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Milk, Mucosal Immunity and the Microbiome: Impact on the Neonate

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Contents

Foreword

There have been considerable advances in science to understand the varied mixture of bioactive components in human milk that influence the immune status of infants not only by providing protection, but also by facilitating development, tolerance, and an appropriate inflammatory response.

Human milk is the communication vehicle between the maternal immune system and the infant, a system actively directing and educating the immune, metabolic, and microfloral systems within the infant. The physiological and protective functions of several immune components in human milk have been studied not only in infants, further evidence has also been obtained from what is known in other species and in vitro models.

The 94th Nestlé Nutrition Institute (NNI) Workshop entitled *Milk, Mucosal Immunity and the Microbiome: Impact on the Neonate*, which took place in Lausanne in September 23–25, 2019, reviewed the latest data on immune development in infants and the role of milk factors. The program was focusing on the current knowledge of how both the "classical" immune and nonimmune ingredients found in human milk support maturation of the immune system, facilitate development of tolerance, and regulate inflammatory responses of infants.

World experts in human milk research and nutrition, i.e., *Pearay L. Ogra* (Professor Emeritus Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, NY, USA); *W. Allan Walker* (Conrad Taff Professor of Nutrition [Emeritus], Professor of Pedictrics, Harvard Medical School); and *Bo Lönnerdal* (Distinguished Professor Emeritus, Department of Nutrition and Internal Medicine, University of California, Davis, CA, USA), have contributed to the Workshop program.

The 94th NNI Workshop was designed with the goal to provide a comprehensive overview on the latest human milk research and its potential in modulating mucosal immunity, the microbiome, and its impact on the neonate. The program was comprised of three sessions. Session I, led by Prof. Pearay L. Ogra, reviewed data on the immunology of milk and lactation. The flow of the topics brought us from a historical perspective to the latest scientific findings in order to understand the complex immunobiology of mammalian milk. Session II, directed by Prof. W. Allan Walker, discussed the microbiology of human milk and lactation

in detail, with a focus on premature infants and necrotizing enterocolitis. The objective of the third and last session, shepherded by Prof. Bo Lönnerdal, was to shed light on the protective factors in human milk, e.g., human milk oligosaccharides, bioactive milk fat components, and lactoferrin, and their role in influencing the neonate's immune system.

The program brought important new insights but also raised unanswered questions. We are only beginning to understand the complex milk composition, functions of different bioactive components, and the most important role of each in human development.

The 94th NNI Workshop and its book are dedicated to an extraordinaire personality: Prof. *Lars A. Hanson* (MD, PhD), who is considered the founder of modern milk immunology by the human milk research community. We believe that this publication and online material will be a great scientific support to all people seeking a deeper understanding of human milk and its immunological properties and enlarge the knowledge of those who have already specialized in human milk research.

We would like to thank the three chairpersons Pearay L. Ogra, W. Allan Walker, and Bo Lönnerdal who designed the scientific program.

Our special thanks also go to all speakers and scientific experts in the audience who contributed to the content of the workshop and scientific discussions.

Finally, we thank Maria Elena Munoz and the NNI team for making this workshop possible.

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The Evolution of Lactation in Mammalian Species

Olav T. Oftedal

Although the evolution of the mammary gland and its secretion products has been the subject of speculation since the time of Charles Darwin [1], it is only recently that it has been possible to compare the development of the mammary gland across diverse taxa to consider the genetic origin and function of specific mammary constituents and to assess mammary evolution within the context of the evolution and paleobiology of ancestral taxa.

Milk constituents of particular evolutionary interest are listed in Table 1. Most constituents are found only in mammary secretions, i.e., they appear to have evolved as components of the mammary system. Some (such as lactalbumin) bear strong similarities (in terms of gene structure, amino acid pattern, and three-dimensional structure) to nonmammary proteins and are believed to derive from them. Another feature is their ubiquitous distribution. From an evolutionary perspective, if a milk protein occurs across the three major mammalian taxa (monotremes, marsupials, and eutherians), this is evidence indicating that it evolved before these groups diverged and that it was inherited from ancestral taxa.

The synapsids (ancestral to mammals) diverged from the sauropsids (ancestral to crocodilians, lizards, and birds, for example) in the Carboniferous, i.e., more than 300 million years ago. Lactation may have first evolved as a source of moisture and antimicrobial compounds for parchment-shelled eggs [2]. If so, there was ample time for the evolution of skin secretions that became milk. The ancestral group immediately prior to mammals (mammaliaforms) had a number of characteristics such as minute size, traits associated with endothermy and high activity, delayed tooth development (including restriction to two sets of teeth), and tiny eggs, which may indicate nutrient provision to hatchling young via lactation.

Among basal mammals (monotremes), each mammary gland develops as a triad in association with a hair follicle and sebaceous gland; in the mature gland, milk is secreted into the infundibulum of a hair follicle; this

Table 1. Mammary constituents and their evolutionary interest

Structure/ function	Specific constituents	Unique to mammary gland	Universal to mammals
Casein micelle	α_{s1}-, α_{s2}-, β-, and κ-caseins	Yes	Yes
MFG membrane	Butyrophilin 1A1	Yes	Yes
	Xanthine oxidoreductase	No	Yes
	Adipophilin	No	Yes
Sugar synthesis	α-Lactalbumin	Yes	No
	β-1,4-Galactosyltransferase 1	No	Yes
	Other glycosyltransferases (for milk oligosaccharide synthesis)	No?	No
Whey proteins	β-Lactoglobulin	Yes	No
	Whey acidic protein	Yes	No

MFG, milk fat globule.

developmental unit is the mammopilosebaceous unit (MPSU). In marsupials, there is a similar triad, but hair follicles are shed during development. Eutherian mammals are more diverse: some demonstrate no association with mammary hairs, whereas others (e.g., the horse) develop MPSUs with mammary hairs and sebaceous glands present in mature mammary glands. The MPSUs bear a lot of similarity to apocrine glands in apopilosebaceous units, which indicates that mammary glands may have evolved from an apopilosebaceous unit-type structure [3].

Caseins present an evolutionary challenge because of their diversity and the large size of the micelles in milk. All studied mammalian milk types contain the four primary types of caseins, α_{s1}-, α_{s2}-, β-, and κ-caseins, which suggests that these derive from a premammalian ancestor; casein genes have also duplicated among diverse mammals. Caseins are members of the secretory calcium-binding phosphoproteins, which have an ancient history in the evolution of mineralized tissues [4]. Based on related genes of secretory calcium-binding phosphoproteins, caseins are also thought to have an ancient origin, perhaps in a protolacteal secretion that delivered calcium to eggs.

Another mammary-specific structure is the milk fat globule (MFG), which is encompassed by an MFG membrane. Several proteins, such as butyrophilin 1A1 and xanthine oxidoreductase, play a major role in maintaining the structure of the MFG membrane and its proximity to the core lipid. Interestingly, in other tissues, these proteins participate in immune function. MFGs also include cytoplasmic crescents, which may be remnants of an ancestral apocrine secretion.

Milk has a species-specific mix of lactose and a variety of neutral and acidic oligosaccharides. Lactose is dominant in most eutherian milk, but oligosaccharides dominate in monotremes, marsupials, and caniform carnivores; human milk has the greatest oligosaccharide diversity. This distribution of oligosaccharides (e.g., in basal species) suggests that oligosaccharides may be ancestral [5].

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Immunology of Human Milk and Lactation: Historical Overview

Pearay L. Ogra

The development of the mammary gland and the process of lactation is an integral component of mammalian evolution, and suckling has been essential for the survival of neonates of most mammalian species [1–5]. The colostrum and milk, the major products of lactation, contain a wealth of biologically active products derived from the immunologic and microbiological experiences in the maternal circulation and in the maternal mucosal surfaces. These include major immunoglobulin isotypes in the maternal circulation, secretory IgA, a variety of soluble proteins, casein, nutritional components, hormones, a large number of cellular elements and their secreted functional products (cytokines and chemokines), several peptides, lipids, poly- and oligosaccharides, and a diverse spectrum of microorganisms. During the past few decades, significant new information has become available about the evolutionary biology of mammalian lactation, the functional characterization of antibodies and cellular immunologic products, the role of oligosaccharides and other proteins and peptides, and about the distribution and biologic functions of the microbiome observed in human products of lactation [5–7]. This workshop explores this information in some detail in a series of presentations. A brief overview of the earlier observations on the immunologic aspects of lactation is presented , and detailed reviews of more recent observations are presented in subsequent presentations in this workshop [7].

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The Mammary Gland as an Integral Component of the Common Mucosal Immune System

Jiri Mestecky

The human mammary gland is an integral effector component of the common mucosal immune system [1–4]. However, from physiological and immunological aspects, it displays several unique features not shared by other mucosal sites [1, 2, 4]. The development, maturation, and activity of the mammary gland exhibits a strong hormonal dependence [1, 4]. Furthermore, in comparison to the intestinal and respiratory tracts, the mammary gland is not colonized by high numbers of bacteria of enormous diversity and does not contain mucosal inductive sites analogous to the intestinal Peyer's patches [1, 4–6]. Consequently, when exposed to antigens, local or generalized immune responses are low or not present [2, 4, 5]. Comparative evaluations of various immunization routes effective in the induction of antibodies in human milk are limited [2, 4, 6]. Systemic immunization induces IgG antibodies in plasma, but due to the low levels of total IgG in human milk, their protective effect remains unknown [3, 5]. Oral or intranasal immunization or infection induces secretory IgA in milk as demonstrated in several studies [1, 2, 7]. Other routes of mucosal immunization such as sublingual or rectal exposure effective in the induction of antibodies in various external secretions have not been explored in the mammary gland. Because secretory IgA in milk displays protective functions [2, 3, 5], alternative immunization routes and antigen-delivery systems should be explored.

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Immunomodulatory Components of Human Colostrum and Milk

Helena Tlaskalová-Hogenová, Miloslav Kverka, and Jiří Hrdý

Breastfeeding represents the continuation of the tight relationship between mother and child after birth. In addition to the nutrients, colostrum (early milk within 4–5 days after parturition) and mature milk contain many immunological components that boost the immune responses of the infant against infection. Due to this complex composition, breastfeeding has numerous beneficial effects for both mother and infant.

Breastfeeding reduces diarrheal diseases and protects against respiratory and urinary infections. Milk components act directly against infectious agents, but they also accelerate the development of the newborn's immune system, increasing its capacity for immune defense and reducing the risk of inflammation. Moreover, breastfeeding exerts long-term effects and decreases the risk of allergy and other immune-related diseases [1, 2]. In this regard, a broad array of cytokines found in colostrum and milk shows the most refined immunomodulatory effects [3], but oligosaccharides, hormones, and other components affect the newborn's immunity as well. Most milk components substantially affect the microbial colonization of the infant mucosa, which in turn influences the development of all parts of the immune system. All these components act primarily locally on mucosal membranes, preventing the penetration of microbes and other antigenic components into the circulation thus ensuring effective defense without the damaging effects of inflammation.

The large quantity of secretory IgA the mother delivers to the infant via colostrum and breast milk reacts to a broad spectrum of antigens, and its resistance to enzymatic digestion guarantees its activity even in the distal parts of the infant's intestine. The common mucosal immune system of the mother ensures the transfer of antibodies to the mammary gland during lactation. These antibodies originate in the gut lymphoid tissue and are thus useful for gut mucosa protection in the immunologically immature infant, because they are directed primarily against gut microbes colonizing the newborn mucosa [4]. However, secretory IgA contains not only antibodies against microbial and food antigens but also natural

autoantibodies reacting with autoantigens of the infant. These autoantibodies are involved in regulatory pathways of the developing immune system.

Human lacteal secretions contain a number of live cells. Although there are no major differences in the cytokine production between allergic and healthy mothers, they are capable to respond to multiple stimuli [5].

By increasing happiness via the stimulation of oxytocin production, boosting the protective immune response by mucosal microbiota of the infant, and decreasing the risk of breast and ovarian cancer, breastfeeding has multiple benefits for the mother as well.

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Breastfeeding, a Personalized Medicine with Influence on Short- and Long-Term Immune Health

Valérie Verhasselt

Compared to adulthood, early postnatal life is a period that is characterized by rapid changes. The neonate's tissues are constantly changing due to the growth process and are exposed to multiple new antigens, which are found in the environment, are present in the diet, or are associated with gut microbiota colonization (Fig. 1). The neonatal immune system has its own reactivity, which profoundly differs from that of the adult. It is neither that of a "small adult" nor is it an immature or tolerance-prone immune system. The neonatal immune system is a different one with specific requirements for activation and regulation. Breast milk is most probably a key condition for physiological (and optimal) functioning and imprinting of the immune system in early life (Fig. 1). Similar to the immune system and environment, which are constantly changing in early life, breast milk composition is constantly evolving. Volume, macronutrients, micronutrients, immunological factors, microbiota, and microbiota-shaping molecules are changing with lactation stages, and are also affected by infant growth and environmental immune challenges [1]. Here, we will focus on factors in breast milk that we – and others – extensively studied and found to actively influence their immune trajectory and long-term immune health. More specifically, we will review the importance of TGF-β, vitamin A, immunoglobulins, and allergens in mucosal immunity in both early life and long-term allergic disease susceptibility. There is strong evidence from rodent studies and epidemiological data that oral exposure to allergens in early life, out of the context of breast milk, is not inducing immune tolerance but, instead, is priming for allergic responses [2]. Nonbreastfed infants are exposed to only a few allergens, such as β-lactoglobulin, which occur at high concentrations. In contrast, breastfed infants are exposed to a wide variety of breast milk allergens that are found at concentrations that are at least 1 million times lower [3]. Importantly, there is evidence from rodent studies that the neonatal immune system requires very-low-dose antigen

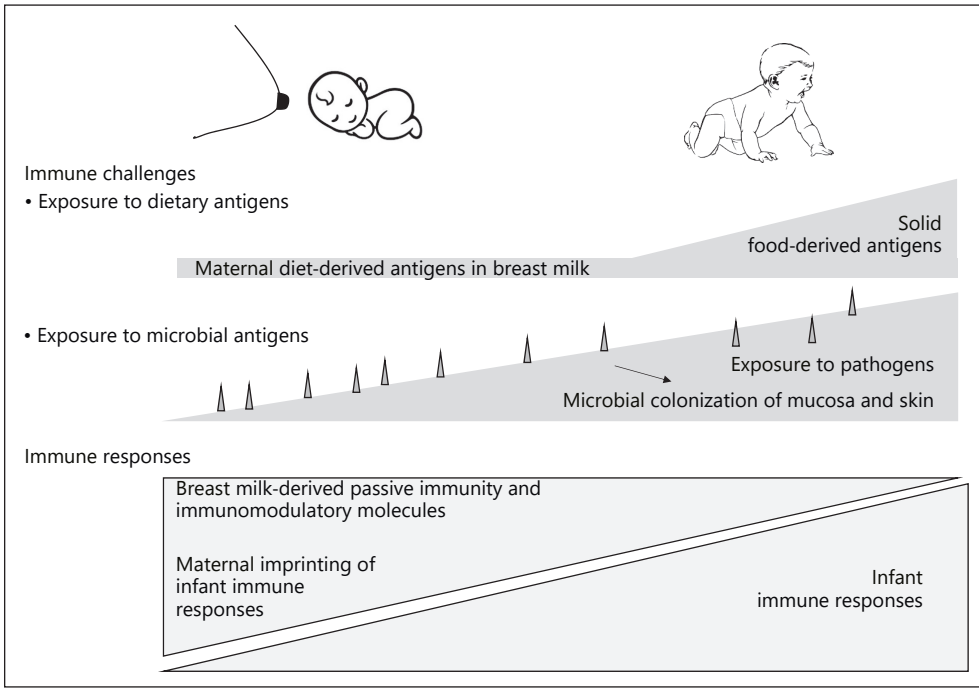

Fig. 1. Evolving maternal-child immune complementarity through breast milk.

exposure for appropriate immune reactivity. Early life is also characterized by a relative lack of TGF-β in the mucosal tissue, a physiological deficiency in vitamin A, and a low mucosal and systemic immunoglobulin secretion, which contribute to the lack of oral tolerance induction in early life in the absence of breast milk. Breast milk is providing infants with these cofactors, which will affect gut epithelium barrier integrity, antigen transfer, and antigen presentation for successful regulatory immune response induction [4]. This will result in a decreased risk for allergic diseases in the long term, as shown for egg allergy both in an experimental mouse model as well as in humans [5].

We are starting to decipher the specific requirements for the neonatal immune system to function optimally, and we are discovering how breast milk fulfills these requirements and guides immune trajectories from early life. Answering these questions will provide the infant with preventive and curative approaches that are tailored to this very specific period of life and will ensure long-term immune health.

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Milk Microbiome and Neonatal Colonization: Overview

Samuli Rautava

Breastfeeding confers the infant long-term health benefits by reducing the risk of obesity and noncommunicable diseases in later life. These effects may at least partially be mediated by the considerable impact breastfeeding has on the developing infant gut microbiome. Human milk contains molecules such as human milk oligosaccharides, one of the main functions of which is to selectively promote the growth of specific bacteria in the infant gut. In addition, human milk has relatively recently been discovered to harbor a distinct microbiome.

The origin, function, and significance of the bacteria in human milk remain poorly understood. The milk microbiome includes obligate anaerobic bacteria, such as bifidobacteria, often considered to be characteristic of the intestinal microbiome (Table 1) [1]. Data from experimental and clinical studies suggest that the bacteria in milk may indeed originate in the maternal gut, and the existence of a specific enteromammary pathway has been suggested based on data from experimental and clinical studies [1, 2]. Increased bacterial translocation from the intestinal lumen to mesenteric lymph nodes has been observed during the perinatal period in mice [2]. In line with this observation, the human milk microbiota in mothers who have delivered by elective cesarean section is reportedly significantly different from that of mothers who have undergone labor (Table 2) [3]. In addition, the milk microbiome is affected by maternal health, metabolic state, and antibiotic use.

The functions of the milk microbiome and particularly its potential contribution to infant gut colonization are an area of active scientific research. The overall composition of the milk microbiome is clearly distinct from that of the infant gut microbiome. Results from clinical studies indicate, however, that specific bacterial taxa are detectable both in human milk and infant feces [1]. The infant gut microbiome resembles more closely the microbiome in the milk of the infant's own mother than that in the milk of an unrelated women [4]. Finally, based on source tracking analyses of samples from 107 mother-infant pairs, approximately 15%

Table 1. Selected studies investigating the association between the human milk microbiome and infant gut colonization

Jost et al. [1] (*n* = 7): several methods including 16S culture and sequencing Members of *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, *Blautia*, *Clostridium*, *Collinsella*, and *Veillonella* were detected in maternal feces, milk, and neonatal feces. Viable *Bifidobacterium breve* was detected in maternal feces and milk as well as in infant feces.
Pannaraj et al. [4] (*n* = 107): 16S sequencing Infant fecal microbiome at the median age of 40 days resembled more closely the microbiome in their own mother's milk than that of nonrelated mothers. As per source tracking analysis, 15% of the fecal microbiome was derived from the bacteria in milk.
Collado et al. [5] (*n* = 15): 16S sequencing The neonatal gut microbiome shifted during the first week of life to resemble more closely that in colostrum.
Grönlund et al. [6] (*n* = 61): qPCR Maternal *Bifidobacterium* frequencies and counts in feces but not in milk correlated with those detected in the infant gut at 1 month of age.
Williams et al. [7] (*n* = 21): 16S sequencing The milk and infant fecal microbiomes have some similarity in early life but become increasingly different over time. Source tracking analyses indicated that on day 2 of life, milk microbes contribute 4.9% to the infant gut microbiome, but this diminishes to 0.3% at 6 months of age.

of the fecal microbiome in predominantly breastfed infants originate from the bacteria in milk during the first 30 days of life [4]. Taken together, these data strongly suggest that the bacteria in human milk are the source of bacteria colonizing the neonatal gut.

The biological or clinical significance of the human milk microbiome and its role in infant gut colonization remain open questions. If the human milk microbiome is shown to contribute to the beneficial effects of breastfeeding, interesting new therapeutic avenues may be discovered. Modulating or mimicking the milk microbiome may provide a novel means to affect early gut colonization and reduce the risk of noncommunicable diseases associated with aberrant gut colonization or suboptimal breastfeeding.

Table 2. Exposure/factors affecting the human milk microbiome

Excessive weight gain during pregnancy
↑ Staphylococci
↓ Bifidobacteria
↑ *Akkermansia muciniphila*-type bacteria
Cesarean section delivery
↓ Diversity
↓ Richness
↓ Bifidobacteria
↑ Staphylococci
Intrapartum antibiotics
↑ Diversity
↑ Richness
↓ Bifidobacteria
Human milk oligosaccharide (HMO) profile
↑ Bifidobacteria with increased HMO total concentration and sialylated HMOs
↑ *Akkermansia muciniphila* with increased fucosylated HMOs

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Human Milk Microbiota: Origin and Potential Uses

Leónides Fernández and Juan M. Rodríguez

The mucosa or epithelial surfaces from diverse human organs that are in direct contact with the host environment (e.g., lungs or mammary glands) had traditionally been considered to be sterile under physiological conditions. At the beginning of the XXI century, microbiological studies on human milk switched from considering it a potential vehicle for pathogens to describing the existence of its own microbiota.

The human milk microbiota in hygienically collected samples from healthy women contains a relatively low bacterial load which is dominated by *Staphylococcus* (mainly *S. epidermidis* and other coagulase-negative species), *Streptococcus* (from *S. mitis* and *salivarius* groups), *Corynebacterium*, *Propionibacterium*, and other gram-positive bacteria, including *Lactobacillus* and *Bifidobacterium* [1]. It is also possible to detect DNA from strict anaerobic bacteria in human milk, but not viable bacterial cells, as they cannot be grown using conventional culture techniques. However, when the milk is collected by pumping, a high concentration of contaminating gram-negative bacteria (e.g., enterobacteria, *Pseudomonas*, or *Stenotrophomonas*) and yeasts may arise from the rinsing water and/or poor hygienic manipulation practices. Culture-independent techniques also reveal the presence of contaminant DNA of bacteria typically associated to soil arising from molecular-biological reagents (i.e., *Acinetobacter*, *Methylobacterium*, *Pseudomonas*, *Sphingobium Sphingomonas*, *Stenotrophomonas*, or *Xanthomonas*). Although the human milk microbiome may be influenced by several factors, the exact triggers or drivers of differences in the composition of the human milk microbiota/microbiome need to be elucidated in the future.

Colostrum and milk bacteria are among the first colonizers of the infant gut and, therefore, may play a key role in driving the development of its microbiota. A vertical mother-infant transfer of human milk microorganisms has been proven using both culture-dependent and -independent techniques. However, studies dealing with the potential functions of such microbiota are scarce. In fact, the complex composition of human

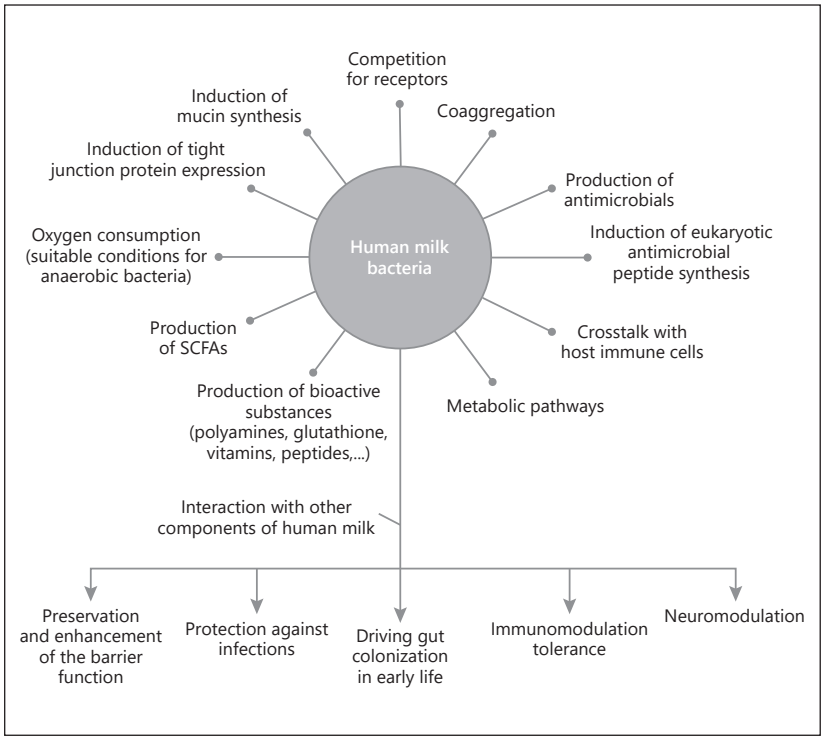

Fig. 1. Potential roles of human milk microbiota. SCFAs, short-chain fatty acids.

milk, including nutrients, bioactive molecules, and cells that may act synergistically, makes it difficult to delimit the specific functions of human milk microbiota. Despite this, several studies, including clinical trials, have shown the ability of bacteria isolated from human milk to inhibit a wide range of pathogenic bacteria by different mechanisms and to reduce the incidence of gastrointestinal and upper respiratory tract infections in infants.

Human milk bacteria may participate in the correct maturation of the infant immune system by modulating both innate and acquired immune responses and enhancing tolerance mechanisms (Fig. 1). Some human milk strains colonize and are metabolically active in the infant intestine. Although *Lactobacillus* and *Bifidobacterium* have attracted much attention from scientists and industries, some staphylococcal and streptococcal strains, which are dominant in human milk, may play important empirical probiotic roles in the breastfed infant.

A well-balanced human milk microbiota is also relevant for maternal breast health. Mastitis, a mammary bacterial dysbiosis, is mostly caused by various bacterial species showing antibiotic resistance and the ability to form biofilms. Probiotic treatment using strains isolated from human milk is a promising alternative or complement to antibiotic therapy in the prevention or treatment of mastitis.

The origin of the bacteria present in human milk still remains largely unknown and is a subject of scientific controversy. The infant's oral cavity and the maternal skin, particularly the external surfaces closer to the exit point of milk, may provide bacteria to milk. Additionally, selected bacteria of the maternal digestive microbiota may access the mammary glands through oral- and enteromammary pathways [2]. Mononuclear cells may be involved in the transport of intestinal bacteria to the mammary glands. The existence of such bacterial oral- and enteromammary pathways would provide new opportunities for manipulating maternal-fetal microbiota, reducing the risk of preterm birth or infant diseases [3].

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Beyond the Bacterial Microbiome: Virome of Human Milk and Effects on the Developing Infant

Sindhu Mohandas and Pia S. Pannaraj

Human milk is an important source of microbes that colonize the infant gut in early life contributing to immune system maturation and protection against pathogen invasion [1, 2]. Emerging evidence shows that human milk viruses are also transmitted from mother to infant via breastfeeding [3]. These viruses include eukaryotic viruses, bacteria-infecting viruses called bacteriophages, and other viral particles. Human milk viruses are instrumental in shaping the infant gut virome and microbiome. Eukaryotic DNA and RNA viruses contribute to pathogenic challenges and protection. The early infant virome is dominated by bacteriophages that likely contribute to a highly dynamic microbiome in early life. These bacteriophages have the ability to kill bacteria or supply them with potentially beneficial gene functions, thereby shaping the microbiome. Thus, the eukaryotic virus, bacteriophages, and bacteria coexist in the infant gut in an interdependent and dynamic relationship [4, 5]. Figure 1 shows the hypothesized trajectory of the microbial diversity during the first 2–3 years of life based on the few existing studies. There is a critical window of early childhood growth with rapid maturation of metabolic, endocrine, neural, and immune pathways. The colonization of microbes in the infant body during this time plays an important role in the establishment and maturation of these pathways. The virome transmitted via breastfeeding may also be particularly important at these critical time points of immune development. More longitudinal studies of mother-infant pairs will help to define the human milk virome and its functional impact on the development of the growing infant better.

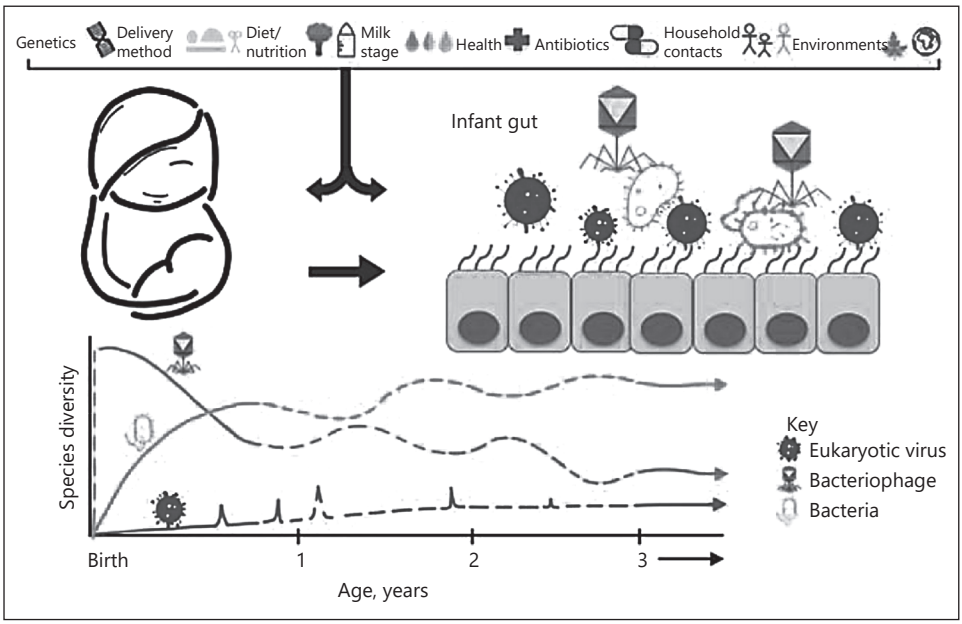

Fig. 1. Breastfeeding transfers milk microbes, including bacterial and viral communities, directly to the infant gut. Multiple factors impact on the diversity and composition of the microbial communities in both mother and infant. The eukaryotic virus, bacteriophages, and bacteria coexist in the infant gut in an interdependent and dynamic relationship. The trajectory of the microbial diversity over time is shown during the dynamic period in the first 2–3 years of life. Dashed lines represent hypothesized trajectories based on limited data.

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Gut Microbiota, Host Gene Expression, and Cell Traffic via Milk

Josef Neu

The human neonate is often born with a nonsterile gastrointestinal tract. The mechanism of this in utero colonization is poorly understood, but potential sources include ascending vaginal microbes, translocation of microbes from the maternal intestine or skin, or hematogenous spread from the maternal oral cavity.

Furthermore, human milk is not sterile. Using both culture and non-culture-based techniques, this fluid has been found to be rich in microbes, and they will eventually colonize or at least be present in the intestine of the developing infant with the potential to elicit numerous immune responses via metabolite production, interaction with the developing infant immune system, and induction of signals for host gene expression.

In addition to the milk microbes, this fluid also provides a source of enzymes such as lipase and alkaline phosphatase. Immunoglobulins, especially IgA, are found at relatively high concentrations. Milk also provides a multitude of biologically active proteins such as lactoferrin and lysozyme. Carbohydrates, including disaccharides such as lactose, when not digested and absorbed by the host can be utilized by microbes that metabolize this sugar to other highly biologically active agents such as short-chain fatty acids, acetate, propionate, and butyrate. Oligosaccharides serve as nutrients for certain microbes that flourish in their presence and have the ability to interact with the intestinal mucosa of the host.

The cellular composition (including immune cells and stem cells) and siRNAs found in milk exosomes also appear to play important roles in terms of immunologic protection, immune modulation, and transcriptional regulation.

Although exciting, much of the information currently available is associational. These agents are present in milk; we have theories about their roles, without a strong body of mechanistic data. We have also the impression that just because they are present in human milk, they

provide specific advantages to the infant who is not fed these components. However, it is clear that despite major efforts, not all babies will be able to receive and benefit directly from their own mothers' milk. A better understanding not only of the composition of human milk – but also the mechanisms of how these components affect the host – should help us optimize milk for a larger number of infants.

Suggested Reading

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Breast Milk and Microbiota in the Premature Gut: A Method of Preventing Necrotizing Enterocolitis

W. Allan Walker and Di Meng

Necrotizing enterocolitis (NEC) is an inflammatory condition of the gastrointestinal tract that affects approximately 10% of newborn infants <1,500 g. It occurs in part due to colonizing bacteria interacting with an immature intestine [1]. The immature intestine evokes an innate immune response with increased TLR-4 expression on its intestinal surface and increased signal molecule and NF-κB levels in enterocytes combined with decreased levels of regulatory molecules (e.g., SIGIRR, IRAK-M, and A20). This results in an inflammatory response rather than immune homeostasis [2].

To counteract this tendency, neonatologists have supported breast milk donation [3]. They have also used a variety of probiotics [4]. A combination of both factors – breast milk and probiotics (symbiosis) – appears to set the best results. Therefore, the mechanism of this effect on newborn infants has attracted increased interest [5].

We have shown that *Bifidobacterium infantis* and breast milk produce a molecule which is effective against NEC. This molecule has been shown to affect inflammation in the immature intestine by inhibiting the transcription factor aryl hydrocarbon (AHR) which stimulates IL-8 production (Fig. 1).

In addition, complex carbohydrates in breast milk interacting with colonizing bacteria such as *B. infantis* can produce short-chain fatty acids (SCFAs) which are able to induce anti-inflammatory effects via G-coupled receptor (GRP 109) and modulate inflammatory responses of immature enterocytes (Fig. 2).

Fixed protocols in clinical trials including a large number of infants are required to confirm the significance of these effects on the newborn intestine regarding NEC prevention and thus to provide a way to prevent NEC in premature infants.

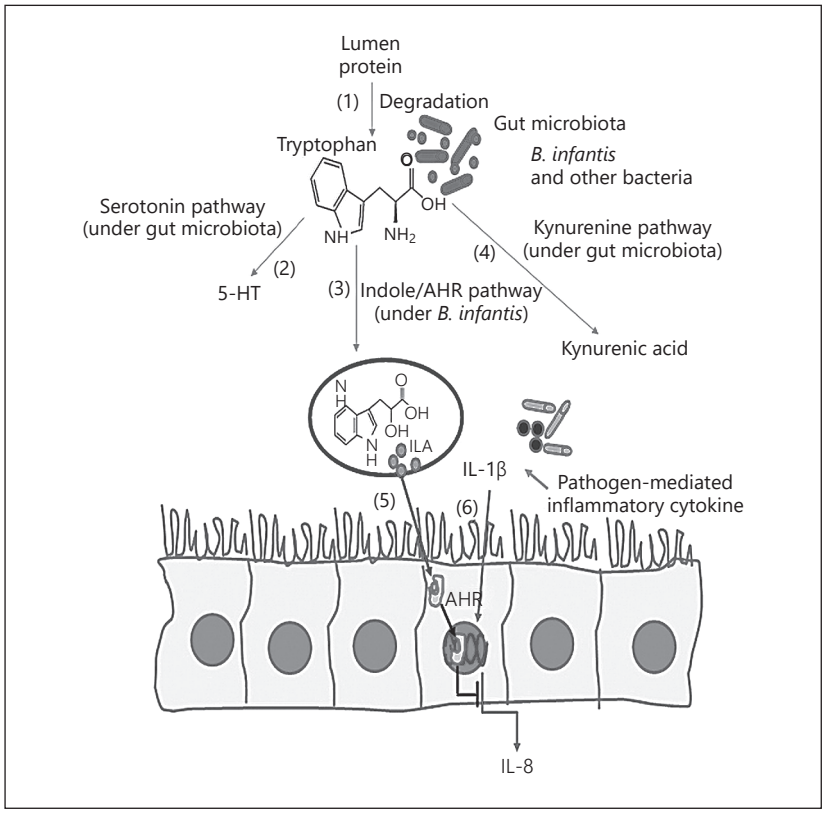

Fig. 1. Cartoon of the suggested mechanism of the anti-inflammatory effect of the tryptophan (Trp) catabolite indole-3-lactic acid (ILA) in *B. infantis* secretions on an intestinal epithelial cell. (1) Degradation of lumen protein leads to Trp release. Under the influence of the gut microbiota, Trp is converted to (2) 5-hydroxytryptamine (5-HT) by the serotonin pathway, (3) ILA by the indole/AHR pathways, and (4) kynurenic acid by the kynurenine pathway. ILA acts on AHR found in fetal enterocytes (5) thereby affecting the innate immune response in a ligand-specific fashion suppressing a pathogen-mediated inflammatory cytokine IL-1β-induced IL-8 secretion (6).

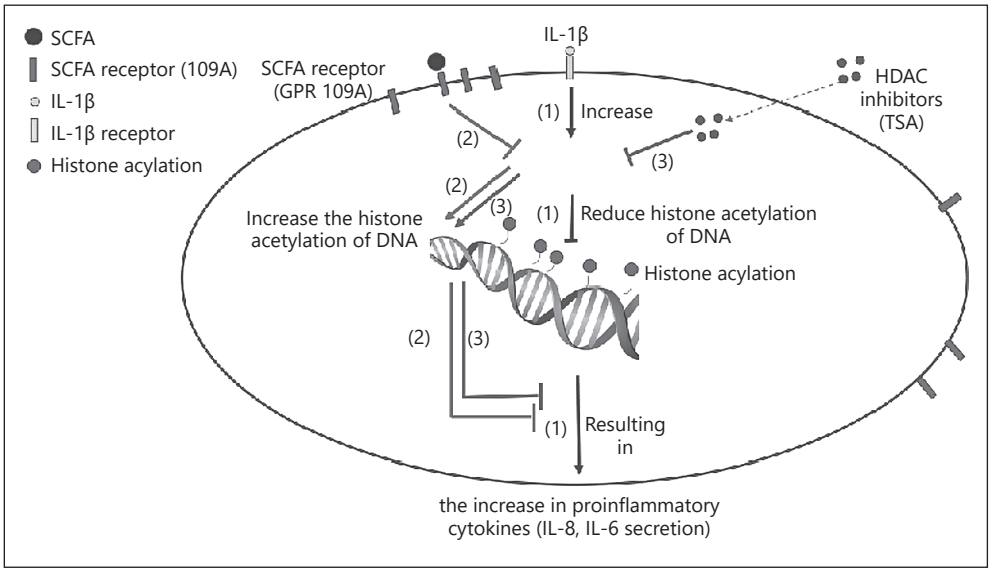

Fig. 2. SCFAs decrease IL-1β-induced IL-8 secretion by inhibition of histone deacetylase (HDAC) activity in immature enterocytes. (1) IL-1β increases HDAC activity that will reduce histone acetylation of DNA of the immature enterocytes resulting in the induction of proinflammatory cytokine secretion such as IL-8 and IL-6. (2) Via SCFA receptor, e.g., GPR 109A, SCFAs inhibit HDAC activity leading to the increase in histone acetylation of DNA resulting in the inhibition of IL-1β-induced IL-8 and IL-6 induction. (3) HDAC inhibitors, e.g., trichostatin A (TSA), can inhibit HDAC activity leading to increased histone acetylation of DNA resulting in inhibition of IL-1β-induced IL-8 and IL-6 induction.

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Human Milk Oligosaccharides: Structure and Functions

Lars Bode

Human milk is unique when it comes to the high concentrations and structural diversity of oligosaccharides. In fact, human milk oligosaccharides (HMOs) are the third most abundant component of human milk after lactose and lipids, often exceeding the total amount of human milk proteins. HMOs are composed of up to 5 different building blocks: glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. More than 150 different HMOs have been identified and characterized so far, and, most intriguingly, the composition varies between women and changes over the course of lactation. Single-nucleotide polymorphisms in genes that encode specific fucosyltransferase enzymes are known to dramatically affect HMO composition. However, how other enzymes and transporters, for example, are involved in HMO biosynthesis remains largely unknown. The combination of genome-wide association studies, human milk transcriptomics, in vitro gene editing, and in silico pathway modeling allows us to reconstruct the HMO biosynthetic pathway and lays the foundation to modulate and optimize it once we fully understand the effects of HMOs on infant and maternal health and development. Once ingested, HMOs resist degradation through the infant, a small percentage is absorbed and reaches the systemic circulation, and the rest reaches the colon where it gets metabolized by the infant gut microbiota or is excreted intact with the feces. HMOs are known to be prebiotics, but they also serve as antimicrobials, antiadhesives, or immune cells modulators – both locally in the gut as well as systemically after absorption. Using new data-mining approaches and leveraging samples and metadata from large mother-infant cohorts enables us to identify associations between individual HMOs or HMO composition profiles with infant and maternal health outcomes. Suitable preclinical models and clinical intervention studies allow us to corroborate the established associations for causal relationships and test for in vivo efficacy in humans. In some cases, individual HMOs alone are effective, and the effects are highly structure specific and dose dependent, suggesting mediation through specific receptors –

either on a host cell or on microbes. For example, we discovered that a specific HMO named disialyllacto-N-tetraose improves survival and reduces pathology scores in an animal model of necrotizing enterocolitis [1]. In human cohort studies, preterm infants that receive human milk with low levels of the same HMO are at higher risk to develop necrotizing enterocolitis [2]. In a second example, specific HMOs increase the infectivity of a specific rotavirus G10P strain in tissue culture. The same HMOs occur at higher concentrations in the milk given to infants that acquire symptomatic rotavirus infections [3]. Both examples highlight the power of combining data generated from suitable preclinical models with data obtained from mother-infant cohorts. In other cases, the mixture and relative abundance of different HMOs to each other is what makes them most effective, suggesting mediation through complex interactions of multiple different HMOs on different molecular targets that shape the composition of gut microbial communities or complex multi-cell immune responses. For example, the ratio of 2′-fucosyllactose and lacto-N-neotetraose in mother's milk is associated with height and weight z-scores in a small Danish pilot study as well as in a large Finnish mother-infant cohort [4]. In a second example, not one single HMO alone is associated with food sensitization when the infant is 1 year of age, but the combination of several HMOs as a specific profile is associated with food sensitization [5]. Overall, the knowledge generated from combining suitable preclinical models, mother-infant cohort association studies, as well as randomized clinical trials will help us establish true structure-function relationships and provide the rigorous evidence required to improve infant health and development.

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Oligosaccharides and Viral Infection: Human Milk Oligosaccharides versus Algal Fucan-Type Polysaccharides

Franz-Georg Hanisch and Cem Aydogan

Norovirus infections belong to the most common causes of human gastroenteritis worldwide, and epidemic outbreaks are responsible for hundreds of thousands deaths annually. In humans, noroviruses are known to bind to gastrointestinal epithelia via recognition of blood group-active mucin-type O-glycans. Besides vaccine-based approaches to combat the virus, one of the alternative strategies to prevent norovirus infections is based on food additives, e.g., human milk oligosaccharides (HMOs).

HMOs represent an ideal source of potential competitors of viral glycan receptors, which mimick the structures of blood group-active mucin-type O-glycans. The trisaccharide 2-fucosyllactose (2′FL) is able to block norovirus binding quite efficiently and has reached market approval as a save food additive. In addition, we could provide evidence for other milk oligosaccharides in the high-mass range that exert even stronger competitive effects on norovirus binding to gastric mucins [1]. During these studies, we observed that oligovalency of fucose in hepta- to decasaccharides promotes competitive effects on norovirus binding. This became most evident when L-fucose cyclodextrin-based dendrimers with varying degrees of substitution were compared with respect to their competitive activity [1]. High valency of α-L-fucose with no relationship to blood group structures is also a feature of natural polysaccharides belonging to the group of polyfucoses or fucans.

Algal fucoidans are present in several orders, mainly Fucales and Laminariales, and they exist either as a homopolymer of fucose or as a heteropolysaccharide. Fucoidans can have their central chains composed of (1→3)-linked α-L-fucose or of Fuc-α(1-3)Gal repeating units as in *Undaria pinnatifida*, or they are composed of repeating (1→3) and (1→4)-linked fucose (*Fucus* species). Sulfate residues are found at high densities, as every second fucose can be substituted at C2 and/or C4.

Among fucoidans, those of the brown algae have previously attracted much attention, as they were claimed to exert a series of health beneficial effects. Like other sulfated polysaccharides, fucoidans can inhibit virus infection of cells. This has been demonstrated for *Herpes simplex*, cytomegalovirus, and human immunodeficiency virus as well as bovine viral diarrhea virus, probably by competing with cell surface heparan sulfate for binding to the virus, a mechanism which is strictly dependent on sulfation of the polysaccharide.

Considering the involvement of α-L-fucose residues in epithelial receptors, their high valency-associated binding avidity far surpasses the low-affinity (though specific) interactions of monovalent blood group-active HMOs, which is reflected in IC_{50} values in the millimolar range (for example, LNFP-I: >50 mM) [1]. We here report for the first time on competitive anti-norovirus effects exerted by α-L-fucose of fucoidans derived from different sources and demonstrate that their inhibitory capacity is retained in desulfated and fragmented low-molecular-weight processing products. Avidity-based increases in efficiency, as reflected in the IC_{50} values, are in the order of 100-fold fucose: >50 mM, 2′FL: 25 mM, fucoidan from *Fucus vesiculosus*: 250 μM. Insight into structural aspects was obtained by X-ray analysis of P-domain dimer oligofucose cocrystals, which confirmed low-affinity interaction of single fucose with the capsid protein. NMR-based structural work on various forms of low-molecular-mass fucoidans prepared from *F. vesiculosus* and *U. pinnatifida* are ongoing.

In an animal model, blocking tests with various fucoidan preparations affecting viral binding to gastrointestinal epithelia have been performed by virion-challenging experiments in oyster. In targeting currently untreatable norovirus infections, this study provides first steps towards a prophylactic food additive that is produced from algal species.

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Milk Fat Globule Membranes: Effects on Microbiome, Metabolome, and Infections in Infants and Children

Olle Hernell, Bo Lönnerdal, and Niklas Timby

The composition of human milk is optimal to meet the nutritional needs for infant growth and development during the first 4–6 months of life. The milk fat globule (MFG) consists of a core of mainly energy-rich triglycerides enveloped by a unique membrane structure, the milk fat globule membrane (MFGM). MFGM is composed of a phospholipid and cholesterol triple layer with incorporated proteins and glycoproteins. Milk phospholipids, sphingomyelins, and gangliosides are largely located on the MFGM [1]. Several studies reported various health benefits of feeding bovine MFGM to humans of different age groups, including infants and children. We found 5 double-blind randomized controlled trials exploring the effects of supplementing the diet of infants and children with bovine MFGM concentrates on infections (Table 1) [2, 3]. Two of these (Peru and Sweden) found a protective effect (on bloody diarrhea and otitis media) in infants, and one reported fewer days with fever (5- to 7-year-old Belgian children) (Table 1).

The Swedish study investigated plasma and erythrocyte lipidome and oral, plasma, and fecal metabolome as well as fecal microbiome. There were significant differences in both plasma and erythrocyte membrane lipidomes between the MFGM-supplemented experimental formula (EF) group and the group fed standard formula (SF) mostly due to differences in sphingomyelin and phosphatidylcholine concentrations but not at 12 months of age [4].

In 30 randomly selected infants from each group (EF, SF, and a breastfed [BF] reference group), plasma metabolomes were analyzed by NMR. Overall differences between the formula-fed groups (FF) and the BF group were much larger than between EF and SF groups. Interestingly, however, the EF group had higher levels of fatty acid oxidation products than the SF group, which is typical for BF infants. These differences disappeared after the introduction of complementary feeding. Thus, MFGM may have a role in directing infant metabolism [5].

Table 1. Double-blind, randomized, controlled trials exploring the effects of MFGM supplementation to the diet of infants or children on infections compared with no supplementation (reviewed by Hernell et al. [2])

Study location	Age	Supplementation	Effects of MFGM on infections
Peru	6–11 months	MFGM (Lacprodan® MFGM-10; Arla Foods Ingredients)	Lower longitudinal prevalence of diarrhea Lower incidence of bloody diarrhea
Belgium	2.5–6 years, during 4 months	MFGM (INPULSE®; Büllinger SA)	Fewer days with fever
India	8–24 months, during 12 weeks	Complex milk lipids (Fonterra Co-operative Ltd)	No difference regarding diarrhea
Sweden	<2–6 months	MFGM (Lacprodan® MFGM-10; Arla Foods Ingredients)	Lower incidence of otitis media
China	<1–4 months	MFGM (Lacprodan® MFGM-10; Arla Foods Ingredients)	No differences regarding fever, diarrhea, or urinary tract infections

The oral microbiota analyzed by Illumina MiSeq multiplex sequencing exhibited moderate effects on oral microbiota. Species richness did not differ between the EF and SF groups, but a few taxa that were significantly associated with being in either group were identified, e.g., a lower level of *Moraxella catarrhalis* in the EF group. Of note, *M. catarrhalis* is one of the major otitis media pathogens, and this finding may thus be associated with a decrease in otitis media seen in the EF group compared to the SF group [6].

Fecal microbiota analysis was performed by 16S rRNA gene sequencing. While the impact of EF on the fecal microbiome was minor compared to the SF group, the fecal metabolome of EF infants showed a significant reduction in several metabolites, including lactate, succinate, amino acids, and their derivatives, compared to SF infants. Again, the introduction of complementary food with either human milk or infant formula reduced the distinct BF or FF characteristics of the fecal microbiome and metabolome profiles in infants. The findings support the hypothesis that higher protein levels in infant formula promote a shift towards amino acid fermentation in the gut, and that MFGM may play a role in shaping gut microbial activity and function [7].

In conclusion, studies have shown that feeding infants MFGM reduces the risk of infections and affects the plasma and erythrocyte membrane lipidomes, which might be reflected by improved neurodevelopment. Further, MFGM also modifies the oral microbiome, which may contribute to less risk of otitis media and exerts a minor impact on the fecal microbiome but drives the metabolic phenotype profile towards fatty acid oxidation, which is typical for BF infants but not FF infants.

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Clinical Trials of Lactoferrin in the Newborn: Effects on Infection and the Gut Microbiome

Nicholas D. Embleton and Janet E. Berrington

Every year, more than 15 million infants are born premature or with a low birth weight globally [1]. Whilst survival rates are improving, infections remain prevalent. In infants born very preterm, late-onset sepsis (LOS) and necrotizing enterocolitis (NEC) are the commonest reasons for death after the first week [2]. The etiology of NEC and LOS is multifactorial, and various strategies have been employed in an attempt to reduce their occurrence, especially the provision of mother's own breast milk, which results in a dose-response reduction in NEC and LOS occurrence.

In recent years, our knowledge of gut microbiota in preterm infants has increased due to next-generation sequencing methodologies. In several studies, the pattern of gut microbial communities differed between preterm and healthy term infants. Furthermore, these patterns appear to change prior to the onset of NEC or LOS, offering hope that earlier disease identification may be possible [3]. However, many healthy preterm infants exhibit gut community patterns that are not dissimilar to those who develop disease, and many infants show rapid changes in microbial patterns without obvious precipitants. Identification of an enteral immunonutrient such as lactoferrin with a range of enterocyte growth activity and immunomodulation might lead to a reduction in NEC.

Human breast milk has bacteriostatic activity against certain bacteria such as *Escherichia coli*. It was originally postulated that much of this activity derived from the presence of lactoferrin, an iron-binding glycoprotein present at high concentrations especially in early colostrum. Lactoferrin may also act as a "prebiotic," and studies also show improved immune cell responses. Typically, preterm infants receive only small amounts of mother's own colostrum in the first 1–2 days and usually take several days to achieve full enteral feeds. Providing supplemental lactoferrin at this stage may, therefore, be beneficial. A large multicenter randomized controlled study conducted in Italy showed that overall rates of

LOS were significantly lower in those receiving lactoferrin, and there was a reduction in NEC [4].

The ELFIN trial, the largest trial of lactoferrin supplementation, enrolled 2,203 infants [5]. Very preterm infants were recruited before 72 h of age and allocated to lactoferrin or placebo. The primary outcome was LOS, and primary outcome data showed no difference in the rate of LOS between lactoferrin (28.9%) and placebo groups (30.7%). The incidence of NEC stage II/III and all-cause mortality also did not differ. The ELFIN trial provides high-quality data that show that routine supplementation with lactoferrin does not improve outcome, contradicting findings suggested by previous studies.

The ELFIN trial highlights the importance of adequately powered high-quality trials to provide the most robust evidence base for clinical practice. This requires collaboration at national and international level. Whilst large-scale pragmatic trials are essential, mechanistic studies are needed in order to better understand disease pathology and treatment effects. However, these are practically and ethically challenging in preterm infants, and studies must be planned, developed, and conducted concomitant with parents and the public. The ELFIN trial reminds us that nutrients do not function in isolation, and that the optimal source of immune nutrients remains fresh human milk. Whilst we search for immune nutrients that improve health and reduce disease, we must continue to better understand how to support mothers to meet their infants' intake needs using their own milk.

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Effects of Milk Osteopontin on Intestine, Neurodevelopment, and Immunity

Rulan Jiang and Bo Lönnerdal

Osteopontin (OPN) is an acidic phosphorylated glycoprotein. It is a multifunctional protein which is involved in cell proliferation and differentiation, biomineralization, immunomodulatory activities, and myelination. OPN contains integrin and CD44 receptor binding sites, and it exerts its pleiotropic functions by binding to its receptors on the cell membrane and triggering cellular signaling pathways. OPN is expressed in various cell types, such as epithelial cells and immune cells, and it is found in most body fluids, including milk and blood. It is present at increased concentration in human milk, but not in cow milk and infant formula. Milk OPN is relatively resistant to gastrointestinal digestion, and it may, therefore, contribute to intestinal development. Additionally, orally ingested OPN appears in the circulatory system, which suggests that milk OPN exerts its multiple functions also systemically (Fig. 1).

Milk OPN is commercially available since methods to isolate OPN from cow milk have been established. Bioactivities of milk OPN were investigated using cell lines, animal models, and randomized clinical trials, and the results from these studies demonstrated that there are three major functions of milk OPN in early life. First, milk OPN contributes to intestinal proliferation and maturation. It stimulates proliferation, differentiation, and immunity of human intestinal epithelial cells [1]. In a study in rhesus monkeys, monkey infants fed formula supplemented with bovine milk OPN for 3 months exhibited a transcriptome more similar to the breastfed group, and OPN upregulated genes involved in cell proliferation, migration, survival, and signaling pathways from integrin and CD44 receptors [2]. Second, milk OPN promotes neurodevelopment in early infancy. It was shown that OPN is abundantly present in the brain and regulated brain myelination. In an established OPN mouse model, wild-type mouse pups nursed by wild-type or OPN knockout dams received milk with abundant OPN or no OPN. The orally ingested milk OPN upregulated endogenous OPN in the brain and thus promoted proliferation and differentiation of NG-2 glia into oligodendrocytes.

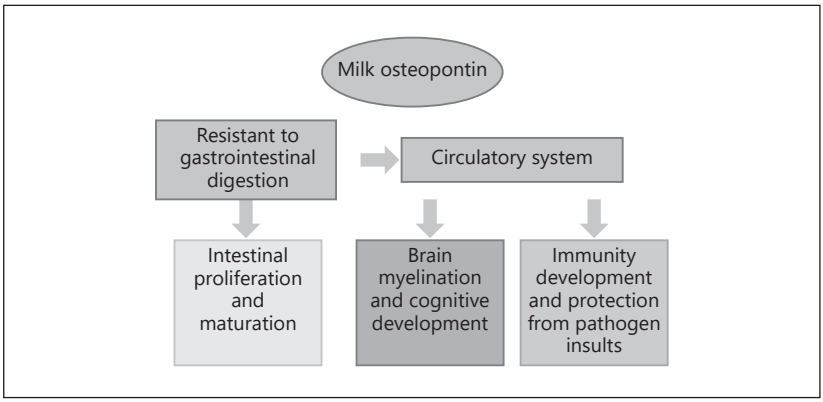

Fig. 1. Milk osteopontin functions in early life.

As a consequence, brain myelination and cognitive development were enhanced [3]. Finally, milk OPN stimulates immune development. In mice with colitis induced by dextran sulfate sodium, oral administration of bovine milk OPN showed reduced inflammatory symptoms. Further, OPN may alter the gut microbiota to fight enterotoxigenic *Escherichia coli* infection in piglets. Additionally, in a randomized clinical trial in infants 1–6 months of age, infants were either breastfed, fed regular formula, or fed OPN-supplemented formula. Infants fed OPN-supplemented formula had significantly lower serum TNF-α levels and fewer days of illness compared with infants fed regular formula, and their immune cell profile was more similar to that of breastfed infants [4, 5].

In conclusion, milk OPN plays important roles in the development of the intestine, brain, and immunity.

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Effects of Milk Secretory Immunoglobulin A on the Commensal Microbiota

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Breast milk contains many components capable of modifying the infant intestinal microbiota, including antimicrobial peptides and bioactive proteins like lactoferrin and lysozyme, oligosaccharides that feed commensal bacteria and provide pathogen decoys, and a high concentration of secretory IgA (SIgA), the primary mucosal antibody responsible for pathogen exclusion at mucosal surfaces. Milk SIgA is derived from a diverse pool of intestinally induced plasma cells homing to the lactating mammary gland. This antibody repertoire is varied, with evidence of both naturally polyreactive and antigen-specific monoclonal immunoglobulins with capacity to bind to enteric pathogens like *Campylobacter*, but also to cross-react with general microbial- and self-antigens [1]. This cross-reactivity may be due to both a low-affinity maturation of some milk immunoglobulins or to the glycan heterogeneity on the SIgA glycoprotein. Milk SIgA is heavily glycosylated with O- and N-linked glycans that bind to select commensals promoting mucosal localization and increasing their colonization potential in the infant gut (Fig. 1).

Recent data from Nakajima et al. [2] suggest that antigen-independent binding of commensal bacteria can occur while the immunoglobulin is bound to its cognate antigen in a process termed "bystander effect." Studies provide evidence that nonspecific binding of SIgA promotes colonization of the commensal bacteria in a unique mucosal niche in the intestine, reducing competition within the complex microbiota and providing benefit to the host. In a mouse model, nonspecific SIgA binding of commensals reduced dextran sulfate sodium-induced colitis whereas both the absence of SIgA in IgA-deficient mice and the absence of polysaccharide production in commensals responsible for the IgA association failed to protect the mouse from the development of colitis [3]. In vitro, nonspecific SIgA binding to commensals reduced the induction of proinflammatory cytokines and increased the expression of tight junction binding

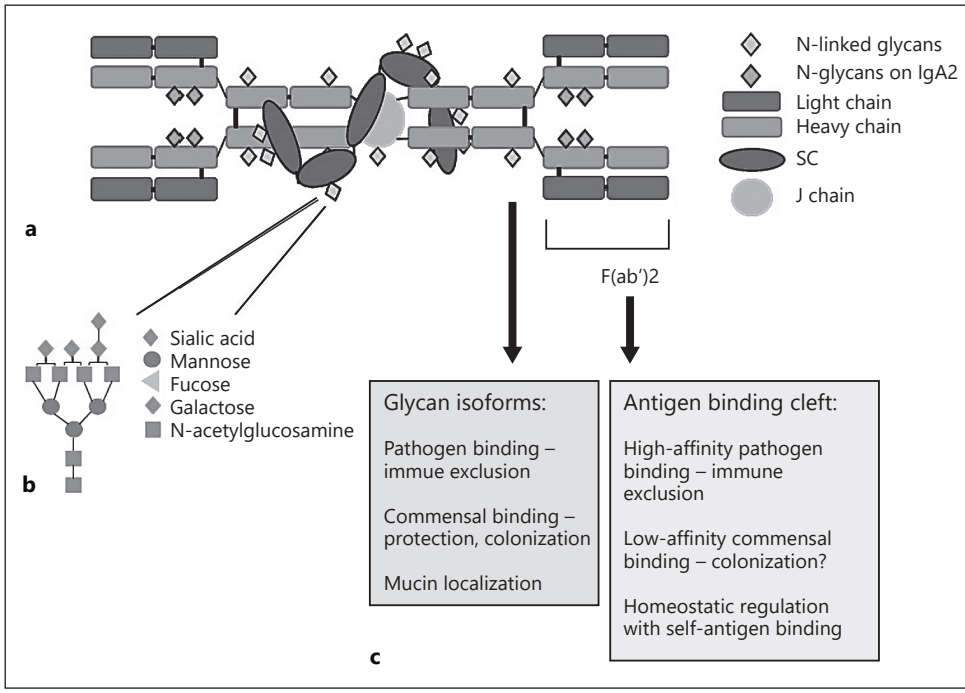

Fig. 1. Schematic representation of the SIgA molecule (**a**), an example of the glycan isoforms (**b**), and some of the functions of the glycans and the antigen-binding region (**c**) on the molecule.

proteins in mammalian colonocytes when the bacteria were added to the cell culture [4]. Our research group has shown that polymeric milk SIgA binding to select commensals protects them from intestinal proteolysis in vitro. In addition, Moor et al. [5] demonstrated the ability of targeted SIgA to remain bound to the bacterial surface throughout the process of cell division in their model of *enchained growth*. Taken together, milk SIgA may retain antipathogenic protective functions with Fab-dependent high-affinity interactions while concurrently maintaining homeostatic commensal colonization in the infant intestinal mucosa through glycan or nonspecific binding that promotes exopolysaccharide production and niche partitioning. These studies and others demonstrate the complex utility of milk SIgA in shaping the intestinal microbiota in the infant, both in the protection from pathogens and in the promotion of commensal colonization.

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