flow may provide an ideal route for the exchange of bacteria from the infant's mouth into the mammary gland but it is also true that breast milk can also be a source of bacteria to the infant's mouth. It should highlighted that ecological niches in the human microbiome are not thought to be isolated environments, but rather a network of interrelated communities experiencing constant exchange (20). Therefore, it is very likely that milk or mammary bacterial communities are no the exception, and that they are constantly influenced by exposure to other microbial populations associated with the mother and her infant. Little is known about the infant human salivary microbiome but investigations on adults have revealed that Streptococcus species are the dominant phylotype in this fluid (76,113) while such dominance is even higher in edentulous infants (19). Streptococcus species are among the most abundant phylotype in colostrum and milk samples (45,46,41), supporting the hypothesis that the maternal milk bacteria may play an important role in establishing salivary bacterial communities and viceversa.

Some bacterial phylotypes usually present on adult skin, such as Staphylococcus, Corynebacteria and Propionibacteria (29,32), are also common in human milk. This presents the possibility that interactions with the maternal skin microbiota may also contribute to shape the composition of milk microbiota. However, a comparison of the bacterial communities detected in milk to those of the sebaceous skin found on the breast indicates that although the two communities share some phylotypes, major differences also exist (41). Such relevant differences between the two communities indicate that bacterial communities in milk are not simply a result of skin contamination (41). Similar conclusions have been obtained recently in a second study of the human milk microbiome (17). Obviously, sampling of breast milk for microbiological analysis must have into account that skin contamination is almost unavoidable and that, logically, doubts on the original location (internal mammary gland or skin) of the isolated bacteria may arise. However, independent studies have shown that lactobacilli or bifidobacteria could not be isolated from breast skin swabs obtained from women that provided milk samples from which such bacteria were isolated (34,69). In addition, bifidobacteria belongs to a strictly anaerobic genus and, therefore, skin is, at least, a very unlikely source. Previously, it has been reported that lactobacilli and enterococcal isolates present in human milk are genotypically different from those isolated from the skin, within a same bacterial species and a same host (63).

These and other findings suggest that at least some of the bacteria present in the maternal gut can reach the mammary gland through an endogenous route (64). As an example, some studies have shown that the presence of live lactic acid bacteria in the bloodstream of healthy human hosts is not an uncommon event (10,43,82,110). It is interesting to note that 125 of the 485 lactobacilli strains deposited in the PROSAFE collection were originally isolated from human blood from healthy individuals, and it is also illustrative that these 125 strains belong to 16 different Lactobacillus species, most of them included among the potentially probiotic ones (110). Other study investigating the influence of the composition of the oral microbiota on pregnancy outcome in 300 pregnant women revealed that some bacteria, such as Actinomyces naselundii, were linked to lower birth weight and earlier delivery while others, including Lactobacillus casei, were associated with a slightly higher

birth weight and normal delivery (23). These authors concluded that oral bacteria can enter the uterine environment through the bloodstream. Globally, these studies indicate that certain intestinal bacteria may have a rather underrated ability to spread from gut to extra-intestinal locations in healthy hosts. Interestingly, a recent study found identical genotypes of Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Enterococcus faecium, Enterococcus faecalis and Pediococcus pentosaceus in the faeces of mothers, her milk and in faeces of her babies (3). These data also sreinforces the hypothesis that there is a vertical transfer of intestinal bacteria from the mother's gut to her milk and through the milk to the infant's gut.

Although the exact pathway and mechanisms that some bacteria could exploit to cross the intestinal epithelium and reach the mammary gland and other locations remains far from elucidated, recent works offer a reasonable explanation. It has been demonstrated that dendritic cells (DCs) can penetrate the gut epithelium to take up non-pathogenic bacteria directly from the gut lumen. DCs are able to open the tight junctions between intestinal epithelial cells, send dendrites outside the epithelium and directly sample bacteria, while preserving the integrity of the epithelial barrier through the expression of tight-junction proteins (94). Using such mechanism, a Salmonella typhimurium strain that was deficient in invasion genes encoded by Salmonella pathogenicity island 1 (SPI1) was still able to reach the spleen after oral administration to mice (94). This mechanism may not be exclusive to DCs, as CD18+ cells, which also include macrophages, have been shown to be essential for extra-intestinal dissemination of non-invasive Salmonella (111). Recently, it has been found that intestinal DCs can retain small numbers of live commensal bacteria for several days in the mesenteric lymph nodes (57).

Hypothetically, DC-associated gut bacteria could spread to other locations since there is a circulation of lymphocytes within the mucosal associated lymphoid system. Antigen-stimulated cells move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of the respiratory and genitourinary tracts, salivary and lachrymal glands, and, most significantly, that of the lactating mammary gland (99). In addition, it is known that, during the lactation period, colonization of the mammary gland by cells of the immune system is a selective process regulated by the lactogenic hormones (13). This process is responsible for the abundance of such cells in human milk. A speculative model to explain how some maternal bacteria could be transferred to the neonatal gut is shown in Fig. 2.

DCs are professional antigen-presenting cells with the ability to migrate and stimulate a primary T-lymphocyte response when they are activated by different stimuli (7,37). They have two distinct functional stages: (a) an immature stage, characterized not only by a high ability to uptake and process antigens but also by a poor T-cell stimulatory function; and (b) a mature stage, with the opposite characteristics (93). Bacteria are potent inducers of DC activation both in vitro and in vivo (22,25,39,93). Therefore, it is not strange that the innate and adaptive immunity to microorganisms is greatly influenced by their interaction with DCs (7). It is thought that microorganisms have played a key role in the evolution of the immune system and, as a result, macrophages have reinforced their phagocytosis function while mature DCs have developed potent sensing and migratory activities (93).

There are strong evidences that bacterial signalling at the mucosal surfaces is dependent of a network of cellular interactions between bacteria, epithelial cells and cells belonging to the immune system, conforming the so-call "cross-talk" process, which may led to specific mucosal and/or systemic effects depending on the bacterial species and strains that conform the gut microbiota (35,85,96).

DCs and macrophages are able to discriminate between harmless and pathogenic compounds through the expression of various pattern-recognition receptors (PRRs). These include toll-like receptors (TLRs), scavenger receptors (SRs) and C-type lectin receptors (CLRs) such as the

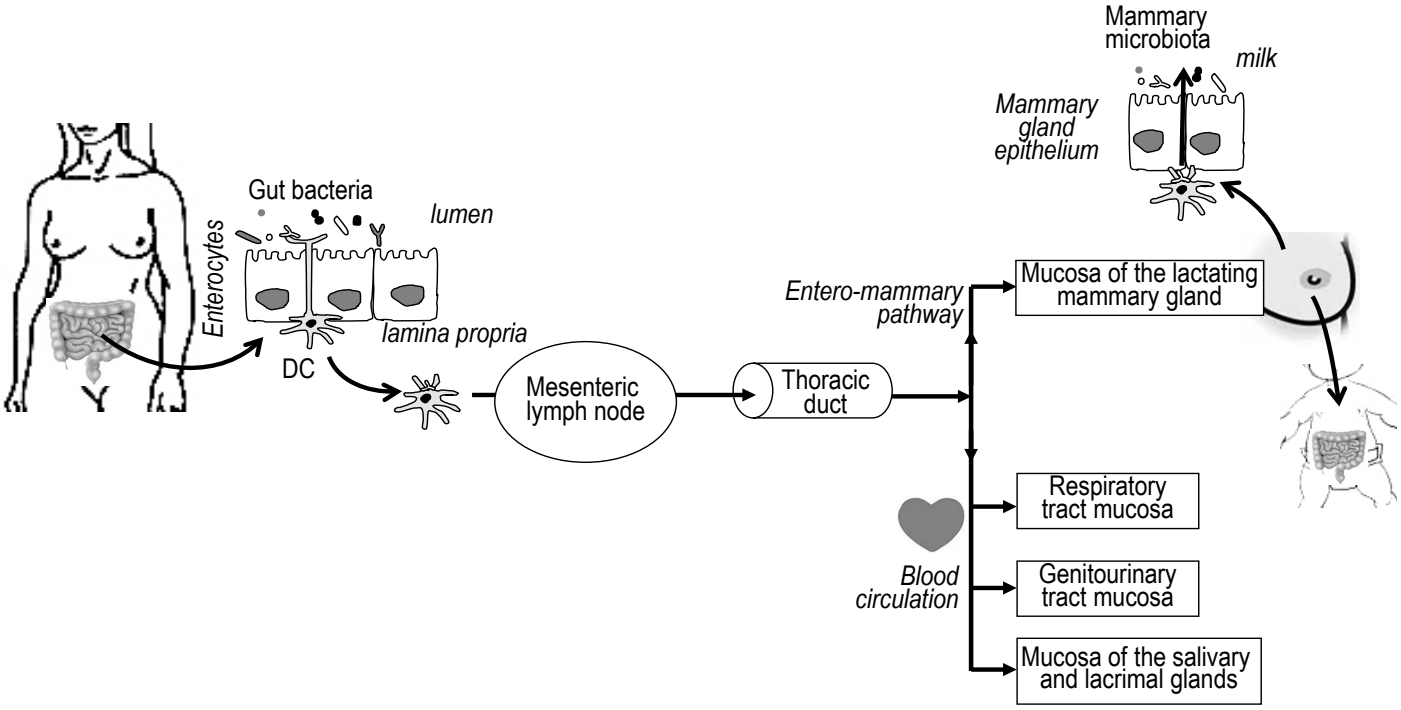

Figure 2. A hypothetical model to explain how some maternal bacteria could be transferred to the mammary gland and, then, to the infant gut. DC, dendritic cell.

DC–specific intercellular adhesion molecule 3–grabbing non-integrin (DC-SIGN) and mannose receptor (MR) (28,87). These receptors can recognise specific pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) in Gram-negatives and peptidoglycans in Gram-positives. In addition, the capacity of these receptors to interact with microbes is highly dependent on the density of the PAMP present on the microbial surface (18). Up to the present, some lactobacilli strains have already been reported to bind to DC-SIGN and to TLR2, TLR4 and TLR9 in a strain-specific manner (48,90,104).

The suggestion that the origin of the live bacteria found in breast milk could be the maternal gut and the bacteria would arrive to the mammary gland through an endogenous route, involving maternal DCs and macrophages, has been confirmed recently by independent research groups. Initially, Langa (52) showed that the exposure of mouse immature DCs to two bacterial strains isolated from human milk, Lactobacillus salivarius CECT 5713 and Enterococcus faecium M1a, led to a high stimulation of two surface markers of DC activation: the class II major histocompatibility complex and the B7.2 protein. It seems clear that bacterial induction of DC maturation is an active process since it has been shown that dead bacteria or inert particles, such as latex beads, can not activate DCs even if they are rapidly phagocytosed (92). On the other hand, Lactobacillus gasseri CECT 5715, other strain isolated from breast milk, showed a high level of binding to DCs and the ability to translocate across Caco-2 cells through a DC- mediated mechanism (52) (Fig. 3).

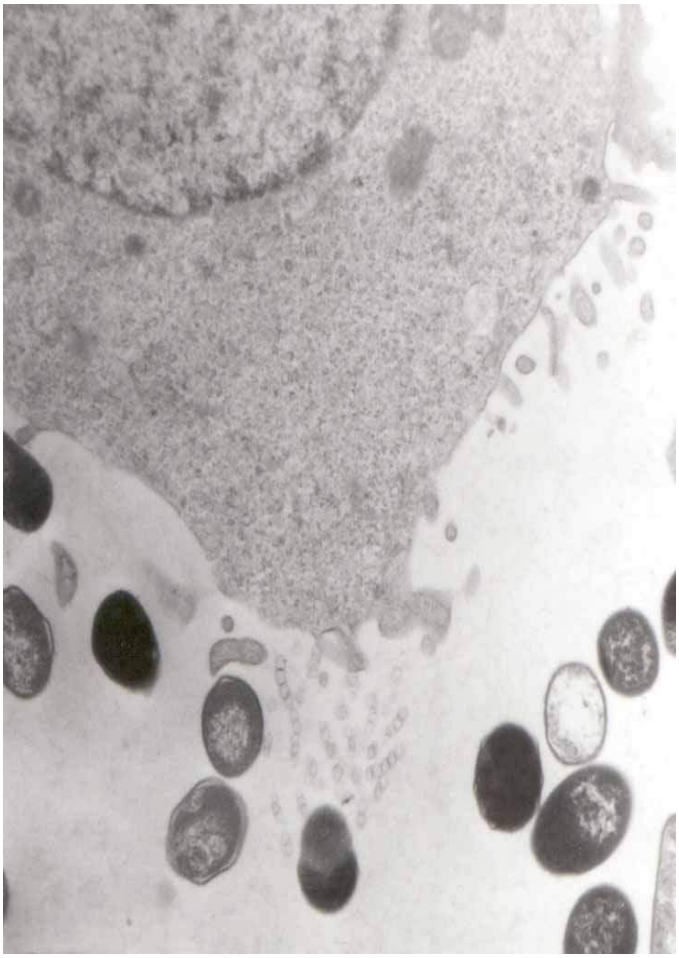

Figure 3. *Lb. gasseri* CECT 5715 cells bound and interacting with a dendritic cell (52).

Parallel, Pérez et al. (88) showed that bacterial translocation from the gut to mesenteric lymph nodes and mammary gland occurred during late pregnancy and lactation in mice. In addition, their work revealed that human breast milk cells contain a number of viable bacteria and a range of bacterial DNA signatures, which are also found in maternal peripheral blood mononuclear cells. Those peripheral blood mononuclear cells showed greater biodiversity than did peripheral blood mononuclear cells from control (neither pregnant or lactating) women. Taken together, their results suggest that intestinally-derived bacteria and bacterial components are transported to the lactating breast within mononuclear cells. They speculated that this programs the neonatal immune system to recognize specific bacterial molecular patterns and to respond appropriately to pathogens and commensal organisms.

More recently, two successive studies focused on the oral administration of three lactobacilli strains isolated from human milk (Lb. salivarius CECT5713, Lb. gasseri CECT5714, Lb. fermentum CECT5716) to treat lactational mastitis have provided new evidence of the existence of a bacterial entero-mammary pathway during lactation (4,47). In these studies, women in the probiotic groups daily ingested the respective probiotic strain for 4 weeks while those in the control one only ingested the excipient or the antibiotic prescribed by their doctor. At the end of the study, the administration of the lactobacilli not only led to the improvement of the condition, but also to the specific detection of the strains in at least 60% of the women of the different probiotic groups.

The mammary gland prepares for lactation through a series of developmental steps that occur during adolescence and pregnancy. The principal feature of mammary growth in pregnancy is a great increase in ducts and alveoli under the influence of many hormones. At the end of this period, the lobules of the alveolar system are maximally developed and small amounts of colostrum may be released for several weeks prior to delivery. Additionally, the nipple and areola markedly enlarges and the sebaceous glands within become more prominent (11). The increased lymph and blood supply to the mammary gland and the oxytocin release that causes contraction of the mioepithelial cells that invest the mammary alveoli may also facilitate the presence of endogenous bacteria in breast milk. These changes provide good conditions for biofilm formation on the mammary areola and/or in the mammary duct system, leading to the formation of a specific and transitory mammary microbiota. At the same time, the whole body (cardiovascular, respiratory, digestive and genitourinary systems, immune system…) experiences a series of physiological adaptations during pregnancy and lactation (14), and most of them are compatible with an increase in the rate of bacterial translocation in the gut.

CONCLUSIONS

Human milk is a source of commensal and potentially beneficial bacteria to the infant gut. In fact, recent studies indicate that this biological fluid contains a specific microbiota. Therefore, it is not strange that the bacterial composition of the faecal microbiota of breastfed infants reflects the bacterial composition of breast milk. Some works suggests that certain bacteria from the maternal gut can use immune cells to colonize, first, the mammary

gland and, later, the infant gut through breast-feeding. If further studies confirm this hypothesis, they would have practical consequences since it would imply that modulation of the maternal intestinal microbiota can have a direct effect on the health of infants. This would open new perspectives for gut colonization, bacteriotherapy and probiotics. Adequate knowledge of the mucosal-associated microorganisms, as well as the events that influence the timing of colonization, may provide opportunities to enhance important functions ranging from postnatal intestinal maturation to maintenance of mucosal barrier and nutrient absorption.

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