

# Human milk and infant intestinal mucosal glycans guide succession of the neonatal intestinal microbiota

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Infants begin acquiring intestinal microbiota at parturition. Initial colonization by pioneer bacteria is followed by active succession toward a dynamic ecosystem. Keystone microbes engage in reciprocal transkingdom communication with the host, which is essential for human homeostasis and health; therefore, these bacteria should be considered mutualists rather than commensals. This review discusses the maternal role in providing infants with functional and stable microbiota. The initial fecal inoculum of microbiota results from the proximity of the birth canal and anus; the biological significance of this anatomic proximity could underlie observed differences in microbiota between vaginal and cesarean birth. Secondary sources of inocula include mouths and skin of kin, animals and objects, and the human milk microbiome, but guiding microbial succession may be a primary role of human milk. The unique glycans of human milk cannot be digested by the infant, but are utilized by mutualist bacteria. These prebiotic glycans support expansion of mutualist microbiota, which manifests as differences in microbiota among breastfed and artificially fed infants. Human milk glycans vary by maternal genotype. Milks of genetically distinct mothers and variations in infant mucosal glycan expression support discrete microbiota. Early colonization may permanently influence microbiota composition and function, with ramifications for health.

## ONTOGENY OF THE GUT MICROBIOTA: OVERVIEW OF SUCCESSION

Appreciation for the roles of gut microbiota in human health is rapidly escalating. This is mostly attributable to metagenomic technologies that allow measurement of the entire microbiome, including microbes that are currently not culturable. Much of the variation in microbiota among individuals is thought to emanate from particular differences in early colonization that have long-term sequelae. At birth, the gut of most infants is largely devoid of bacteria. The inoculation of the gut and its succession into the adult microbiota is a complex process strongly influenced by maternal factors, and the initial inoculum most closely resembles the maternal fecal microbiota. Continued exposure to microbes occurs throughout early development. The selection of microbes is dependent upon the glycan phenotype expressed at the mucosal surface,

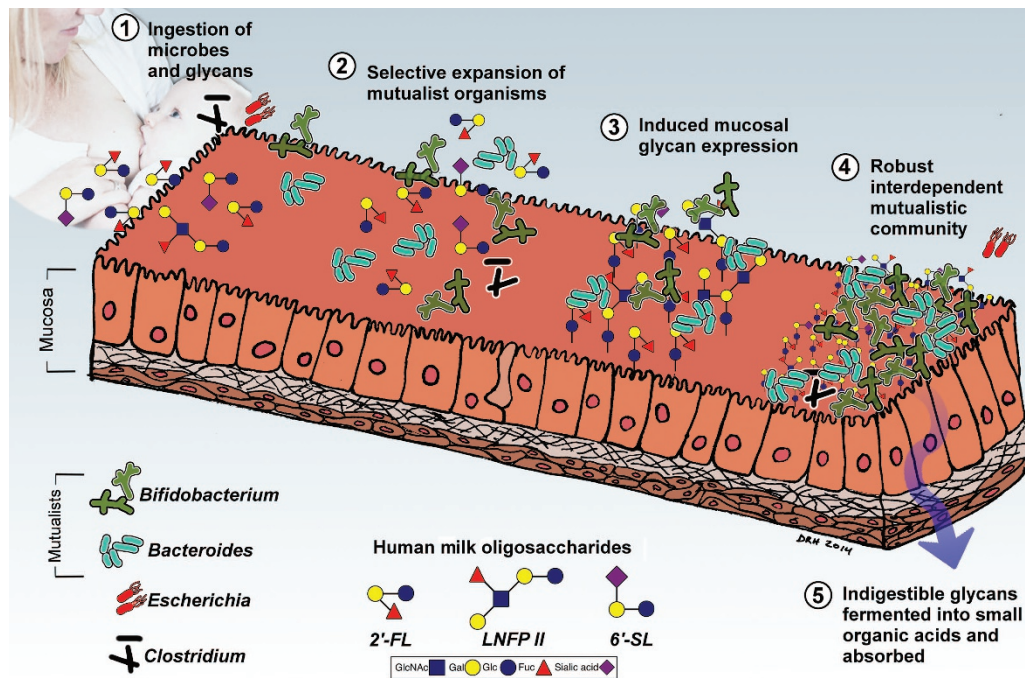
which reflects the genotype of the glycosyltransferases of the infant, and on the oligosaccharides and glycans of the milk, which reflects the genotype of the maternal glycosyltransferases. The focus of this review is the maternal influences on the ontogeny of the gut microbiota, that is, how specific human milk components affect succession in the bacterial ecosystem of the infant alimentary canal. Accordingly, we will discuss the human milk microbiota, the human milk oligosaccharides (HMOS), and their interaction with the infant gut mucosa and microbiota (Figure 1).

## DEFINING HUMAN MILK MICROBIOTA

The presence of bacteria in breast milk was noted in the 1950s (1,2), with a focus on pathogenic microorganisms. Enterobacteriaceae, enterococci, and staphylococci (2–4) were the most frequently identified using the plate count technology of the time. Interest in human milk bacteria blossomed in the 1970s when the use of raw breast milk, especially within neonatal units, raised concerns about the possibility of milk transmitting pathogenic bacteria (5,6). Scrupulous procedures for milk collection and storage are important to achieve “low counts” (below  $10^6$  colony-forming units/ml (4,7)). *Staphylococcus aureus*, a typical skin inhabitant, was found to be the predominant organism in freshly expressed milk, while poor storage of the milk was responsible for high counts of enterobacteria and other Gram-negative rods. The distribution of bacteria was reported for 688 human milk samples (8). In milks with a quantitative count of <2,500 organisms/ml, the aerobic skin bacteria such as micrococci, staphylococci, and streptococci (believed safe for consumption) represented 67% of the sample. Milks with either a total count >2,500 colony forming units/ml or with a potential pathogen, such as *Staphylococcus aureus*,  $\beta$ -hemolytic streptococci, *Pseudomonas* spp., *Proteus* spp., or *Enterococcus faecalis*, were deemed to require pasteurization prior to use by infants. This distribution of bacteria did not differ significantly between milks collected at home or in hospital environments. A subsequent bacterial assessment of milk from 810 individual mothers and 303 pools (9) found that 22% of individual samples did not contain detectable bacteria. However, pools contain the sum of all microbes from each individual sample, and 87% of pools contained *Staphylococcus*; the

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**Figure 1.** Human milk glycans and microbiota influence the ontogeny of neonatal mucosal mutualism. Exposure to maternal and environmental microorganisms at birth coincides with ingestion of maternal glycans in colostrum, especially human milk oligosaccharides (HMOS), which have prebiotic, antiadhesive, and anti-inflammatory activities. HMOS facilitate the selective expansion of mutualist microorganisms, particularly bacteria from the genera *Bacteroides* and *Bifidobacterium*, and inhibit growth and adhesion of opportunistic and obligate pathogens at the gut mucosal surface. The expanding mutualistic microbiota stimulate the induction of mucin expression and glycosyltransferase activity (e.g., *fut2*). Mucosal glycans support a robust, stable microbiota, which benefits the mature host through conversion of dietary components into critical metabolites (e.g., short chain fatty acids) and competitive exclusion of enteric pathogens and occupation of mucosal niches. 2'-FL is 2'-fucosyllactose; LNFP II is lacto-*N*-fucosylpentaose II; and 6'-SL is 6'-sialyllactose.

Gram-positive organisms detected were *Enterococcus* (16%), *α-Streptococcus* (8%), and *S. aureus* (4%). In addition, 60% of pools contained Gram-negative bacteria. Pasteurization kills most bacteria, resulting in 93% of pooled milk samples being negative. The presence of skin microbes in cultures of human milk led the authors to conclude that the skin microbiota of the mother provides the inoculum to breast milk. In a study on late-onset neonatal group B *Streptococcus* infection, the molecular typing of the group B streptococcus in the infant was identical to that found in the milk samples taken after the infection was detected, suggesting that milk can transmit pathogens to infants (10), or alternatively, that infected infants can transmit bacteria into their mother's breast.

These reports conclude that human milk can carry a substantial inoculum of skin-related bacteria, including some pathogens. It is considered prudent to reduce microbes in milk with disinfection of the skin and freeze-drying, or, most effectively, through pasteurization followed by microbiologic testing (11–13). However, pasteurization compromises other aspects of human milk, including the purported presence of microbes of potential benefit to the infant that have been more recently detected through DNA-based culture independent techniques.

DNA-based techniques suggest a diverse human milk microbiota. While these techniques eliminate the bias of only measuring what can be cultured, specific DNA techniques can

introduce other forms of bias; however, for the most part, modern techniques produce results consistent with earlier conclusions. Denaturing gradient gel electrophoresis analysis after polymerase chain reaction, when used in conjunction with libraries of 16S rRNA gene sequences, found that the composition of the microbiome of milk from four mothers (14) was consistent with the ability of breast milk to provide an inoculum of bacteria to the infant gut. DNA was identified from all skin associated species that had been previously identified in human milk by plating, including staphylococci and streptococci. DNA from lactobacilli and gut microbiota were also detected, but not DNA from *Lactobacillus gasseri* and bifidobacteria, bacteria whose presence had been reported previously from the same group when using classical plating techniques. Later, quantitative polymerase chain reaction using specific primers detected DNA of streptococci, staphylococci, lactic acid bacteria, and bifidobacteria in milk collected from 50 mothers (15). Overall, the data from these various techniques confirm the presence and composition of a human milk microbiome, and are consistent with human milk providing bacteria to the infant.

The bacterial composition of milk was assessed at three different time points from 18 mothers: colostrum obtained within 2 d, at 1 mo, and 6 mo after mothers gave birth. Milk microbe composition varied by mode of delivery as well as BMI and weight gain, as indicated by pyrosequencing and quantitative polymerase chain reaction (16). Colostrum is inhabited

mainly by *Weisella* and *Leuconostoc*, which are lactic acid bacteria not usually associated with the intestinal microbiome, and by *Staphylococcus*, *Streptococcus*, and *Lactococcus*. In 1- and 6-mo milk samples, lactic acid bacteria genera were the most abundant, but genera normally inhabiting the oral cavity (i.e., *Veillonella*, *Leptotrichia*, and *Prevotella*) were significantly present. Milk microbiota differs when collected from mothers delivering by cesarean section or vaginally (16).

Milk samples from mothers who gained excessive weight during pregnancy contained more *Lactobacillus* in colostrum and fewer *Bifidobacterium* in breast milk 6 mo after delivery relative to milk from mothers with normal weight gain, and were also associated with higher amounts of staphylococci (16). Although these results were limited to a comparison of 10 obese women with a BMI of 30 kg/m<sup>2</sup> and 8 normal-weight mothers with a BMI less than 25 kg/m<sup>2</sup>, these observations suggest a new line of inquiry.

Overall, human milk contains many microbes that are associated with the human gut microbiota, suggesting a potential role for milk as an intermediate between the maternal intestinal microbiota and that of her breastfed infant.

If maternal enteric microbiota influence human milk microbiota, and human milk microbes influence colonization and succession of infant enteric microbiota, strategies to adjust maternal gut with selected bacteria (17) should confer benefits to the offspring. The connection between maternal microbiota and milk microbiota is discussed above; the connection between milk microbes and infant gut is addressed below. The genera *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* occur in breast milk and infant fecal samples in paired samples (18), but stronger evidence of transfer requires identifying bacteria by strain. In 20 mother–infant pairs, colonies representing each morphological type were subject to selective plating at the strain level (19). The identity of each strain was confirmed by genetic assays such as pulse field gel electrophoresis. Most of the 20 pairs shared two identical strains of *Staphylococcus*, *Lactobacillus*, and/or *Bifidobacterium* in breast milk and infant feces, while 2 mother–infant pairs shared four bacterial strains.

Thus, breastfeeding seems to contribute to bacterial transfer from the mother to the infant and, therefore to colonization of the infant gut colonization.

#### HUMAN MILK GLYCANS INHIBIT INFECTION BY PATHOGENS

Infants fed human milk have lower risk of enteric and other infectious disease (20). Major sources of this defense include many biologically active human milk glycans. Milk components classically attributed to defense of the breastfed infant, such as secretory antibodies and lactoferrin, are glycosylated. More recently, complex glycans whose carbohydrate moieties *per se* protect the infant have been identified, including mucins, glycosaminoglycans, glycoproteins, and particularly the HMOs, whose complex glycans are attached to lactose. Specific complex milk carbohydrates inhibit the adhesion of pathogens to the cell surface receptors of their target cells, an essential first step in pathogenesis. These glycans inhibit

pathogen binding irrespective of the pathogen source, and therefore are a natural defense against ingested pathogens for the duration of breastfeeding, including those introduced directly by human milk.

Overall protection by human milk involves hundreds of glycans, both in the free monovalent oligosaccharide form and as multivalent and polyvalent glycoconjugates, such as in mucins, glycosaminoglycans, glycoproteins, glycopeptides, and glycolipids (glycolipids are functionally multivalent in lipid rafts). Pathogens whose binding is inhibited by HMOs include *Campylobacter jejuni*, *Escherichia coli*, and enteropathogenic *E. coli*, and those inhibited by the more complex glycoconjugates of human milk include *Entamoeba histolytica* (21), rotavirus, norovirus, salmonella, and HIV. The heterogeneity of HMOs expression in conjunction with the fastidious specificity of many pathogens suggests highly specific functions for the known hundreds, and postulated thousands, of individual HMOs and larger glycoforms. Individual HMOs and larger glycoconjugates may also function synergistically, suggesting that the mixtures of HMOs and glycoconjugates in human milk may be especially potent inhibitors of pathogens. Human milk substitutes do not contain these protective glycans, consistent with the higher risk of enteric disease in formula fed infants and neonates (22,23), despite formula ostensibly having a lower bacterial load than human milk.

#### POTENTIALLY BENEFICIAL BACTERIA IN HUMAN MILK

In the late 1970s, lactobacilli were reported in human milk at 10<sup>6</sup> per ml (21). Yeasts were also reported at these concentrations, and *Bacilli* at 10<sup>7</sup> per ml, but the reports did not provide the microbiologic procedures used. A report of “commensal” bacteria by Carrol *et al.* (6) in 1978 was lacking in details regarding microbiological media, incubations conditions, and other details essential to clearly identify their nature.

In 2003, the lactobacilli content of milk, mammary areola, and breast skin of eight Spanish healthy lactating mothers, as well as the oral cavity and feces of their infants, were detected using selective culture media (24). Isolates from each dyad of mother and infant were characterized at the strain level by randomly amplified polymorphic DNA (RAPD) polymerase chain reaction. Isolates that displayed identical RAPD patterns were identified at the species level by 16S rDNA sequencing. Rod-shaped lactic acid bacteria sharing the same RAPD profile were isolated from mammary areola and breast milk of the mother, and oral swabs and feces of their infants, but not from the skin of the mothers. All were identified as *Lactobacillus gasseri* species, with the exception that *Lactobacillus fermentum* was isolated only from breast milk. The coccoid lactic acid bacteria were identified as *Enterococcus faecium*, and those isolated from these samples displayed identical RAPD patterns. Although earlier studies had concluded that many milk microbes could have origins in maternal skin, the inability of these authors to detect these milk isolates in the mothers’ skin prompted them to conclude: “Lactic acid bacteria present in milk may have an endogenous origin and may not be the result of contamination from the surrounding breast skin”.

This group also cultured bifidobacteria from 8 milk samples and 21 fecal samples of the 23 mother/infant pairs, but not from any breast skin (25). The species isolated from milk were *Bifidobacterium breve*, *B. adolescentis*, and *B. bifidum*, and from infant feces *B. breve*, *B. adolescentis*, *B. bifidum*, *B. longum*, and *B. pseudocatenulatum*.

Bacteria were also detected in sow (26) and canine milk (27), including *Lactobacillus reuteri*, *Lb. salivarius*, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. brevis*, and *Weissella paramesenteroides*. Sow milk also contained *Lactobacillus fermentum*, *Lactobacillus murinus*, *Lactobacillus animalis*, and canine milk *Weissella viridescens*.

The identification of bifidobacteria in human milk (27) is consistent with the longstanding observation of a much higher content of bifidobacteria in breastfed feces than in those fed artificial formula. The same Spanish laboratory followed up their observations by demonstrating the use of strains isolated from breast milk as potential probiotic bacteria including treatment of mastitis and inhibition of mother-to-infant transfer of HIV (28).

These data strongly supported the presence of bacterial DNA in milk, but the concentrations were imprecise, as the first few microliters of milk contained much higher concentrations of bacteria than the milk samples further along in the pumping. Also, with the exclusion of skin as the origin, the source of the bacteria in milk remained uncertain. To address these issues, viable bacteria were cultured by plating;  $10^3$ /ml colony forming units were found in breast milk aseptically collected from healthy lactating mothers (29). Temporal temperature gradient gel electrophoresis indicated the presence of DNA from a diverse pool of enteric bacteria in maternal blood and milk. Parallel investigations performed in mice showed that bacterial translocation increased during pregnancy and lactation. Further studies demonstrated endogenous transport of enteric bacterial components by dendritic cells, suggesting a key role in neonatal immune imprinting, but also providing a carrier mechanism for bacterial components assumed to be destined for the lactating mammary gland (30).

In summary, a preponderance of data indicates that breast milk can provide a small inoculum of viable beneficial microbiota to the suckling gut, that it may initiate immunological priming by bacterial debris, and that the source of the debris and perhaps the small amount of viable beneficial bacteria may be maternal intestinal microbiota.

### PREBIOTIC ROLE OF HUMAN MILK

The inoculation of the neonatal gut at birth and bacteria in human milk can provide only limited numbers of bacteria, and some that are provided are not beneficial. A selection process is necessary to assemble an interdependent ecosystem that becomes the microbiota. Two distinct sources of selection by the host qualify as providing much of this interkingdom interaction. The glycans expressed on the surface of the infant intestinal mucosa have been demonstrated to select for distinct bacteria; specifically, the fucosylated glycans of secretors select for microbes that utilize fucose (31). This is complemented

by the prebiotic effect of the dominant fucosylated oligosaccharides of human milk, which are prebiotic, and specifically stimulate the growth of bifidobacteria and bacteriodes, predominant mutualists in healthy microbiota (32,33).

Prebiotics are indigestible glycans whose consumption modifies the intestinal microbiota and confers health benefits. In addition to their carbohydrate nature and oral ingestion, prebiotics are defined by minimal digestion or absorption in the proximal gastrointestinal tract (34), and selective fermentation by mutualist microbes in the distal gut. The HMOS fraction of human milk is prebiotic to human fecal slurries cultured anaerobically. HMOS support an increase in bifidobacteria, and they are fermented by microbiota to produce organic acids that acidify the milieu. During this fermentation, three principal components of HMOS, 2'-fucosyllactose (2'-FL), lactodifucotetraose, and 3-fucosyllactose (3-FL), were consumed. Each of these was tested individually in pure form for prebiotic utilization by four individual representative strains of infant gut microbes. The mutualists *B. longum* JCM7007 and *B. infantis* ATCC15697 efficiently consumed the oligosaccharides, and produced abundant lactate and short chain fatty acids, resulting in significant pH reduction. *E. coli* K12 and *C. perfringens*, which are not mutualists, did not utilize appreciable fucosylated oligosaccharide. Moreover, a typical mixture of organic acid fermentation product produced by the mutualists inhibited the growth of these nonmutualists (33). Thus, HMOS and their major fucosylated components are prebiotic to the mutualists and the mutualist fermentation products inhibit pathogens and opportunists. Moreover, HMOSs, and especially fucosylated HMOSs could promote the growth of specific pioneering or keystone species of human microbiota, thereby shaping birth and milk inocula into a mutualist symbiotic microbiota (32). This is a potential mechanism whereby human milk could confer long term beneficial effects on infant health well after weaning.

### GENETIC VARIATION IN GLYCAN PRODUCTION: SECRETOR STATUS

Expression of individual molecules of human milk differs among mothers (35), by stage of lactation, by diet, and by other biological variables. The expression of genes underlying production of human milk glycans is especially heterogeneous (8,35); major differences in milk glycans are apparent in mothers whose glycosyltransferase alleles differ. Of these, most prevalent are differences in expression products of fucosyltransferase 2. In populations of European, Asian, and African ancestry, 15–25% can be homozygous recessive for expression of the *FUT 2* gene; they are nonsecretors, that is, unable to secrete  $\alpha$ 1,2-linked fucosylated glycans in their secretions, including milk (36). Specific  $\alpha$ 1,2-fucosylated HMOSs, including 2'-fucosyllactose, inhibit binding by specific pathogens to their host target. The milk of nonsecretor mothers lacks 2'-fucosyllactose and related fucosylglycans, and is less able to protect the infant from enteropathogens that bind  $\alpha$ 1,2-fucose (37). Thus, heterogeneous glycan expression in milk implies that some milk will protect poorly against one pathogen, but may protect more strongly against another.

This heterogeneous expression of glycans, especially those related to the secretor status, also implies that milk varies by mother and lactation period in quantity and quality of its prebiotic components. This is consistent with recent findings that the infant microbiota varies among individuals and changes as the infant matures (unpublished data, D.S.N.). Perhaps individual variation in HMOS directs colonization by specific bacteria, leading to systematic differences in microbiota. If so, this phenomenon could contribute toward the differential risk of disease that has been observed in breastfed infants of genetically distinct mothers (36).

This genetic variation in glycosyltransferase genes within the infant manifests as distinct differences in glycosylation of the infant intestinal mucosa. Such dissimilarities are consistent with differences in gut colonization and differential risk of enteric inflammatory disease (36).

These considerations predict that certain combinations of breastfeeding dyad, such as a nonsecretor mother nursing a nonsecretor infant, may leave the infant at especially high risk of specific enteric diseases.

#### HEALTH RAMIFICATIONS OF DIVERSITY OF HUMAN MILK AND GUT GLYCOSYLATION

The early inputs into formation of the microbiota, including those provided by the mother, are thought to have lifelong consequences on its ultimate composition and stability. Research on interactions between microbes and host is suggesting dysbiosis as underlying several chronic diseases, including antibiotic-associated diarrhea and irritable bowel syndrome (38). Altered gut microbiota, with concomitant abnormal communication between bacteria in the gut and the mucosal immune system, seems to underlie inflammatory bowel disease, that is, Crohn's disease or ulcerative colitis (38). A strong risk factor for necrotizing enterocolitis, a severe gut inflammation most common in premature infants, is early dysbiosis (36). Unregulated inflammation and cell turnover can eventually lead to colorectal cancer, the third most common cause of cancer mortality (39).

The pathobiology underlying an association between microbial dysbiosis and cystic fibrosis, autism, atopic dermatitis, insulin resistance, and obesity (40,41) is less obvious. However, bacterial colonization influences development of the immune and endocrine systems, which could have such diverse consequences. Of course, associations between changes in microbiota and disease cannot differentiate between causes or consequences of pathology.

That notwithstanding, gut microbiota play a major role in intestinal health and disease, and, therefore, early maternal influences may have lifelong ramifications. Accordingly, the human milk glycans, especially the oligosaccharides, and the human milk microbes should be considered major components of an innate immune system by which breastfeeding mothers protect their infants from disease. The absence of this innate immune system may account for the higher risk for enteric disease among infants fed artificial formula. As synthetic HMOSs become available for human use, they

may ameliorate a portion of this deficit of artificial milk substitutes through strengthening the development of a healthier microbiota of infants fed formula (42). Overall, the interplay between maternal glycan and microbial input with infant gut colonization strongly argues for the promotion of breastfeeding whenever feasible.

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