Prebiotics in Infant Nutrition

EDITED BY:

SHARON DONOVAN, PhD
UNIVERSITY OF ILLINOIS—URBANA, ILLINOIS

GLENN GIBSON, PhD
THE UNIVERSITY OF READING—READING, UNITED KINGDOM

DAVID NEWBURG, PhD
MASSACHUSETTS GENERAL HOSPITAL—BOSTON, MASSACHUSETTS

Table of Contents

Executive Summary

Human milk fulfills many nutritive and immunoprotective functions for the neonate, especially within the neonatal gastrointestinal (GI) tract. Although it is not possible to produce infant formula that exactly replicates the composition of human milk, one goal of infant formula manufacturers is to offer products that can simulate the composition and biological effects of human milk as closely as possible. A very important area is the effect of human milk on content, composition and activity of the gut microbiota.

Normal development and function of the GI tract critically depends on the presence of complex microbiota, which perform numerous metabolic, growth-promoting, and protective roles. The composition of the GI microbiota is influenced by ingestion of food substances that enhance or inhibit growth of selected genera/species in the intestinal tract, mainly the colon.

Infants fed human milk have a predominance of bifidobacteria, a genus known to have health-promoting functions, in their lower GI tract. The GI microbiota of breastfed infants also contain low populations of potentially pathogenic groups like clostridia. The gut microbiota of formula-fed infants, in contrast, are more diverse than those of breastfed infants, with similar or lower levels of bifidobacteria, but greater diversity and higher levels of potential pathogens and commensal bacteria. No single genus is thought to predominate in the formula-fed infant gut. Clinical and epidemiological investigations have demonstrated that breastfed infants have lower rates of intestinal infection than formula-fed counterparts, leading to the hypothesis that their decreased rates of infection compared to formula-fed infants may be related in part to the higher concentration of bifidobacteria, which are known to be inhibitory to pathogens. Differences in GI microbiota between breastfed and formula-fed infants also result in different stool and laxation characteristics. Breastfed infants typically have larger, softer, and more frequent stools than those who are formula-fed.

Although the microbiota present in the formula-fed infant may not necessarily be harmful, it is desirable for infant formula to more closely match the functional activities of breast milk, and investigators are currently seeking ways to promote the establishment of a similar intestinal environment in formula-fed infants. Efforts to improve the performance of infant formula include investigating the use of novel ingredients that have the potential to shift the GI microbiota of formula-fed infants to more closely resemble those of breastfed infants.

Human milk oligosaccharides (HMOS) are a structurally diverse and highly variable group of bioactive carbohydrates. HMOS resist digestion by enzymes in the human small intestine (including those supplied from the pancreas) and thereby reach the lower GI tract largely intact. HMOS have several key beneficial effects on the development of the neonatal intestine, including promotion of intestinal colonization by beneficial microbes (e.g., bifidobacteria), stimulation of intestinal adaptation to the extrauterine environment, compensation for developmental immaturity of the intestine, and protection against colonization by pathogenic bacteria.

Infant formula manufacturers have evaluated numerous ingredients as part of continuing efforts to more closely approximate the nutritional and functional properties of human milk, including those of HMOS. Numerous studies have suggested that *prebiotics* can be used to alter the gut microbiota of formula-fed infants to more closely simulate those of breastfed infants. A prebiotic has been defined as a nondigestible food ingredient that brings about specific changes in the composition and/or activity of the gastrointestinal microbiota which confer benefits upon host well-being and health. Prebiotic ingredients are typically nondigestible oligosaccharides. Although it is impossible to fully match the diverse and dynamic nature of oligosaccharides in human milk, a number of food-grade oligosaccharides have demonstrated capacity to increase levels of selected beneficial bacteria (principally bifidobacteria) and improve stool characteristics in formula-fed infants.

Commercial oligosaccharides studied for use in infant formula include galactooligosaccharides (GOS), polydextrose (PDX), lactulose (LOS), inulin, and fructooligosaccharides (FOS), as well as combinations of these products. The effects of prebiotics on infant growth, GI microbiota, stool characteristics, mineral absorption and biomarkers of immune function have been assessed.

Current clinical evidence indicates that infant formulas supplemented with prebiotic carbohydrates support normal growth and that prebiotics are capable of mimicking some of the effects of HMOS, particularly in the areas of intestinal microbiota and stool characteristics. Clinical studies in infants have shown that ingestion of GOS, LOS, inulin, and/or FOS can result in positive effects on the composition of the GI microbiota. However, not all clinical trials have demonstrated a positive effect of prebiotics on gut levels of beneficial bacteria. This may be due to differing methodologies used to ascertain effects. As such, more studies are needed to establish a direct relationship between alterations of infant GI microbiota and improved measures of GI health. Some studies have assessed the effect of prebiotics on stool pH and short-chain fatty acid (SCFA) levels as a measure of the health of the GI tract of infants. An additional goal of prebiotic supplementation is to produce stool characteristics in formula-fed infants similar to those observed in breastfed infants. Studies in infants have shown beneficial effects of prebiotic ingredients on stool characteristics.

Much of the evidence for the beneficial effects of GOS, PDX, LOS, or FOS on mineral absorption is based on animal studies, which have shown that the absorption of calcium, magnesium, potassium, and phosphorus was increased following dietary prebiotic supplementation. Although mineral absorption in humans is positively affected with prebiotic supplementation, research has been largely conducted in adolescents and postmenopausal women. Few clinical studies have evaluated the effect of prebiotics on mineral absorption in infants and young children. In order to define the role of prebiotics on mineral absorption, additional studies are needed—including a determination of the mechanism of effect.

Because prebiotics influence the composition and activity of the gastrointestinal microbiota, which in turn have a major effect on the immune system (mainly the gut-associated lymphoid tissue, or GALT), it is reasonable to expect prebiotics to indirectly affect the immune system. Indeed, data from animal studies suggest that dietary supplementation with prebiotics can influence certain markers of immunity and affect the incidence of allergy and disease. However, the critical association between prebiotic supplementation and improved immunologic function in humans remains to be fully defined. Few studies have been conducted in humans, and most of these have been in adults. More studies are needed to understand whether prebiotic ingredients in infant formulas lead to sustained positive effects on infant immunity.

In summary, trials conducted in experimental animals and humans have shown beneficial effects of prebiotics on the composition of GI microbiota, measures of GI health and immunity, laxation, and mineral absorption. Mead Johnson will continue to evaluate the evolving science supporting the use of prebiotic ingredients in infant formula; however, further research is needed in order to provide state-of-the-science nutrition to formula-fed infants.

Introduction

Breast milk is widely regarded as the gold standard for infant feeding, and numerous health organizations endorse breastfeeding as the optimal form of nutrition for infants during the first year of life. However, over 80% of US infants receive formula at some time during the first 12 months of life.[1] For these infants, iron-fortified formula is recommended.[2] One goal for infant formula manufacturers is to offer formulas that simulate human milk as closely as possible.

The gastrointestinal (GI) microbiota play a critical role in supporting health. For many years, scientists have known that the composition of GI microbiota is influenced by dietary substances that enhance or inhibit the growth of selected microbiota in the intestine. Numerous studies have suggested that the gut microbiota of formula-fed infants can be altered to be more like those of breastfed infants through the addition of ingredients called *prebiotics*. A prebiotic is a nondigestible food ingredient that brings about specific changes in the composition and/or activity of the gastrointestinal microbiota which confer benefits upon host well-being and health.[3] A number of potential prebiotic ingredients such as galactooligosaccharides (GOS), polydextrose (PDX), lactulose (LOS), inulin, and fructooligosaccharides (FOS) has been evaluated for use in infant formulas.

Sidebar: Distinguishing Prebiotics from Probiotics

Prebiotics are food ingredients (typically oligosaccharides) that are selectively fermented by beneficial bacteria in the gut (such as *Bifidobacterium*), stimulating the growth and/or activity of those bacteria and thereby contributing to host health and well-being. Prebiotics are resistant to gastric acidity, enzymes and absorption. Their purpose in infant formula is to stimulate the growth and colonization of naturally occurring beneficial bacteria.

Probiotics are bacteria that pass through the GI tract and have beneficial effects on the health of the host. Probiotic bacteria typically have a history of safe consumption; examples include *Lactobacillus rhamnosus GG* and *Bifidobacterium animalis ssp. lactis*. Their purpose in infant formula is to substitute for naturally occurring beneficial bacteria and thereby influence the mucosal immune system.

Development of Intestinal Microbiota in Infants

The mature GI tract contains a large quantity and diversity of microbiota that play a critical role in protecting and promoting infant health. Normal development and functionality of the human GI tract depend on the presence of complex gastrointestinal microbiota, as these microorganisms perform numerous metabolic, growth-promoting, and protective roles.[4,5]

Colonization of the GI tract

The infant GI tract is essentially sterile prior to birth, but is immediately exposed to maternal and environmental bacteria during delivery. The subsequent colonization process is influenced by the genetics of the host[5] and several environmental factors, including mode of delivery, gestational age, hospitalization, antibiotic use by the mother or infant, and type of infant feeding.[6] Studies using standard microbiological methods have shown that during the first few days of life, infants' intestinal microbiota are comprised almost entirely of enterobacteria and Gram-positive cocci, obtained predominantly during the birth process.[7-9] Studies suggest that these facultatively anaerobic bacteria generate conditions that favor the subsequent establishment of strict anaerobes such as bacteroides, bifidobacteria, and clostridia.[10,11]

The method of delivery at birth is important in determining the initial composition of intestinal microbiota since infants' GI tracts are first exposed to microorganisms during the delivery process. Infants born by the vaginal route acquire their initial intestinal microbiota from maternal vaginal and fecal microbiota, and it is recognized that the newborn infant's fecal flora closely resemble those of the mother. Infants born by Caesarean section show a different pattern of GI microbiota development, which may also be influenced by other factors such as reduced gestational age, increased exposure to the hospital environment, and prophylactic use of antibiotics.[12,13] Generally, development of the GI microbiota is delayed for up to several weeks in Caesarean-delivered infants, who have slower acquisition of *Bifidobacterium* and *Bacteroides*.[12,13] In preterm infants, intestinal colonization with bifidobacteria is delayed.[14,15] This has been attributed to several factors including delayed oral feeding, parenteral feeding, aggressive antibiotic therapy[16] and reduced gut maturation due to early gestational age.[17]

Diet has a particularly crucial influence on the composition of intestinal bacteria, especially in the first few months of life.[18] The intestinal microbiota of breastfed and formula-fed infants are comparable for the first 3 to 4 days of life, but substantial differences between the two groups develop over time.[19] Another shift in GI microbiota composition typically occurs around 4-6 months of age with the introduction of non-milk foods and complex carbohydrates to the diet. As weaning progresses, both intestinal function and fermentation capacity continue to mature; composition of the intestinal microbiota approximates that of adults by the second year of life.[20]

The predominant microorganisms in the adult human GI tract are highly individualized and, once established, remain relatively stable over time.[21] It is widely believed that microorganisms introduced to the neonatal GI tract have a higher likelihood of becoming established compared to introducing the same microorganisms (e.g., as probiotics) into the adult GI tract. By adulthood, the intestinal microbiota are extremely complex, with total numbers reaching as high as 10^{12} bacterial cells per gram feces and representing as many as 1000 different species.[22,23]

Sidebar: Host-Microbe Interactions at Mucosal Surfaces: Key to Balancing Immune Tolerance vs. Inflammation

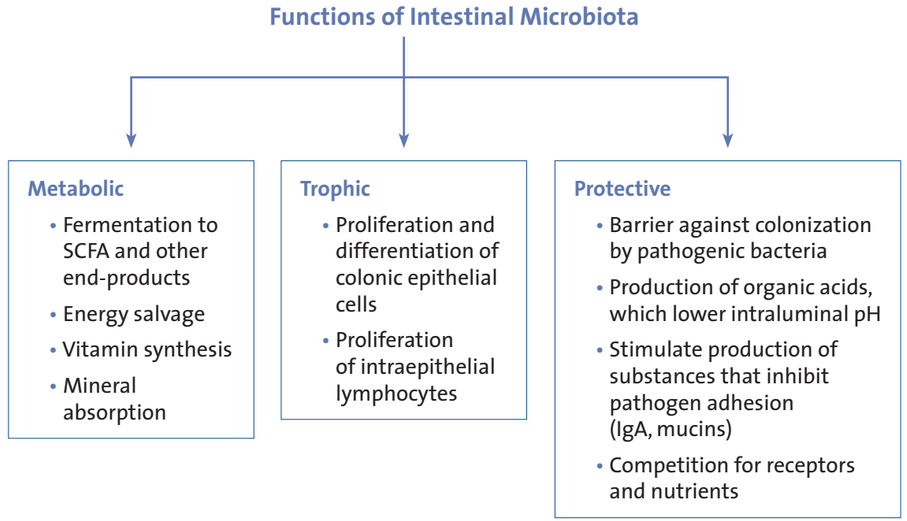
It has long been appreciated that mutually beneficial relationships between animals and microbes are a dominant theme of life; however, the extent to which our commensal microbiota affect human health has been realized only recently. Human cells are outnumbered ten-fold by the microbiota resident in the human GI tract, and the collective microbial genome (microbiome) contains at least 100 times as many genes as our own.[24] Humans have co-evolved with these microbiota, which provide genetic and metabolic attributes that humans do not possess, including the ability to harvest otherwise inaccessible nutrients.[5] In reality, the intestinal microbiota have a metabolic activity equal to a virtual "organ within an organ,"[25] and humans could be viewed as "superorganisms whose metabolism represents an amalgamation of microbial and human attributes."[24] Beneficial microorganisms also partially protect against pathogens by competing for metabolites, producing antimicrobial peptides, occupying epithelial/mucous niches, and preventing host-pathogen interactions; thus, it is in humans' interest to tolerate them.[26,27]

At the same time, however, immunity to dangerous microorganisms has to be initiated. There are several ways in which the immune system may discriminate between commensal and pathogenic microorganisms.[27-29] Immune cells could discriminate between the two via engagement of different pattern-recognition receptors (PRRs). Second, intestinal epithelial cells could "sense" the presence of noninvasive (commensal) versus invasive (pathogenic) microorganisms and transmit this information to antigen-presenting cells (APCs), such as dendritic cells. Finally, APCs could be programmed to perform different tasks according to their tissue of origin, such that dendritic cells or macrophages that are resident in the gut mucosa and regularly encounter commensal bacteria are tolerogenic, whereas cells that are recruited to the gut in response to pathogenic insult activate the immune response.[27]

Beneficial functions of intestinal microbiota

The primary functions of intestinal microbiota can be categorized as metabolic, trophic and protective (Figure 1). Metabolic functions include fermentation of some carbohydrates to produce short-chain fatty acids (SCFAs) and other end products,[4, 20] with subsequent effects on luminal pH, synthesis of vitamins and absorption of minerals.[30-35] Trophic effects of the intestinal microbiota include stimulating the proliferation and differentiation of colonic epithelial cells and proliferation of specialized immune cells.[36-46] One key protective function of the intestinal microbiota is to prevent adhesion and colonization by pathogens.[4, 47] The net effect of these functional properties of the intestinal microbiota is to protect the infant from infection, stimulate developmental maturation of the gastrointestinal digestive and immune systems and promote growth and development.

Figure 1: Beneficial Functions of Intestinal Microbiota in Infants



Adapted from references 4 and 25.

Metabolic effects

One important function of the intestinal microbiota is to transform complex dietary and host substances, such as mucin, into simpler compounds that can be used as energy sources by other microorganisms and/or the host. Most notable is fermentation of non-digestible dietary carbohydrates.[4, 20] The primary end products of this metabolic activity are SCFAs (primarily acetate, propionate, and butyrate). Other end products include carbon dioxide, methane, and hydrogen. Many of the beneficial effects of dietary fiber are attributed to SCFAs.[34] These compounds are readily metabolized by the intestinal epithelium, enhance small bowel digestion and absorption, stimulate sodium and water absorption in the colon, and affect the immune function of the gastrointestinal tract. SCFAs lower the gut pH, creating a more acidic environment that favors the growth of certain beneficial bacteria and inhibits the growth of gram-negative and other potential pathogens.[4, 20, 30] The SCFAs are also readily absorbed and metabolized within the liver and contribute calories to the host. Because of these effects, SCFAs have been the focus of much research in GI health.[31, 32, 35] Additional metabolic effects of the gut microbiota include stimulation of the absorption of calcium, magnesium, and iron, and metabolism of other dietary ingredients.[33]

Trophic effects

Intestinal microbiota increase the surface area of the intestinal lining by modulating crypt-villus structure, epithelial turnover, GI motility, and development of the GALT.[36] These trophic effects appear to be mediated by SCFAs, which are readily absorbed by intestinal mucosal cells and have been shown to increase epithelial proliferation and mucosal growth in the intestinal tract.[31, 41-43, 45] These and other changes in the colonic epithelial cells and in immune tissues and lymphocytes work synergistically to decrease the likelihood of microbial and antigen translocation across the gut epithelial lining. The SCFAs, particularly butyrate, stimulate the secretion of glucagon-like peptide-2 (GLP-2).[34, 38] GLP-2 is a pluripotent hormone, which may represent a common mediator of SCFA-associated intestinal proliferation, motility and nutrient absorption.[39]

The presence of a complex microbial community is integral to many functional processes in the human GI tract. For example, colonization of germ-free mice with *Bacteroides thetaiotaomicron*, which is a normal constituent of both human and mouse intestinal microbiota, significantly altered the expression of host genes related to nutrient absorption, mucosal barrier function, xenobiotic metabolism, angiogenesis and maturation.[40] Others have shown that components of the intestinal microbiota stimulate angiogenesis (vascularization),[46] provide protection against epithelial cell injury,[44] and affect host fat storage.[37] Because of ethical issues involved in human studies, much of our understanding about how colonizing microorganisms affect infant GI development and function has been derived from animal studies.

Protective effects

One of the key functions of the intestinal microbiota is to provide a natural barrier against colonization and proliferation of opportunistic pathogens, thereby decreasing the risk of intestinal infection and disease.[48] The ability of certain intestinal bacteria, such as bifidobacteria and lactobacilli, to interfere with colonization by exogenous and possibly disease-causing microorganisms is called "colonization resistance".[10] Selective stimulation of beneficial genera in the infant GI tract at the expense of harmful bacteria may also be referred to as "competitive exclusion." It is widely reported that consumption of breast milk by infants can promote the growth of beneficial bacteria such as *Bifidobacterium* spp[16] and/or reduce colonization by less beneficial or potentially harmful species, such as *Escherichia coli (E. coli)* and *Clostridium difficile (C. difficile)*.[49] This method of inhibiting colonization

by undesirable bacteria has been proposed as one protective mechanism that helps improve the resistance of breastfed infants to infection.

Indigenous intestinal microbiota can inhibit the growth of potentially pathogenic exogenous microorganisms in several ways, such as:

- Competing with pathogens for available nutrients;
- Producing organic acids, which lower intraluminal pH to a level that inhibits pathogen growth;
- Stimulating the production of substances (mucins and IgA) that inhibit pathogen adhesion to the gut wall or block their sites of colonization[30];
- Excreting antimicrobial factors such as peptides; and
- Compromising the virulence of potential pathogens by preventing attachment to epithelial cells, competing for nutrients and producing bacteriocins[4]

Sidebar: Methodological Issues with Evaluating GI Microbiota

Advances in molecular biology have made it possible to more fully understand the effects of diet on GI microbiota. Classical microbiology techniques attempt to recover specific bacterial species using partially selective artificial media containing complex blends of nutrients, growth factors, antibiotics, and other ingredients. Such methods have been reported to recover as few as 10% of total fecal bacteria,[50] partly due to the strictly anaerobic growth environment of nearly all target microorganisms.[11] However, these methods are now seen as flawed because of a lack of media selectivity, an inability to recover non-culturable diversity and the subjective nature of colony descriptions.

More recent non-culture-based methods that identify bacteria by specific ribosomal DNA (rDNA) sequences have proven essential in gaining a more accurate representation of the infant GI microbiota.[51] Four main approaches have been used:

- The first involves amplification of rDNA target sequences of all bacteria in a stool sample by polymerase chain reaction (PCR). Separation of the resulting bands by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) and sequence analysis make it possible to reveal the biodiversity of the intestinal microbiota.[11]
- The terminal restriction fragment length polymorphism (T-RFLP) approach involves amplifying and fluorescently labeling bacterial rDNA and then digesting amplicons with restriction enzymes to generate terminal-restriction fragments (T-RFs). The T-RFs are separated by gel electrophoresis and sequenced and the sequences aligned with known bacterial sequences.[52]
- In addition, 16S rRNA sequences, species- and group-specific primers can also be used to directly identify a microbe of interest by quantitative real time PCR (RT-PCR).[49]
- Lastly, bacteria in stool samples can be identified by fluorescence in situ hybridization (FISH) analysis using fluorescently-labeled, species-specific rDNA primers.[53]

Molecular methods have been particularly useful in evaluating beneficial microbiota such as bifidobacteria at the genus and species levels, where classical microbiology can provide little or no information.

Differences between microbiota of breastfed and formula-fed infants

Both conventional and molecular microbiology techniques have shown that infants fed human milk have a GI microbiota profile containing a predominance of bifidobacteria,[54] which are known to have health-promoting functions. Bifidobacteria are particularly predominant among infant GI microbiota, consisting primarily of *B. breve, B. longum,* and *B. infantis*, along with an emerging but significant contribution of other genera.[51] DNA-based techniques have shown that *Bifidobacterium* species appeared in the feces of newborns by 5 or 6 days postpartum in breastfed and formula-fed infants, respectively.[51] In general, results also suggest that the GI microbiota of breastfed infants contain reduced populations of potentially pathogenic species such as *C. difficile* and *E. coli*.[49]

In contrast, the gut microbiota of formula-fed infants are more diverse and similar to those observed in adults, with lower levels of bifidobacteria[55], but greater diversity and higher levels of other potentially pathogenic groups, including *Bacteroides, Clostridium,* and Enterobacteriaceae.[9,16,56-59] While not all studies are in agreement, many have confirmed these overall patterns, including more recent studies using reliable and accurate molecular-based methods.[50,60]

Sidebar: What Makes Bacteria Harmful in the Body?

It is normal for the human GI tract to contain both beneficial and potentially harmful types of microbes. Their effects on host health depend on the balance of these types of microbes, the health of the host and numerous factors within the environment of the GI tract. When beneficial bacteria are predominant, potentially pathogenic microbes are less likely to multiply to levels at which they could cause illness.

Major mechanisms by which pathogenic bacteria can become harmful include attachment to epithelial cells via specific receptors and/or secretion of toxins (e.g., Cholera toxin). Interaction of bacteria with host cells triggers an immunological response in the host, including the secretion of proinflammatory cytokines at the site of infection. When the intestinal barrier function is impaired, bacteria may translocate across the intestine and cause septicemia.

Results from several studies have been compared to determine whether patterns of infant fecal bacteria are reflective of feeding regimen.[22] Fecal samples from breast- and formula-fed infants were not different in overall levels of bifidobacteria or lactobacilli. However, higher levels of clostridia were observed in the feces of formula-fed infants than breastfed infants. Fecal levels of enterobacteria and streptococci also tended to be lower in breastfed infants. In a more recent study, RT-PCR techniques were used to monitor the GI microbiota of exclusively breastfed and formula-fed infants.[49] All of the infants were colonized by *Bifidobacterium* species, and mean values were comparable among the breastfed and formula-fed groups. In contrast, the prevalence and counts of both *C. difficile* and *E. coli* were significantly lower in breastfed infants.

This finding is of clinical relevance because pathogenic bacteria have been strongly associated with disease in infants. For example, *C. difficile* is responsible for 20-40% of cases of antibiotic-associated diarrhea[61] and has been implicated in necrotizing enterocolitis.[62] There are extensive data demonstrating the protective role of the intestinal microbiota against *C. difficile*.[48, 63-65] In fact, it is thought that the normal flora suppress the activity of *C. difficile* and that this suppression becomes compromised during antibiotic intake.

Differences in GI microbiota between breastfed and formula-fed infants also result in varying stool and laxation characteristics. Breastfed infants have larger, softer, and more frequent stools than formula-fed infants, which is attributed to differences in bacterial cell mass, fecal pH, and other factors.

Although the microbiota present in the formula-fed infant may not necessarily be harmful, it is highly desirable for infant formula to function more like breast milk, and investigators are currently seeking ways to create a similar intestinal environment in formula-fed infants. Efforts to improve the performance of infant formula include the use of novel ingredients that have the potential to shift the GI microbiota of formula-fed infants to more closely resemble that of breastfed infants.

Bioactive Components in Human Milk

Human milk is a complex physiological fluid; its components fulfill many nutritive, developmental, and immunoprotective functions in infant nutrition and within the neonatal GI tract. Various protein, lipid, and carbohydrate components of human milk are involved in stimulating growth and maturation of cells in the GI tract, aiding in the establishment of the gastrointestinal microbiota, enhancing mucosal barrier function, and stimulating development of the GALT, which functions as a crucial interface between intestinal contents and the infant's developing immune system.[66] Bioactive components of human milk are thought to provide protection while the infant develops greater immunological and developmental maturity.[67] This concept is supported by clinical studies demonstrating that the incidence and severity of infectious diseases are lower in breastfed infants compared to formula-fed counterparts.[55, 68-73]

Proteins, lipids, carbohydrates, and other bioactive components in human milk provide essential nutrients and energy; they also function to support growth and development in other ways. Categories of bioactive compounds in human milk include immune factors (such as secretory IgA, or sIgA), hormones, growth factors, proteins and peptides, lipids, carbohydrates, and other components.[55] It has been suggested that the bioactive substances in human milk represent an evolutionary strategy for protecting infant health and promoting gastrointestinal development during the vulnerable newborn period.[74] Beneficial functions associated with bioactive compounds in human milk include:

- Compensating for developmental immaturity;
- Protecting against infection by potential pathogens;
- Aiding intestinal adaptation to extrauterine life;
- Reducing inflammation of the GI tract;
- Protecting bioactive substances from digestion;
- Aiding the establishment of a beneficial microbial population.[74]

A review of the literature describing bioactive components typically identified in human milk offers a number of insights into how they might influence neonatal GI development. For example, the whey protein fraction of human milk contains high concentrations of hormones, growth factors and numerous antimicrobial proteins.[66] The nutritional, developmental and protective contributions of human milk whey proteins have been investigated for several years. Scientists have only recently begun evaluating the contributions of oligosaccharide components in human milk. The importance of HMOS is evidenced by their relative prominence; HMOS constitute the third-largest component of human milk, following lactose and lipid, and are thought to play significant developmental and protective roles.[75]

Human milk oligosaccharides

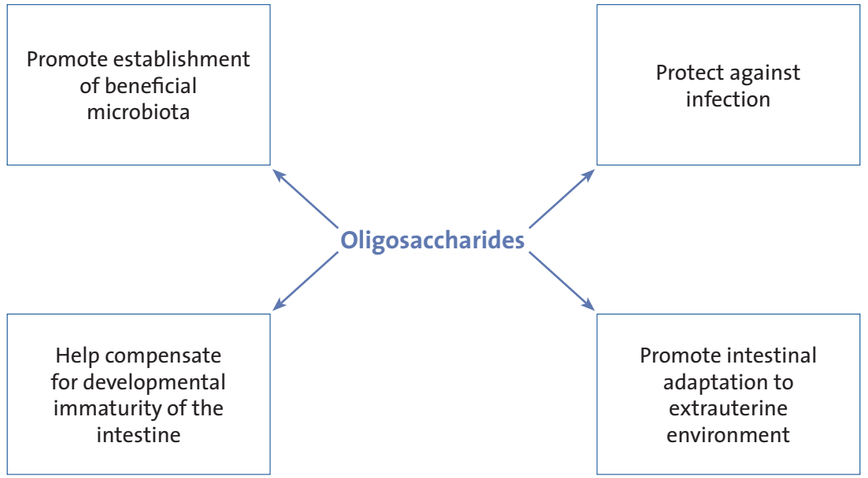
Human milk oligosaccharides are a structurally diverse and highly variable group of bioactive carbohydrates. HMOS are comprised of a mixture of oligosaccharides that differ in molecular weight and structure, constituent sugar or sugar derivatives, and charge.[76] Structurally, HMOS are composed of combinations of five monosaccharide building blocks: D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid. Some of these monosaccharides are also found in the milk of other mammalian species.[77] HMOS resist digestion by enzymes in the stomach and small intestine and reach the lower GI tract largely intact. They also can be absorbed into the circulatory system and transported to other sites, such as the urinary tract, where they are thought to function systemically by blocking pathogen adhesion[77] as many of the structures present in HMOS resemble typical pathogen binding sites (i.e., the HMOS thereby act in a 'decoy' manner).

Variability occurs in both the concentration and composition of HMOS during the normal course of lactation.[78] Unbound HMOS are found in mature milk at levels ranging from 5-10 g/L and are present at higher levels in colostrum.[77] They have been estimated to vary in size from 3 to 32 sugars.[75] Current analytical methods have detected approximately 200 distinct molecular species of HMOS,[79] though this may represent only a small fraction of their true structural diversity.[80] Further, HMOS concentrations vary considerably among women and during lactation. As a result of this complexity, it is not possible to replicate the HMOS content or composition of human milk in infant formula.

HMOS influence GI function and immune protection

A significant proportion of HMOS pass undigested into the lower gastrointestinal tract,[81] where they can be selectively metabolized by beneficial microorganisms such as the bifidobacteria.[82] The bifidogenic capacity of HMOS is frequently cited as an important reason why the gastrointestinal microbiota of breastfed infants contain proportionally more bifidobacteria than those of formula-fed infants.[54] In fact, the original "bifidus factor" is human breast milk. Human milk oligosaccharides have several key beneficial effects on the development of the neonatal intestine (Figure 2). [66]

Figure 2: Beneficial Effects of Human Milk Oligosaccharides on Neonatal Intestinal Development



Adapted from reference 66.

Oligosaccharides are constituents of the innate immune system in human milk that provides breastfed infants with protection from potential pathogens. The significant immunological protection afforded to infants by human milk has been attributed, in part, to the presence of α(1-2)-linked fucosylated oligosaccharides.[75] This is supported by observations that fucosylated oligosaccharides are capable of preventing diarrheal illness through diverse mechanisms, including: 1) inhibition of *E. coli* stable toxin activity by binding and blocking access to target receptors; 2) prevention of *Campylobacter* adhesion to intestinal cells; and 3) competitive inhibition of the binding of Norovirus to the intestinal epithelium.[83]

In contrast to human milk, cow's milk contains only trace amounts of oligosaccharides. Infant formulas made from cow's milk are essentially devoid of oligosaccharides, unless they are provided exogenously. Although it is not possible to add oligosaccharides that are structurally identical to HMOS to infant formula, researchers have evaluated the feasibility and desirability of adding food-grade prebiotic ingredients to partially mimic the functional properties of HMOS. The intended benefit of such prebiotic ingredients in infant nutrition is to bring the biological responses of infants to formulas into closer alignment with breast milk, particularly with regard to development of positive intestinal microbial populations and associated effects on immunity and stooling patterns.

Use of Prebiotic Carbohydrates in Infant Formulas

Given the many benefits purported to be associated with the intestinal microbiota present in breastfed infants, it is not surprising that researchers are interested in determining ways to promote similar microbiota in formula-fed infants. The addition of prebiotics to infant formula has the potential to mimic some of the beneficial effects of HMOS in formula-fed infants. One goal of using prebiotics in the diet is to modify the intestinal microbiota such that their beneficial activities are enhanced and detrimental activities suppressed. To be effective, prebiotics must be resistant to digestion until they are specifically fermented by the intestinal microbiota. Also, by definition, prebiotic substances must have health-promoting properties.[3] Postulated benefits of prebiotic ingredients for infants include increased production of SCFAs, stimulation of growth and/or activity of lactic acid bacteria, improved stool characteristics (laxation) and improved bioavailability of minerals.[84]

Infant formula manufacturers have evaluated numerous ingredients as part of their continuing effort to more closely approximate the nutritional and functional properties of human milk. Although it is impossible to completely match the diverse and dynamic nature of HMOS, a number of food-grade oligosaccharides have demonstrated capacity to increase levels of select beneficial bacteria (typically bifidobacteria) and improve stool characteristics in formula-fed infants.[19]

Prebiotics evaluated in infant feeding

Several food-grade oligosaccharides have been evaluated for use as prebiotics in infant formula, including galactooligosaccharides (GOS), polydextrose (PDX), lactulose (LOS), inulin, and fructooligosaccharides (FOS). Combinations of these products have also been evaluated. Oligosaccharide ingredients may be commercially produced by bacterial synthesis, enzymatic synthesis or extraction from natural sources.[85] Dietary components most commonly investigated as potential prebiotics are nondigestible carbohydrates, primarily oligosaccharides, consisting of 2 to 20 saccharide units.[86] Dietary oligosaccharides are present in fruits such as bananas and in vegetables such as asparagus, leeks, and artichokes. Many naturally-occurring oligosaccharides are relatively resistant to digestion by intestinal enzymes and subsequently fermented by bacteria in the colon. There has been considerable interest in supplementing foods with prebiotic ingredients because the levels of naturally-occurring oligosaccharides in diets are considered inadequate to produce substantial effects on health.[87]

Selected characteristics of these prebiotics and human milk oligosaccharides are shown in Table 1.

"The addition of certain mixtures of oligosaccharides in infant formula bring infant formula, the second choice infant feeding, one step closer to the gold standard, breast-feeding."[19]

Table 1: Select Characteristics of Potential Prebiotic Carbohydrates and Human Milk Oligosaccharides

Prebiotic Carbohydrate	Abbreviation	Chemical Structure*	Degree of Polymerization†
Galactooligosaccharides	GOS	α-D-Glu-(1→4)-[β-D-Gal-(1→6)]$_n$	2-8 2-4 Average
Polydextrose	PDX	Random, highly branched glucose polymer + sorbitol	3-30 12 Average
Lactulose	LOS	4-O-β-D-Gal-D-Fru	2 (Disaccharide)
Inulin	IN	Glu-[β-Fru-(2→1)]$_n$	3-60 10-12 Average
Fructooligosaccharides	FOS	Glu-[β-Fru-(2→1)]$_n$ Fru-[β-Fru-(2→1)]$_n$	2-8 4 Average
Human Milk Oligosaccharides	HMOS	Complex and highly variable	3-32 (Estimated)

* Abbreviations: Glu=glucose; Gal=galactose; Fru=fructose; O=oxygen.
† Degree of polymerization=number of repeating carbohydrate units (represented by n)

Given that human milk contains a wide variety of oligosaccharides, it has been postulated that the benefits of HMOS on infant health are more likely associated with a mixture of oligosaccharide structures rather than a single oligosaccharide.[88] A combination of GOS and PDX was shown to result in a range of oligosaccharide chain lengths within the range reported for HMOS.[75] Blends of GOS and FOS have been widely studied and found to be effective in stimulating the growth of bifidobacteria and promoting the establishment of intestinal microbiota similar to that of breastfed infants.[89]

In vitro studies measuring carbohydrate utilization patterns and the production of short-chain fatty acids and gas by infant fecal bacteria have shown that larger, more complex carbohydrates, such as PDX and inulin, are fermented more slowly and less completely than short-chain materials such as LOS, FOS, and GOS.[90] Thus, a combination of specific long-chain and short-chain carbohydrates may allow for slower fermentation by fecal bacteria, a process that may translate into a more sustained effect during gastrointestinal transit. This is a desirable trait as the distal (left) side of the large intestine has low saccharolytic activity compared to more proximal areas and is also more frequently affected by intestinal disorders. Further, *in vitro* data suggest that blends of prebiotic carbohydrates would be more likely to stimulate fermentation by a broader array of gastrointestinal bacteria, resulting in greater SCFA production and reduced pH—both conditions that are considered unfavorable for pathogens.

Prebiotic use in commercial products

There appears to be broad support for the use of prebiotics in infant formulas in many countries, judging by the commercial availability of prebiotic-supplemented formulas. Prebiotic carbohydrates have been added to infant formulas in Japan for over 20 years, and 90% of infant formulas in Japan are purported to contain prebiotics.[88] Within the past decade, prebiotics have been introduced into formulas in Europe as well.[88] Prebiotic-supplemented infant formula is a relatively new concept in the United States. In recent years, however, there has been an increase in the number of foods available for children and adults containing prebiotics, including yogurt and cereals.

Evidence Supporting Use of Prebiotics in Infant Feeding

More than 30 research articles have described results of infant clinical trials with prebiotics. A key finding of a majority of trials is that infants consuming prebiotic-supplemented formulas have significantly increased levels of bifidobacteria compared to those consuming control formulas. The results have been equivocal, however, as a bifidogenic response has not always been demonstrated, even in studies using the most reliable (molecular-based) methodologies.[91] Studies that evaluated growth have shown that formulas containing prebiotics support growth comparable to that observed in infants fed control formulas. Another consistent finding with prebiotic supplementation is beneficial changes in stooling patterns (i.e., increased frequency and softness).

A study in term infants (n=226) found that infants fed formula supplemented with a mixture of prebiotics had similar growth up to 120 days of age as those fed unsupplemented (control) formula.[92] Infants in one study group received formula supplemented with PDX and GOS (50:50 ratio) at a level of 4 g/L; infants in a second study group received formula with 8 g/L of PDX, GOS, and LOS (50:33:17 ratio). There were no significant differences in weight or length growth rate between the supplemented and control formula groups from 14 to 30, 60, 90 or 120 days. Studies with other prebiotics and prebiotic blends have likewise noted no differences in growth between infants fed supplemented or unsupplemented formulas.[93-95]

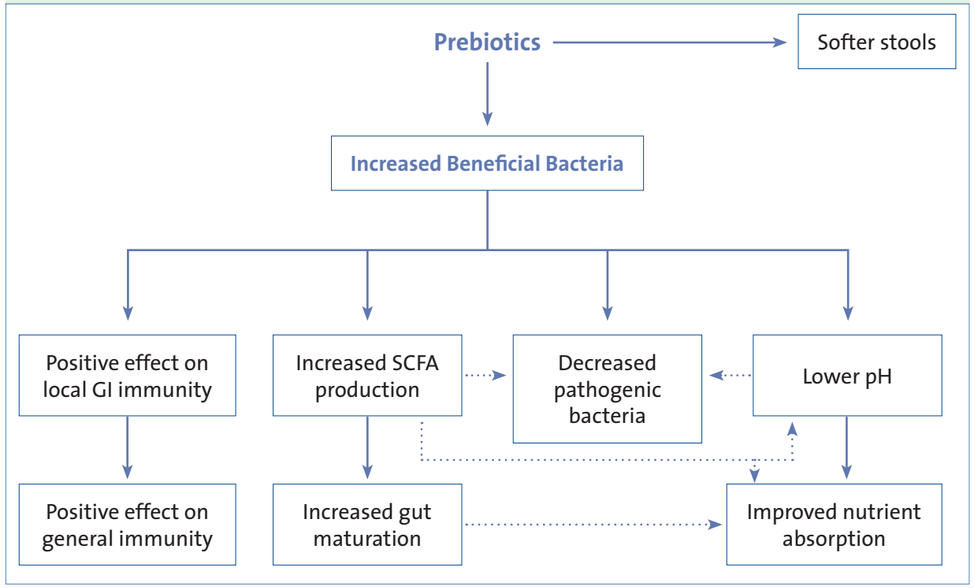
Effects of prebiotics on GI microbiota

Clinical studies in infants suggest that prebiotics have positive effects on measures of gastrointestinal health (Figure 3). Individual prebiotic ingredients and blends of prebiotic ingredients have been evaluated. More studies in infants are needed to establish a relationship between alterations in GI microbiota and improved measures of GI health.

Composition

A few studies have evaluated the effectiveness of individual prebiotics on gut microbial composition. In one study with 271 term infants, subjects who consumed formula with GOS added (2.4 g/L) had significantly increased fecal levels of bifidobacteria and lactobacilli after 3 months and 6 months of feeding.[96] Importantly, there were no significant differences in levels of bifidobacteria or lactobacilli between GOS-supplemented infants and those in a breastfed control group. Another study in preterm infants evaluated the effect of inulin on microbiota.[97] In this study, 56 preterm infants received formula with or without inulin (4 g/L) for 7 days. Fecal bacterial counts showed that the microbiota of supplemented infants were more heavily colonized with bifidobacteria and had fewer potentially pathogenic bacteria (*E. coli* and *Enterococcus*) compared to infants fed unsupplemented control formula.

Figure 3: Potential Beneficial Effects of Prebiotics on Infant Health



A larger number of studies in infants has evaluated the effects of blends of 90% (w/v) GOS and 10% (w/v) FOS or inulin.[98] In many of the studies, FOS and inulin are used interchangeably, as inulin is long-chain FOS. The majority of these studies showed a positive correlation between prebiotic supplementation and enhanced populations of bifidobacteria[18, 94, 99-104] and lactobacilli.[100, 103, 105] Many of the studies also demonstrated decreased levels of potentially pathogenic bacteria with prebiotic supplementation. In a few studies, the investigators used techniques that allowed analysis of the microbial populations at a species-specific level, which demonstrated that infants fed the GOS- and FOS-supplemented formulas had increased total numbers of beneficial bacteria (both *Lactobacillus* and *Bifidobacterium*) as well as a species-specific profile that more closely resembled that of breastfed infants.[101, 103, 105] These clinical trials suggest that combinations of prebiotics may be helpful in altering the composition of gut microbiota to be more like that of breastfed infants.

A clinical trial of 90 infants examined the effects of a blend of 90% (w/v) GOS and 10% (w/v) FOS on bacterial growth.[106, 107] Infants were randomized to receive control formula or formula supplemented with either 4 g/L or 8 g/L of the GOS:FOS blend for 4 weeks. Bifidobacteria counts were significantly increased in both supplemented groups compared to the control group at the end of the treatment period; this effect was dose-dependent and significantly different between the supplemented groups ($P<0.01$). Lactobacilli were also significantly increased in the supplemented infants compared to the control group, but there was no statistically significant difference between the two supplemented groups.

Not all clinical trials have demonstrated a positive effect of prebiotics on gut levels of beneficial bacteria, however. In one study of 72 infants supplemented with FOS at 1.5 g/L or 3.0 g/L for 7 days, only mild prebiotic effects were reported.[108] In another study with 76 infants, supplementation with FOS at 2 g/L for 13 weeks did not result in significant increases in fecal levels of bifidobacteria and lactobacilli compared to control formula.[91]

Sidebar: Understanding Differences in Study Outcomes

Infant feeding studies with prebiotic ingredients are not always consistent in their results. Differences in study design can have important effects on the outcomes observed, and such differences should be considered when comparing study results. Key elements of study design that can impact outcomes include:

- Length of gestation and postnatal age of infants being evaluated
- Mode of infants' delivery
- Duration of supplementation
- Type and level of prebiotic ingredients used
- Methods for measuring study outcomes, especially gut microflora changes
- Use of medications or supplemental feeds

Differences in one or more of these study parameters can lead to outcomes that appear to be contradictory. In fact, gastrointestinal microbiota exist in a complex environment that is affected by many different variables. Also, it is important to keep in mind that genetic differences among infants are likely to also influence initial colonization and response to prebiotics.

GI health

In addition to evaluating composition of the GI microbiota, some studies have assessed the pH and SCFA levels of infant stools as a determinant of GI tract health. Prebiotic supplementation resulted in lower stool pH[18, 53, 100] and increased levels of fecal SCFAs.[18, 53] In one of these studies,[18] 53 infants were randomly assigned to receive either control formula or formula supplemented with GOS and FOS (9:1 ratio, 8 g/L) for 6 weeks. Stool pH of the supplemented group was significantly lower than that of the control group (pH of 5.7 vs. 6.3; $P<0.001$) and did not differ from that of a breastfed reference group. Further, the SCFA profiles of the supplemented and breastfed infants were similar, but the SCFA profiles of supplemented and control infants differed.

Effects of prebiotics on measures of barrier function in the GI tract are difficult to evaluate in infant studies. However, prebiotic oligosaccharides have been shown to block pathogen adhesion *in vitro*. Shoaf and coworkers evaluated the capacity of a range of commercially available prebiotic oligosaccharides to block the attachment of *E. coli* to certain human-derived cell lines. Purified GOS exhibited the greatest activity of the oligosaccharides evaluated, inhibiting *E. coli* adherence to intestinal epithelial cells by up to 70%.[109]

Effects of prebiotics on the digestive system

Studies in animals and humans have demonstrated that supplementation with prebiotics has positive effects on the digestive system, including improved laxation and increased mineral absorption.[33]

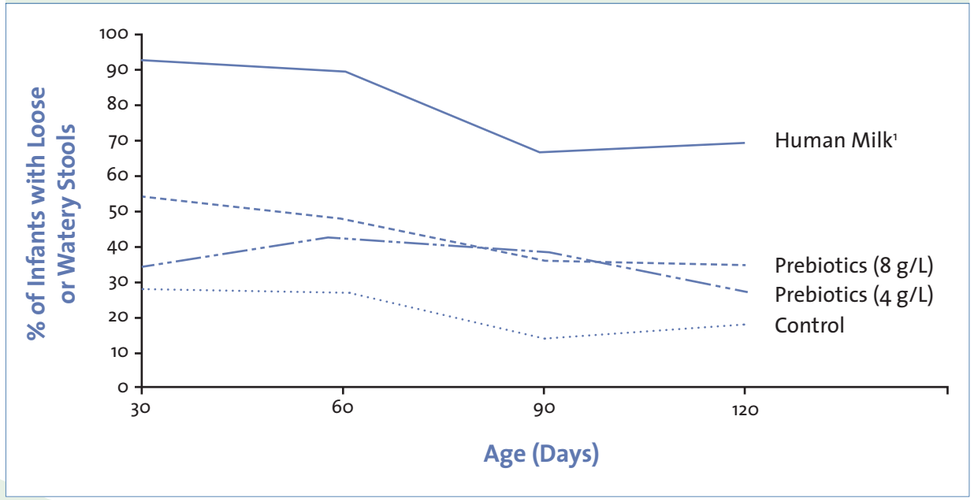
Laxation

An overall goal of prebiotic supplementation is to produce stool characteristics in formula-fed infants similar to those observed in breastfed infants. A number of studies in infants have shown beneficial effects of prebiotic ingredients on stool characteristics. Positive results have been observed with GOS (softer stool consistency)[96] and FOS (more frequent stools and softer stools).[110]

Prebiotic blends have also demonstrated positive results. In a study of more than 200 infants, formulas were supplemented with either 4 g/L of PDX and GOS (50:50 ratio) or 8 g/L of PDX, GOS and LOS (50:33:17 ratio) and fed through 120 days of age.[92] Prebiotic supplementation with either amount resulted in a stool consistency that was intermediate between that of infants fed unsupplemented formula[92] and that of infants in another study who were fed breast milk (Data on file, 2005) (Figure 4).

Positive effects on infant stool consistency and frequency have also been observed with prebiotic blends composed of 90% (w/v) GOS and 10% (w/v) FOS. These effects include fewer symptoms of constipation[111]; increased stool frequency[106, 112, 113] and softer stool consistency.[106, 112, 114] Several studies showed that infants who received the prebiotic blend had stool consistency similar to that of breastfed infants.[106, 112, 114]

Figure 4: Stool Consistency of Infants Fed Prebiotic-supplemented Formula Compared to Infants Fed Human Milk or Control Formula



¹Formula data are from Zeigler et al. (92); Human milk data are from Mead Johnson (Data on file, 2005).

Mineral absorption

Researchers are interested in the potential for prebiotic ingredients to increase absorption of minerals. Even though most minerals are absorbed primarily in the small intestine, it is thought that a lower pH in the colon, resulting from by-products of bacterial fermentation (including SCFA), increases the solubility of some minerals thereby potentially increasing their absorption from the colon.[33, 115]

Studies evaluating the effect of prebiotics on mineral absorption primarily have been conducted in animals. Most of the evidence for the beneficial effects of GOS, PDX, LOS, or FOS on mineral absorption is based on animal studies; these have shown that the absorption of calcium, magnesium, potassium and phosphorus was increased after prebiotic diet supplementation.[116-124] Animal studies also suggest that supplementation with GOS and LOS may increase intestinal absorption of calcium and magnesium; results of a study conducted with PDX showed an increase in calcium absorption and bone mineralization.[125] The applicability of these studies to infants and children has not yet been determined.

Although studies of mineral absorption in humans have shown positive results with prebiotic supplementation, they have been conducted primarily in adolescents and postmenopausal women. One study with children in India suggested a positive effect of GOS on iron absorption, but confounding study variables make it difficult to conclude that the effect was due to the supplement.[126] In a clinical trial with 30 preterm infants, urine calcium concentrations tended to be higher in infants fed a GOS- and FOS-supplemented formula compared to a placebo-supplemented formula, despite equal dietary calcium intake.[127]

In summary, few clinical studies have evaluated the effect of prebiotics on mineral absorption in infants and young children, and it is difficult to extrapolate results from studies in adolescents and adults to infants due to differences in their GI systems. Further, because prebiotics may also decrease GI transit time, there is a possibility that nutrient absorption may be diminished if the prebiotic level is too high. In order to define the role of prebiotics in mineral absorption, additional studies are needed to address issues such as optimal intake, duration of supplementation and the impact of dietary calcium intake.

Effects of prebiotics on immunity

The gut microbiota of breastfed infants are dominated by bifidobacteria, and it has long been suggested that they play a role in enhancing infant resistance to infection.[106] Analyses of fecal bacteria using species-specific probes have demonstrated that infants fed prebiotics develop microbiota more similar to those of breastfed infants.[101, 105] Because prebiotics influence the composition and activity of the gastrointestinal microbiota, and because the microbiota are known to have a major effect on the immune system, it is reasonable to expect that prebiotics can be used indirectly to modulate the immune system. Data from animal studies suggest that dietary supplementation with prebiotics can influence certain markers of immunity and affect the incidence of allergy and disease. However, the critical association between prebiotic supplementation and improved immunologic function in humans remains to be defined.

The effects on immunity of ingesting GOS, LOS, PDX, or blends thereof have not hitherto been examined in infants or children. There is a need for further studies in humans to evaluate the effect of prebiotic ingredients on the gut immune response and subsequent effects for overall health.

Biomarkers

Most of the evidence for the beneficial effects of prebiotics on immunity is based on animal studies. These studies have evaluated the effectiveness of prebiotic ingredients in reducing intestinal infection by decreasing the translocation of bacteria (particularly pathogens) from the intestine to the liver and mesenteric lymph nodes. Various markers of immune function have been used to measure this effect, including concentrations of endotoxin, TNF-α, interleukin-1, interleukin-10, and IgA; levels of pathogenic bacteria; inflammation; and incidence of intestinal infections. *In vitro* and animal studies have suggested that the addition of complex fermentable carbohydrates to the diet can modulate the type and function of cells from different regions of the gut-associated lymphoid tissue,[128-130] increase immunoglobulin production in the small intestine and cecal mucosa[131], and alter the profile of inflammatory cytokines in plasma and intestinal cells.[132-134]

Few studies in infants have specifically evaluated the effect of prebiotic supplements on markers of immune function. One study with 57 infants evaluated the effect of formula supplemented with FOS and GOS (6 g/L for 32 weeks) on fecal levels of IgA.[135] Supplemented infants displayed a trend toward higher fecal sIgA compared to unsupplemented infants, but this difference reached statistical significance only at week 16.

Incidence of allergy

Exclusive breastfeeding is strongly recommended for newborn infants with a family history of allergy, as breastfeeding reduces the likelihood that the infant will develop atopic disease. One study in infants directly evaluated whether infant formula supplementation with prebiotics could replicate the protective effect of breastfeeding. In this study, 259 infants at risk for atopic disease were randomized to receive either control formula or formula supplemented with a blend of GOS and FOS (9:1 ratio; 8 g/L). [136] Fecal microbiota were analyzed in a subgroup of 98 infants. Levels of bifidobacteria were significantly higher among infants who received the prebiotics compared to control infants; levels of lactobacilli did not differ between the groups. Over the 6-month course of the study, fewer infants in the supplemented group developed atopic dermatitis compared to the control group, suggesting that prebiotics may modulate postnatal immune development in part by altering the GI microbiota; there may be other mechanisms as well. This study supports a potentially positive role for prebiotics in managing symptoms of allergy during infancy, but additional studies are still needed.

Incidence of disease

Breastfed infants are often reported to experience a lower incidence of disease than formula-fed infants.[137] This effect is attributed to multiple factors associated with breastfeeding, including the presence of maternal antibodies[138] and oligosaccharides in human milk.[75] Although scientists are hopeful that the use of prebiotics and other ingredients can affect markers of disease in infants, few studies have directly evaluated the relationship between prebiotic supplementation and incidence of disease or reduction of risk. One clinical study in children suggested that supplementation with FOS (2 g/d) for 21 days could improve their immunological status, based on a lower incidence of fever, vomiting, and diarrhea in those taking the supplement.[139] Another study, conducted among breastfed infants living in a community near Lima, Peru with a high prevalence of GI and other infections, found that feeding infant cereal supplemented with oligofructose at 0.55 g/15 g cereal for 6 months was not associated with change in incidence of diarrhea, use of health care resources, or response to a flu vaccination.[140] A high prevalence of breastfeeding in the study population was thought to contribute to the lack of effect with prebiotics observed. Further studies in infants and children are needed to clarify these findings.

Conclusion

Oligosaccharides in human milk are constituents of an innate immune system that provides breastfed infants with protection from potential pathogens. Oligosaccharides are prominent in human milk, as the third largest component, and are thought to play significant developmental and protective roles.[75] Human milk oligosaccharides are active in the development of intestinal microbiota in breastfed infants, leading to increased gut levels of beneficial bacteria, particularly bifidobacteria, and reduced levels of potentially pathogenic bacteria. The bifidogenic potential of HMOS is thought to be one important reason why the gastrointestinal tract of breastfed infants contains proportionally more bifidobacteria than that of formula-fed infants.[54]

Because of their complexity and variability, it is not possible to duplicate HMOS for use in infant formula. As an alternative, the safety and efficacy of naturally occurring and manufactured oligosaccharides in infant feeding have been evaluated. Findings from animal and human studies suggest that prebiotic ingredients can have some effects similar to those of HMOS in the GI tract. Postulated benefits in infants include increased production of SCFAs, inhibition of growth and/or activity of pathogenic bacteria, stimulation of growth and/or activity of beneficial bacteria, improved stool characteristics, and improved bioavailability of minerals.[84] Studies have demonstrated that infants fed prebiotics have stool characteristics more similar to those of breastfed infants, suggesting a positive effect of prebiotics on the digestive system. Preliminary evidence also suggests that prebiotics may positively affect mineral absorption and the infant immune system.

The neonatal period is a critical period of development, when potentially long-term effects of manipulation of the intestinal microbiota could result.[141] While the clinical data are promising, more studies are needed to understand the conditions under which prebiotic formula ingredients can positively influence infant growth and development. Mead Johnson continues to evaluate the science supporting the use of prebiotic ingredients in infant formula with the goal of providing state-of-the-science nutrition to formula-fed infants.

References

1. Li R, Darling N, Maurice E, Barker L, Grummer-Strawn LM. Breastfeeding rates in the United States by characteristics of the child, mother, or family: the 2002 National Immunization Survey. *Pediatrics.* 2005;115:e31-e37.
2. American Academy of Pediatrics. Iron fortification of infant formulas. American Academy of Pediatrics. Committee on Nutrition. *Pediatrics.* 1999;104:119-123.
3. Roberfroid M. Prebiotics: the concept revisited. *J Nutr.* 2007;137:830S-837S.
4. Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet.* 2003;361:512-519.
5. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. *Science.* 2005;307:1915-1920.
6. Penders J, Thijs C, Vink C, et al. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics.* 2006;118:511-521.
7. Cooperstock MS, Zedd AJ. Intestinal flora of infants. In: Hentges DJ, ed. *Human Intestinal Microflora in Health and Disease.* New York, NY: Academic Press; 1983:79-99.
8. Conway PL. Development of the intestinal microbiota. In: Mackie R, White B, Isaacson R, eds. *Gastrointestinal Microbiology Vol II: Gastrointestinal Microbes and Host Interactions.* New York, NY: Chapman & Hall; 1997:3-38.
9. Cooperstock MS. Indigenous flora in pathogenesis. In: Feigin RD, Cherry JD, eds. *Pediatric Infectious Diseases.* Philadelphia, PA: W. B. Saunders; 1987:106-133.
10. Dai D, Walker WA. Protective nutrients and bacterial colonization in the immature human gut. *Adv Pediatr.* 1999;46:353-382.
11. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr.* 1999;69:1035S-1045S.
12. Gronlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr.* 1999;28:19-25.
13. Fryklund B, Tullus K, Berglund B, Burman LG. Importance of the environment and the faecal flora of infants, nursing staff and parents as sources of gram-negative bacteria colonizing newborns in three neonatal wards. *Infection.* 1992;20:253-257.
14. Sakata H, Yoshioka H, Fujita K. Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. *Eur J Pediatr.* 1985;144:186-190.
15. Blakey JL, Lubitz L, Barnes GL, Bishop RF, Campbell NT, Gillam GL. Development of gut colonisation in pre-term neonates. *J Med Microbiol.* 1982;15:519-529.
16. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. *Acta Paediatr Suppl.* 2003;91:48-55.
17. Butel MJ, Suau A, Campeotto F, et al. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J Pediatr Gastroenterol Nutr.* 2007;44:577-582.
18. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. *J Pediatr Gastroenterol Nutr.* 2005;40:36-42.
19. Vandenplas Y. Oligosaccharides in infant formula. *Br J Nutr.* 2002;87(suppl 2):S293-S296.
20. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.* 1995;125:1401-1412.
21. Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol.* 1998;64:3854-3859.
22. Tannock GW. The acquisition of the normal microflora of the gastrointestinal tract. In: Gibson SA, ed. *Human Health. The Contribution of Microorganisms.* London: Springer-Verlag; 1994:1-16.
23. Zoetendal EG, Vaughan EE, de Vos WM. A microbial world within us. *Mol Microbiol.* 2006;59:1639-1650.
24. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. *Science.* 2006;312:1355-1359.
25. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep.* 2006;7:688-693.
26. Corthésy B, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. *J Nutr.* 2007;137(3 suppl 2):781S-790S.
27. Iliev ID, Matteoli G, Rescigno M. The yin and yang of intestinal epithelial cells in controlling dendritic cell function. *J Exp Med.* 2007;204:2253-2257.
28. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol.* 2004;4:478-485.
29. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol.* 2003;3:331-341.
30. Agostoni C, Axelsson I, Goulet O, et al. Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr.* 2004;39:465-473.
31. Frankel WL, Zhang W, Singh A, et al. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. *Gastroenterology.* 1994;106:375-380.
32. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr.* 2002;87 (suppl 2):S221-S230.
33. Scholz-Ahrens KE, Schrezenmeir J. Inulin and oligofructose and mineral metabolism: the evidence from animal trials. *J Nutr.* 2007;137(suppl 11):2513S-2523S.

34. Tappenden KA, Albin DM, Bartholome AL, Mangian HF. Glucagon-like peptide-2 and short-chain fatty acids: a new twist to an old story. *J Nutr.* 2003;133:3717-3720.

35. Tappenden KA, Thomson AB, Wild GE, McBurney MI. Short-chain fatty acids increase proglucagon and ornithine decarboxylase messenger RNAs after intestinal resection in rats. *JPEN J Parenter Enteral Nutr.* 1996;20:357-362.

36. Abrams GD. Impact of the intestinal microflora on intestinal structure and function. In: Hentges DJ, ed. *Human Intestinal Microflora in Health and Disease.* New York, NY: Academic Press; 1983:291-310.

37. Bäckhed F, Ding H, Wang T, et al. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA.* 2004;101:15718-15723.

38. Bartholome AL, Albin DM, Baker DH, Holst JJ, Tappenden KA. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. *JPEN J Parenter Enteral Nutr.* 2004;28:210-222; discussion 222-223.

39. Dubé PE, Brubaker PL. Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators. *Am J Physiol Endocrinol Metab.* 2007;293:E460-E465.

40. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science.* 2001;291:881-884.

41. Kripke SA, De Paula JA, Berman JM, Fox AD, Rombeau JL, Settle RG. Experimental short-bowel syndrome: effect of an elemental diet supplemented with short-chain triglycerides. *Am J Clin Nutr.* 1991;53:954-962.

42. Kripke SA, Fox AD, Berman JM, et al. Inhibition of TPN-associated intestinal mucosal atrophy with monoacetoacetin. *J Surg Res.* 1988;44:436-444.

43. Kripke SA, Fox AD, Berman JM, Settle RG, Rombeau JL. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. *JPEN J Parenter Enteral Nutr.* 1989;13:109-116.

44. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell.* 2004;118:229-241.

45. Sakata S, Tonooka T, Ishizeki S, et al. Culture-independent analysis of fecal microbiota in infants, with special reference to Bifidobacterium species. *FEMS Microbiol Lett.* 2005;243:417-423.

46. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci USA.* 2002;99:15451-15455.

47. Salminen S, Isolauri E. Intestinal colonization, microbiota, and probiotics. *J Pediatr.* 2006;149:S115-120.

48. Rolfe R. Colonization resistance. In: Mackie RW, White B, Isaacson RE, eds. *Gastrointestinal Microbiology Vol II: Gastrointestinal Microbes and Host Interactions.* New York, NY: Chapman Hall; 1997:501-536.

49. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett.* 2005;243:141-147.

50. Harmsen HJ, Gibson GR, Elfferich P, et al. Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. *FEMS Microbiol Lett.* 2000;183:125-129.

51. Favier CF, de Vos WM, Akkermans AD. Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe.* 2003;9:219-229.

52. Collado MC, Calabuig M, Sanz Y. Differences between the fecal microbiota of coeliac infants and healthy controls. *Curr Issues Intest Microbiol.* 2007;8:9-14.

53. Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, Bindels JG. Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the intestinal microflora during the first 4 months of life. *Br J Nutr.* 2005;94:783-790.

54. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr.* 2000;30:61-67.

55. Mountzouris KC, McCartney AL, Gibson GR. Intestinal microflora of human infants and current trends for its nutritional modulation. *Br J Nutr.* 2002;87:405-420.

56. Edwards CA, Parrett AM. Intestinal flora during the first months of life: new perspectives. *Br J Nutr.* 2002;88 (suppl 1):S11-S18.

57. Rastall RA, Maitin V. Prebiotics and synbiotics: towards the next generation. *Curr Opin Biotechnol.* 2002;13:490-496.

58. Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *J Med Microbiol.* 1982;15:189-203.

59. Tuohy KM, Probert HM, Smejkal CW, Gibson GR. Using probiotics and prebiotics to improve gut health. *Drug Discov Today.* 2003;8:692-700.

60. Martin PJ, Savage AH, et al. Investigation of bacterial colonization of the colon in breast-fed infants using novel techniques. *Proc Nutr Soc.* 2000;59:64A.

61. Fekety R, Shah AB. Diagnosis and treatment of *Clostridium difficile* colitis. *JAMA.* 1993;269:71-75.

62. Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL. Enteric colonization in sporadic neonatal necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr.* 1985;4:591-595.

63. Borriello SP, Barclay FE. Evaluation of the predictive capability of an in-vitro model of colonization resistance to *Clostridium difficile* infection. *Microb Ecol Health Dis*. 1988;1:61-64.

64. Gorbach SL, Barza M, Giuliano M, Jacobus NV. Colonization resistance of the human intestinal microflora: testing the hypothesis in normal volunteers. *Eur J Clin Microbiol Infect Dis*. 1988;7:98-102.

65. Rolfe R. Asymptomatic colonization by *Clostridium difficile*. In: Rolfe RF, ed. Clostridium difficile: *Its Role in Intestinal Disease*. New York, NY: Academic Press; 1988:201-225.

66. Donovan SM. Role of human milk components in gastrointestinal development: current knowledge and future needs. *J Pediatr.* 2006;149(suppl 3):S49-S61.

67. Newburg DS, Walker WA. Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatr Res.* 2007;61:2-8.

68. López-Alarcón M, Villalpando S, Fajardo A. Breast-feeding lowers the frequency and duration of acute respiratory infection and diarrhea in infants under six months of age. *J Nutr.* 1997;127:436-443.

69. Newburg DS. Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J Nutr.* 1997;127(suppl 5):980S-984S.

70. Levy J. Immunonutrition: the pediatric experience. *Nutrition.* 1998;14:641-647.

71. Popkin BM, Adair L, Akin JS, Black R, Briscoe J, Flieger W. Breast-feeding and diarrheal morbidity. *Pediatrics.* 1990;86:874-882.

72. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. *Pediatrics.* 1993;91:867-872.

73. Istre GR, Conner JS, Broome CV, Hightower A, Hopkins RS. Risk factors for primary invasive Haemophilus influenzae disease: increased risk from day care attendance and school-aged household members. *J Pediatr.* 1985;106:190-195.

74. Goldman AS. Modulation of the gastrointestinal tract of infants by human milk. Interfaces and interactions. An evolutionary perspective. *J Nutr.* 2000;130(suppl 2S):256S-431S.

75. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr.* 2005;135:1304-1307.

76. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr.* 2000;20:699-722.

77. Bode L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. *J Nutr.* 2006;136:2127-2130.

78. Chaturvedi P, Warren CD, Altaye M, et al. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology.* 2001;11:365-372.

79. Ninonuevo MR, Park Y, Yin H, et al. A strategy for annotating the human milk glycome. *J Agric Food Chem.* 2006;54:7471-7480.

80. Stahl B, Thurl S, Zeng J, et al. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem.* 1994;223:218-226.

81. Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS. Survival of human milk oligosaccharides in the intestine of infants. *Adv Exp Med Biol.* 2001;501:315-323.

82. Ward RE, Ninonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*. *Appl Environ Microbiol*. 2006;72:4497-4499.

83. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr.* 2005;25:37-58.

84. Simmering R, Blaut M. Pro- and prebiotics--the tasty guardian angels? *Appl Microbiol Biotechnol*. 2001;55:19-28.

85. Van Loo J, Cummings J, Delzenne N, et al. Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). *Br J Nutr.* 1999;81:121-132.

86. Priebe MG, Vonk RJ, Sun X, He T, Harmsen HJ, Welling GW. The physiology of colonic metabolism. Possibilities for interventions with pre- and probiotics. *Eur J Nutr.* 2002;41(suppl 1):I2-I10.

87. Manning TS, Gibson GR. Microbial-gut interactions in health and disease. Prebiotics. *Best Pract Res Clin Gastroenterol.* 2004;18:287-298.

88. Ghisolfi J. Dietary fibre and prebiotics in infant formulas. *Proc Nutr Soc.* 2003;62:183-185.

89. Boehm G, Jelinek J, Stahl B, et al. Prebiotics in infant formulas. *J Clin Gastroenterol.* 2004;38:S76-S79.

90. Mead Johnson. Data on file.

91. Brunser O, Figueroa G, Gotteland M, et al. Effects of probiotic or prebiotic supplemented milk formulas on fecal microbiota composition of infants. *Asia Pac J Clin Nutr.* 2006;15:368-376.

92. Ziegler E, Vanderhoof JA, Petschow B, et al. Term infants fed formula supplemented with selected blends of prebiotics grow normally and have soft stools similar to those reported for breast-fed infants. *J Pediatr Gastroenterol Nutr.* 2007;44:359-364.

93. Bettler J, Euler AR. An evaluation of the growth of term infants fed formula supplemented with fructo-oligosaccharide. *Int J Probiotics Prebiotics.* 2006;1:19-26.

94. Decsi T, Arató A, Balogh M, et al. [Randomised placebo controlled double blind study on the effect of prebiotic oligosaccharides on intestinal flora in healthy infants]. *Orv Hetil.* 2005;146:2445-2450.

95. Puccio G, Cajozzo C, Meli F, Rochat F, Grathwohl D, Steenhout P. Clinical evaluation of a new starter formula for infants containing live *Bifidobacterium longum* BL999 and prebiotics. *Nutrition.* 2007;23:1-8.

96. Ben X-M, Zhou X-Y, Zhao W-H, et al. Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. *Chin Med J (Engl).* 2004;117:927-931.

97. Kapiki A, Costalos C, Oikonomidou C, Triantafyllidou A, Loukatou E, Pertrohilou V. The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. *Early Hum Dev.* 2007;83:335-339.

98. Veereman G. Pediatric applications of inulin and oligofructose. *J Nutr.* 2007;137(suppl 11): 2585S-2589S.

99. Boehm G, Fanaro S, Jelinek J, Stahl B, Marini A. Prebiotic concept for infant nutrition. *Acta Paediatr Suppl.* 2003;91:64-67.

100. Fanaro S, Jelinek J, Stahl B, Boehm G, Kock R, Vigi V. Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: effect on intestinal flora, stool characteristics, and pH. *J Pediatr Gastroenterol Nutr.* 2005;41:186-190.

101. Haarman M, Knol J. Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol.* 2005;71:2318-2324.

102. Moro GE, Stahl B, Fanaro S, Jelinek J, Boehm G, Coppa GV. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr Suppl.* 2005;94:27-30.

103. Rinne MM, Gueimonde M, Kalliomäki M, Hoppu U, Salminen SJ, Isolauri E. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. *FEMS Immunol Med Microbiol.* 2005;43:59-65.

104. Scholtens PA, Alles MS, Bindels JG, van der Linde EG, Tolboom JJ, Knol J. Bifidogenic effects of solid weaning foods with added prebiotic oligosaccharides: a randomised controlled clinical trial. *J Pediatr Gastroenterol Nutr.* 2006;42:553-559.

105. Haarman M, Knol J. Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol.* 2006;72:2359-2365.

106. Moro G, Minoli I, Mosca M, et al. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr.* 2002;34:291-295.

107. Moro GE, Mosca F, Miniello V, et al. Effects of a new mixture of prebiotics on faecal flora and stools in term infants. *Acta Paediatr Suppl.* 2003;91:77-79.

108. Euler AR, Mitchell DK, Kline R, Pickering LK. Prebiotic effect of fructo-oligosaccharide supplemented term infant formula at two concentrations compared with unsupplemented formula and human milk. *J Pediatr Gastroenterol Nutr.* 2005;40:157-164.

109. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect Immun.* 2006;74:6920-6928.

110. Moore N, Chao C, Yang LP, Storm H, Oliva-Hemker M, Saavedra JM. Effects of fructo-oligosaccharide-supplemented infant cereal: a double-blind, randomized trial. *Br J Nutr.* 2003;90:581-587.

111. Savino F, Maccario S, Castagno E, et al. Advances in the management of digestive problems during the first months of life. *Acta Paediatr Suppl.* 2005;94:120-124.

112. Boehm G, Lidestri M, Casetta P, et al. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed.* 2002;86:F178-F181.

113. Savino F, Cresi F, Maccario S, et al. "Minor" feeding problems during the first months of life: effect of a partially hydrolysed milk formula containing fructo- and galacto-oligosaccharides. *Acta Paediatr Suppl.* 2003;91:86-90.

114. Schmelzle H, Wirth S, Skopnik H, et al. Randomized double-blind study of the nutritional efficacy and bifidogenicity of a new infant formula containing partially hydrolyzed protein, a high beta-palmitic acid level, and nondigestible oligosaccharides. *J Pediatr Gastroenterol Nutr.* 2003;36:343-351.

115. Cámara-Martos F, Amaro-López MA. Influence of dietary factors on calcium bioavailability: a brief review. *Biol Trace Elem Res.* 2002;89:43-52.

116. Beynen AC, Kappert HJ, Yu S. Dietary lactulose decreases apparent nitrogen absorption and increases apparent calcium and magnesium absorption in healthy dogs. *J Anim Physiol Anim Nutr.* 2001;85:67-72.

117. Brommage R, Binacua C, Antille S, Carrié AL. Intestinal calcium absorption in rats is stimulated by dietary lactulose and other resistant sugars. *J Nutr.* 1993;123:2186-2194.

118. Demigné C, Levrat MA, Rémésy C. Effects of feeding fermentable carbohydrates on the cecal concentrations of minerals and their fluxes between the cecum and blood plasma in the rat. *J Nutr.* 1989;119:1625-1630.

119. Heijnen AM, Brink EJ, Lemmens AG, Beynen AC. Ileal pH and apparent absorption of magnesium in rats fed on diets containing either lactose or lactulose. *Br J Nutr.* 1993;70:747-756.

120. Delzenne N, Aertssens J, Verplaetse H, Roccaro M, Roberfroid M. Effect of fermentable fructo-oligosaccharides on mineral, nitrogen and energy digestive balance in the rat. *Life Sci.* 1995;57:1579-1587.

121. Lobo AR, Colli C, Filisetti TM. Fructooligosaccharides improve bone mass and biomechanical properties in rats. *Nutr Res.* 2006;26:413-420.

122. Lopez HW, Coudray C, Levrat-Verny MA, Feillet-Coudray C, Demigné C, Rémésy C. Fructooligosaccharides enhance mineral apparent absorption and counteract the deleterious effects of phytic acid on mineral homeostasis in rats. *J Nutr Biochem.* 2000;11:500-508.

123. Morohashi T, Sano T, Ohta A, Yamada S. True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats. *J Nutr.* 1998;128:1815-1818.

124. Ohta A, Ohtsuki M, Baba S, Adachi T, Sakata T, Sakaguchi E. Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *J Nutr.* 1995;125:2417-2424.

125. Hara H, Suzuki T, Aoyama Y. Ingestion of the soluble dietary fibre, polydextrose, increases calcium absorption and bone mineralization in normal and total-gastrectomized rats. *Br J Nutr.* 2000;84:655-661.

126. Sazawal S, Dhingra U, Sarkar A, et al. Efficacy of milk fortified with a probiotic *Bifidobacterium lactis* (DR-10™) and prebiotic galacto-oligosaccharides in prevention of morbidity and on nutritional status. *Asia Pac J Clin Nutr.* 2004;13:S28.

127. Lidestri M, Agosti M, Marini A, Boehm G. Oligosaccharides might stimulate calcium absorption in formula-fed preterm infants. *Acta Paediatr Suppl.* 2003;91:91-92.

128. Field CJ, McBurney MI, Massimino S, Hayek MG, Sunvold GD. The fermentable fiber content of the diet alters the function and composition of canine gut associated lymphoid tissue. *Vet Immunol Immunopathol.* 1999;72:325-341.

129. Lim BO, Yamada K, Nonaka M, Kuramoto Y, Hung P, Sugano M. Dietary fibers modulate indices of intestinal immune function in rats. *J Nutr.* 1997;127:663-667.

130. Nagai T, Ishizuka S, Hara H, Aoyama Y. Dietary sugar beet fiber prevents the increase in aberrant crypt foci induced by gamma-irradiation in the colorectum of rats treated with an immunosuppressant. *J Nutr.* 2000;130:1682-1687.

131. Kudoh K, Shimizu J, Wada M, Takita T, Kanke Y, Innami S. Effect of indigestible saccharides on B lymphocyte response of intestinal mucosa and cecal fermentation in rats. *J Nutr Sci Vitaminol.* (Tokyo) 1998;44:103-112.

132. Kanauchi O, Andoh A, Iwanaga T, et al. Germinated barley foodstuffs attenuate colonic mucosal damage and mucosal nuclear factor kappa B activity in a spontaneous colitis model. *J Gastroenterol Hepatol.* 1999;14:1173-1179.

133. Milo LA, Reardon KA, Tappenden KA. Effects of short-chain fatty acid-supplemented total parenteral nutrition on intestinal pro-inflammatory cytokine abundance. *Dig Dis Sci.* 2002;47:2049-2055.

134. Segain JP, Raingeard de la Blétière D, Bourreille A, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut.* 2000;47:397-403.

135. Bakker-Zierikzee AM, Tol EA, Kroes H, Alles MS, Kok FJ, Bindels JG. Faecal SIgA secretion in infants fed on pre- or probiotic infant formula. *Pediatr Allergy Immunol.* 2006;17:134-140.

136. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child.* 2006;91:814-819.

137. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. *Pediatrics.* 2007;119:e837-e842.

138. Sadeharju K, Knip M, Virtanen SM, et al. Maternal antibodies in breast milk protect the child from enterovirus infections. *Pediatrics.* 2007;119:941-946.

139. Waligora-Dupriet AJ, Campeotto F, Nicolis I, et al. Effect of oligofructose supplementation on gut microflora and well-being in young children attending a day care centre. *Int J Food Microbiol.* 2007;113:108-113.

140. Duggan C, Penny ME, Hibberd P, et al. Oligofructose-supplemented infant cereal: 2 randomized, blinded, community-based trials in Peruvian infants. *Am J Clin Nutr.* 2003;77:937-942.

141. Neu J. Perinatal and neonatal manipulation of the intestinal microbiome: a note of caution. *Nutr Rev.* 2007;65:282-285.