

Immune Gut Tolerance in Infant NutritionPrebiotics, Probiotics, Postbiotics from Emerging Concept to Application

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Summary

The increasing knowledge about the composition and activities of the microflora has shown the close link between the bacteria and the health of the human organism. For this reason, it has focused attention on the possibility of modulating the gut flora. The use of probiotics and prebiotics has increased enormously in recent years, more for real beneficial effects demonstrated in patients than for their safety profiles. However, it is recorded an indiscriminate use also in conditions in which there are no scientific evidence to support.

The first objective of infant formulas is to ensure the healthy growth of neonates and infants, as the sole complete food source during the first months of life when a child cannot be breastfed. Beyond this nutritional aspect, infant nutrition companies also try to mimic breast milk in its unique immuno-modulating properties. Numerous studies have demonstrated that the intestinal microbiota under the influence of diet shapes the maturation of the immune system and influences the risk of atopic diseases in infants. A new challenge for dairy industries is, therefore, to develop infant formulas inducing the maturation of immunity and the microbiota that can be observed in breastfed delivered vaginally, representing reference infants. *Streptococcus thermophilus*, *Lactobacillus reuteri* DSM 17938, *Bifidobacterium breve* (BC50), *Bifidobacterium lactis* Bb12, *Lactobacillus fermentum* (CECT5716), and *Lactobacillus rhamnosus* GG (LGG) are some of the probiotics added to infant formula, according to a literature review of the past 10 years. The most frequently used prebiotics in published clinical trials are Fructo-Oligosaccharides (FOSs), Galacto-Oligosaccharides (GOSs), and Human Milk Oligosaccharides (HMOs). This review sums up the expected benefits and effects for infants of pre-, pro-, syn-, and postbiotics added to infant formula regarding the microbiota, immunity, and allergies.

Keywords: Prebiotic; Probiotic; HMO; Postbiotic; Infant Formula; Immunity; Atopy; Microbiota; NEC

Introduction

A host and its commensal microbiota live in symbiosis, allowing both the establishment of local immunity and maturation of the intestinal epithelium [1,2]. The development of the intestinal microbiota at birth is progressive and sequential. The microbiota matures during the first years of life until reaching a kind of "status-quo" after 3 years. The main characteristic of the primo-colonizing pattern at birth is high inter-individual variability, reflecting the fragile acquisition of a diverse ecosystem. Colonization becomes massive after birth [3]. It starts with Enterobacteriaceae, then *Bifidobacterium*, *Bacteroides*, and *Clostridium* [3,4].

Depending on the type of birth, the early microbiota of infants differs, with a gut microbiota close to the mother's vaginal mi-

crobiota in the case of vaginal delivery and one close to the mother's skin microbiota for cesarean births [3,5]. Infants born via C-section have more *Clostridium* and pathogenic potential bacteria and less *Bifidobacteria* and *Bacteroides* [4]. Significant variations in the microbiota due to the type of birth disappear between 6 and 14 months [6].

Breastfeeding remains the strongest factor influencing the digestive microbiota of infants in the first year of life [6]. *Bifidobacteria* usually represent the dominant taxon (up to 90%) in breastfed infants delivered vaginally [4]. Breastfeeding provides *Bifidobacterium* sp., *Lactobacillus* sp., and *Staphylococcus* sp. naturally present in mothers' milk. More importantly, breastfeeding promotes the implantation of *Bifidobacteria* thanks to the richness and high diversity of Human Milk

Oligosaccharides (HMOs), which are uniquely metabolized by the bacteria. In a virtuous circle, endogenous synthesis of secretory IgA (sIgA) by the intestinal mucosal lymphocytes into the lumen is also conditioned by the presence of microbiota, particularly Bifidobacteria [7], after the first weeks of life when sIgA can only be provided by breastmilk. sIgA is an important weapon in immune defense against pathogens and toxins [2].

Conversely, formula-fed infants have a faster maturation of their gut microbiota compared to breastfed infants. Indeed, microbiota from formula-fed infants is diversified earlier, resulting in an enrichment in anaerobic bacteria, such as Bacteroides and Clostridium, with a lower representation of so-called “beneficial” bacteria, such as Bifidobacteria and Lactobacilli [8].

Overall, a lower abundance of Bifidobacteria, as observed in cesarean-born or formula-fed infants, is a risk factor for impaired metabolism of Short-Chain Fatty Acids (SCFAs), an increase in stool pH, and a weakening of the intestinal barrier function. As a result, the dialogue between the microbiota and the host is disturbed, the risk of colonization by pathogens is greater, and digestive inflammation can be observed. All these parameters may also participate in altered immune system programming and metabolic disorders. These infants have an increased risk of developing immune-related disease, such as allergic diseases, autoimmune diseases, or other chronic digestive or extradigestive diseases [4,5].

For several reasons, some newborns and infants cannot benefit from breastfeeding. The objective of dairy industries is then to ensure that infant formulas are as close as possible to breastmilk, both in its composition and its physiological properties. Some breastmilk bioactive components are unique and specific to human milk, and some, such as cytokines and growth factors, are associated with health outcomes in infancy (e.g., food allergies [9]). However, their addition to infant formula is not planned to date (due to cost and stability). On the other hand, supplementation with prebiotics or health-promoting (live) bacteria seems a more rational and easier approach to improve the health-promoting capacity of formulas. Since breastfed infants have more Bifidobacterium in their microbiota, the first strategy was to add probiotics and, in particular, Bifidobacteria directly into infant formulas, followed by prebiotics and, more recently, synbiotics and postbiotics for their bifidogenic effects, as well as for their own positive expected effects on immunity. Nowadays, more than half of formula-fed infants consume probiotic-enriched formula in France [10]. The goal of this review is to sum up the pre-, pro-, syn-, and postbiotics (named “-biotics” in this review) used in infant formulas and the expected and proven clinical benefits for infants regarding microbiota composition, immunity, and allergies.

Definitions

Probiotic: An oral supplement or a food product that contains a sufficient number of viable microorganisms to alter the microflora of the host and has the potential for beneficial health effects [11].

Prebiotic: A nondigestible food ingredient that benefits the host by selectively stimulating the favourable growth and/or activity of 1 or more indigenous probiotic bacteria [12].

Symbiotic: A product that contains both probiotics and prebiotics. Evidence for synergy of a specific prebiotic for a probi-

otic in the product is not essential. Symbiotics may be separate supplements or may exist in functional foods as food additives [11,12].

Postbiotic: A metabolic by product generated by a probiotic microorganism that influences the host's biological functions [13,14].

Functional food: Any modified food or food ingredient that provides a health benefit beyond that ascribed to any specific nutrient/nutrients it contains. It must remain a food, and it must demonstrate its effect in amounts normally expected to be consumed in the diet. Benefits may include functions relevant to improving health and well-being and/or reduction of risk of disease. Any food that contains probiotics or prebiotics is a functional food. An example of a functional food is live-culture yogurt that contains probiotic bacteria, prebiotics, and other dietary nutrients. Human milk may also be considered a functional food; it contains substantial amounts of oligosaccharides (prebiotics) and may contain some naturally occurring probiotic bacteria (103 of bifidobacteria per mL of expressed human milk [17]).

What are Probiotics?

Probiotic microorganisms are typically members of the genera Lactobacillus, Bifidobacterium, and Streptococcus [11,12]. These bacteria are formative, obligatory, or facultative anaerobic organisms, which are typically nonmotile and of varying shapes. They typically produce lactic acid. Their inherent biological features enable them to predominate and prevail over potential pathogenic microorganisms in the human digestive tract. It is currently hypothesized that these microbes generate small molecular metabolic by products that exert beneficial regulatory influence on host biological functions, including short-chain fatty acids such as butyrate. These metabolic by products are sometimes referred to as “postbiotics” and may function biologically as immune modulators [13,14]. The most studied probiotic bacteria to date include Lactobacillus rhamnosus GG (LGG), Bifidobacterium lactis, and Streptococcus thermophilus. These probiotic bacteria are biologically different from the Gram-negative, motile, non-lactic-acid-producing bacteria such as Klebsiella, Pseudomonas, Serratia, and Proteus species, which also may be prominent flora in the human digestive system. These potentially harmful bacteria may translocate across the intestinal epithelium and could result in disease in humans [18,19]. Some yeasts and yeast byproducts have also been studied and have been frequently used as probiotic agents, such as the yeast Saccharomyces boulardii. Probiotic bacteria can be delivered and ingested separately as medicinals or supplements. They can also be mixed with, added to, or naturally exist in functional foods.

What are Prebiotics?

Prebiotics are usually in the form of oligosaccharides, which may occur naturally but can also be added as dietary supplements to foods, beverages, and infant formula [14]. Although indigestible by humans, their presence in the digestive system selectively enhances proliferation of certain probiotic bacteria in the colon, especially Bifidobacterial species. Prebiotic oligosaccharides often contain fructose chains with a terminal glucose and typically consist of 10 or fewer sugar molecules. Examples of prebiotic oligosaccharides include Fructo-Oligosaccharides (FOSs), inulin, Galacto-Oligosaccharides (GOSs), and soybean oligosaccharides. Inulin is a composite oligosac-

charide that contains several FOS molecules. The complex polysaccharides that constitute dietary fiber can also be considered to be prebiotic agents.

Although dietary nucleotides do not fit the exact definition of a prebiotic, they are prebiotic-like agents and have immunomodulating and direct intestinal biological properties [19]. Some infant formulas contain a limited amount of added free nucleotides (7–20 mg/dL) [19]. Human milk, on the other hand, contains a substantial but variable amount of oligosaccharides (14 g/L) as well as free nucleotides (up to 20% of nonprotein nitrogen) [20]. Some infant-formula manufacturers now add prebiotic oligosaccharides to their products.

Beverages and nutritional supplements marketed for older infants, children, and adults contain oligosaccharides and nucleotide additives in varying amounts.

Intestinal Bacterial Colonization and Development of the Intestinal Mucosal Defence System

Similar to the fetus, an infant at the time of birth has a sterile gastrointestinal tract, but bacterial colonization occurs rapidly [21–23]. The new born infant's gestational age, mode of delivery, and diet seem to have significant effects on this process. Neonates who are born by Caesarian delivery, born preterm, and/or exposed to perinatal or postnatal antibiotics have a delay in intestinal commensal probiotic bacterial colonization. When delivered vaginally, breastfed infants and formula-fed infants have a similar pattern of bacterial colonization at 48 hours of age. However, by 7 days of age, approximately two-thirds of formula-fed infants have a predominance of *Bacteroides fragilis*, compared with only 22% of breastfed infants [21]. Toward the end of the first month of life in developing countries, breastfed infants are found to have *Bifidobacteria*-predominant colonization, whereas formula-fed infants have equal colonization with *Bacteroides* and *Bifidobacteria* species. In resource-rich countries, however, differences are less pronounced between breastfed and formula-fed infants.

The composition of intestinal microflora does not change significantly after infancy. Therefore, the composition of fecal flora in older children and adults is less variable and not as dependent on diet. In fact, beyond infancy, bacterial concentrations in the colon are typically 10¹² colony-forming units per mL of intestinal contents (10-fold the total number of human cells in the human body), and anaerobic bacteria far outnumber aerobic coliforms [24]. Typically, 500 different bacterial species contribute to an adult's colonic microflora, but 99% of the microflora are accounted for by 30 to 40 species [24]. The descriptive terms of “microbiota” and “microbiome” are newer terms that are replacing such terms as “microflora” in an attempt by researchers in the field to better define one's microbial environment [25]. “Microbiota” refers to a population of microscopic organisms that inhabit a bodily organ or portion of a person's body, and human “microbiome” refers to the unique entire population of microorganisms and their complete genetic elements that inhabit one's body.

The intestinal mucosal defense system is an integral part of a sophisticated immunoregulatory network that includes the intestinal microflora [22–25]. Recognition of self- and non-self-antigens begin early in life, perhaps even in utero, and is significantly influenced by events that occur within the digestive system soon after birth. The immunoresponsiveness of

the digestive system is significantly affected by the young infant's diet, state of bacterial colonization, and early exposure to potential infectious pathogens and antibiotics as well as the infant's genotype. It is thought that the occurrence of many diseases, both intestinal and nonintestinal, can be related to dysregulation or interference with the early development of the intestinal mucosal defense system [26,27]. Examples of these diseases include those thought to be atopic (asthma, eczema, and allergic rhinitis) or autoimmune (multiple sclerosis, type 1 diabetes mellitus, and chronic inflammatory bowel disease [IBD]) [26]. Certainly, the overriding determining factor in development of the immune system is one's genetic predisposition [23].

The molecular basis for innate and acquired immunity is thought to reside in the recognition and response of mature T lymphocytes to trigger molecules, such as those derived from dietary and bacterial-breakdown products within the intestinal tract [27]. Trigger molecules also include dietary nucleotides and oligosaccharides. Toll-like receptors located in the surface membrane of T lymphocytes facilitate recognition of these trigger molecules, which eventually leads to specialized T-lymphocyte recognition and response to subsequent exposure to the same or very similar molecules. Thus, T-lymphocyte recognition of specific oligosaccharides bound to intestinal pathogens plays an important role in preventing gastrointestinal illness.

Given these important influences on intestinal microflora colonization and immune function, the infant's early diet and intestinal microbial environment are thought to serve as pivotal factors in overall health. Probiotic bacteria, postbiotic bacterial byproducts, and dietary prebiotics are believed to exert positive effects on the development of the mucosal immune system. It is also believed that exposure to “nonbeneficial” microorganisms and antimicrobial agents in the newborn period may result in immune dysregulation in susceptible individuals and may lead to some chronic disease states. There is evidence that human milk contains mononuclear cells that traffic intestinally derived bacterial components from the mother to her infant. The ingested human milk containing the bacterial components derived from the mother are thought to influence her young infant's developing immune system. This process is termed “bacterial imprinting,” and its overall biological effect requires further study [28].

Prebiotics

Definition

Prebiotics are indigestible substrates for humans but are metabolized by host microorganisms and exert a beneficial effect on health [28,29]. They can selectively stimulate the growth or activity of specific bacteria and, thus, promote the production of SCFAs, which have pleiotropic effects both locally, i.e., in the intestinal tract, and at distance on other tissues [29,30]. European regulations do not allow the mention of prebiotics on food packaging and the related health claim without an established and proven effect by clinical studies. In the USA, prebiotics have no legal definition from the FDA (Food and Drug Administration).

Prebiotics are naturally present in many fiber-rich foods. The most common prebiotics are carbohydrate-based, such as resistant starch, cellulose, pectin, and fructan, as well as oligosaccharides structured in Fructo-Oligosaccharides (FOSs) and

Galacto-Oligosaccharides (GOS). Breastmilk also contains a large number of natural prebiotics, i.e., Human Milk Oligosaccharides (HMOs). Dietary fibers have numerous demonstrated direct and indirect health benefits through the fiber–microbiota–immune relationship. The main bacterial metabolites coming from the fermentation of fibers are SCFAs (mostly acetate, butyrate, and propionate), which are potent immunomodulators associated notably with allergy protection [31]. Prebiotics added in adequate levels to infant formula are well-tolerated and ensure normal growth [32]. Adverse events can be observed at high levels of consumption.

HMOs

HMOs are the third most prevalent component of human milk, after lactose and lipids (33). They are indigestible carbohydrates that selectively stimulate the colonic growth of HMO-consuming bacteria, including Bifidobacteria [35,36]. More than 200 different HMOs have been identified in human milk, with up to 130 for an individual mother. HMO composition is highly influenced by the genetic status of the mother, i.e., secretor and Lewis's statuses determining the expressions of FUT2 and FUT3 fructosyltransferases, respectively. As a result of FUT2 activity, 2'-fucosyllactose (2'FL) is the most abundant HMO in breastmilk from secretor mothers (70–90% depending on country), representing 20–40% of the total HMO concentration in colostrum [36]. HMOs promote intestinal barrier function, prevent adhesion of pathogens to epithelial cells, act as decoy receptors, and stimulate the development of an infant's immune system either directly or through a microbiota-mediated effect [37]. Globally, HMOs may then help in preventing infections and diseases related to immune dysregulation, such as allergic and autoimmune diseases [35,37]. It is still unclear whether the protective effect of HMOs is specific to certain classes of HMOs [45] or if it relies on their high diversity and synergic actions. To date, due to technical difficulties and cost issues, only a few HMOs have been synthesized, i.e., 2'FL, 3-Fucosyllactose (3FL), 3'-Sialyllactose (3'SL), 6'-Sialyllactose (6'SL), and Lacto-N-neotetraose (LNnT), for use as supplements in infant formulas.

In vitro studies evidenced that 2'FL increased the relative proportions of Bifidobacterium adolescentis and other bacteria that produce butyrate, a beneficial SCFA [47]. 2'FL also reduced the adhesion of pathogens such as Clostridium difficile, Campylobacter jejuni, enteropathogenic E. coli, and Pseudomonas aeruginosa to epithelial cells [37]. In infants, supplementation with 2'FL promoted the growth of Bifidobacterium species and limited the colonization of opportunistic pathogens, such as C. difficile and K. pneumonia [36].

Feeding with a formula supplemented with 2'FL and GOS (2.4 g total oligosaccharides/L: 2'FL at 0.2 g/L with GOS at 2.2 g/L (n = 54) or 2'FL at 1 g/L with GOS at 1.4 g/L (n = 48)) for 6 weeks resulted in inflammatory cytokine profiles in the plasma that were intermediate between that of infants fed with control infant formula (GOS only, 2.4 g/L, n = 48) and that of exclusively breastfed infants (n = 51) [38].

In healthy infants, the use of infant formulas enriched with 2'FL (1 g/L) and LNnT (0.5 g/L) (n = 88, vs. n = 87 in the control group) during the first 6 months of life was associated with a decrease in lower respiratory infections and with the use of antibiotics and antipyretics before the age of 1 year, but these results were the secondary endpoints of a tolerance study [39]. At

3 months, fecal microbiota compositions (alpha diversity; beta diversity; relative abundance of Bifidobacterium, Escherichia, unclassified Peptostreptococcaceae, and Streptococcus) of infants supplemented with HMOs were closer to that of breastfed children than that of the control group. HMOs increased the proportion of infants with a fecal community type characterized by high abundance of Bifidobacteriaceae compared to the control group. The formula-fed group with the higher abundance of Bifidobacteriaceae required less frequent antibiotics during the first year than infants with other fecal community types. These results suggested that the anti-infectious effect of HMOs is linked to the composition of the microbiota [40].

In another trial, infants were fed from 14 days to 4 months of age with an experimental formula with a five-HMO mix (2'FL at 2.99 g/L, LNnT at 1.5 g/L, 3FL at 0.75 g/L, 6'SL at 0.28 g/L, and 3'SL at 0.23 g/L) (n = 103) or a control formula (n = 104). In the safety outcomes, no differences were shown regarding infections and infestations [41].

Another randomized study with a similar formula (2'FL at 3 g/L, LNnT at 1.5 g/L, 3FL at 0.8 g/L, 6'SL at 0.3 g/L, and 3'SL at 0.2 g/L) showed that the experimental-formula-fed infants (n = 130) had less recourse to healthcare professionals for illness than the control group (n = 129) before 3 months of age (secondary outcomes) [42].

From 1 to 2.5 years of age (n = 461), the incidence of upper respiratory tract infections was similar between randomized infants receiving four different young-child formulas containing GOS (4 g/L), TGF- β (9.9 or 15 μ g/L), lactoferrin (0 to 1.7 g/L), immunoglobulins (0 to 1 g/L), milk fat (0.5 to 17 g/L), and 2'FL (0 or 3 g/L). However, according to the secondary outcomes of the study, children supplemented with 2'FL had longer durations of upper respiratory tract infections and more episodes of coughs and runny noses than the group with the similar formula without 2'FL (p < 0.05 and p < 0.01, respectively). Fever episodes were less frequent, but gastrointestinal tract infections occurred more often in the group supplemented with 2'FL, immunoglobulins, and lactoferrin than in the group fed with formula without these components (p < 0.01 each) [43].

Whey-based extensive hydrolyzates with added HMOs (2'FL at 1 g/L and LNnT at 0.5 g/L) are free of residual milk proteins and were well-tolerated by infants allergic to cow's milk [45]. Cow's-milk-allergic infants in the HMO group (n = 94) and in the control group (n = 96, same formula without HMOs) had similar incidences of upper and lower respiratory tract infections, gastrointestinal infections, other viral infections, and urinary tract infections between enrollment (from 0 to 6 months) and 1 year of age. In a subanalysis, the authors evidenced a significant reduction in the frequency of upper respiratory tracts infections compared to the control group (hazard ratio: 0.58; 95% CI: [0.41–0.83]). There was a slight reduction in the occurrence of otitis media during the follow up in the HMO group. The overall uses of antibiotics and antipyretics were similar in both groups, but between the visits at 4 months for follow-up and 12 months of age, infants in the HMO group required fewer antipyretics (p = 0.02) [44]. There are currently no published clinical studies evidencing acceleration of the acquisition of tolerance to cow's milk [44].

To summarize, results about the prevention of infections

through HMO supplementation of infant formula are divergent, and the potential benefits of such interventions should be further studied.

GOSs

Galacto-Oligosaccharides (GOSs) are prebiotics that are more easily synthesized than HMOs, explaining why they are more frequently used in infant formulas. In vitro, they limit the adhesion of pathogens to epithelial cells and stimulate the Treg (IL10) and Th1 (increase in IFN- γ and decrease in TNF- α) pathways, inducing anti-inflammatory and regulatory effects [55].

In animals, GOSs promoted an increase in SCFAs and stimulated intestinal barrier function [56]. In infants, GOS supplementation (4.4 to 5 g/L) ($n = 44$, vs. $n = 37$ in the control group without GOS) decreased fecal pH and butyric acid concentration, whereas the effect on fecal sIgA was limited [45]. They also had bifidogenic effects [47,55,56] and reduced the gastrointestinal colonization of *Clostridium* ($n = 83$ fed with the study formula vs. $n = 79$ in the control group) [46].

Bozensky et al. studied the effect of GOS supplementation (5 g/L) in a partially hydrolyzed formula on atopic dermatitis in infants with a family history of atopy and moderate eczema at recruitment ($n = 52$ in the intervention group vs. $n = 51$ in the control group). Supplementation was provided from 6 weeks to 6 months. The SCORAD index decreased in both groups (supplemented or not), with no significant differences between the groups [47].

GOSs associated with polydextrose (PDX) (total of 4 g/L; 1:1 ratio) also had a bifidogenic effect ($n = 91$ PDX/GOS group; $n = 91$ control group; $n = 83$ breastfed group) [58] and was evidenced in increased counts of *Lactobacilli*, particularly in *L. rhamnosus*, in supplemented infants ($n = 77$), thus showing a gut microbiota closer to that of breastfed infants ($n = 71$) than to non-supplemented infants ($n = 80$) [48].

In young infants at risk of atopy, GOS/PDX supplementation (total of 4 g/L; 1:1 ratio) ($n = 201$) prevented respiratory infections in the first two years of life, with a rate similar to that observed in breastfed infants ($n = 140$) [49]. In this study, supplementation induced differences in fecal microbiota at 9–12 months of life, with increases in *Bifidobacteria* and *Clostridium* cluster I. The supplementation did not prevent atopic dermatitis, but the increased load of fecal *Bifidobacteria* at 9–12 months was associated with protection against respiratory infection. Atopic-dermatitis-free infants had higher colonization with *Clostridium* postintervention [50].

FOSs

Fructo-oligosaccharides (FOSs) derived from inulin are also known to be bifidogenic [61–63], despite controversies [51]. In vitro, FOSs limit the adhesion of pathogens to intestinal cells, strengthen the intestinal barrier, and stimulate the Th1 immune pathway, as observed for GOSs [52]. Gut inflammation monitored with fecal calprotectin was not affected after 8 weeks of supplementation (3 g/L) ($n = 10$ –12 infants per group; prebiotic formula, control formula, and human milk) [61] or after a 12 months of supplementation (short- and long-chain FOS and inulin combination, total of 8 g/L) ($n = 14$ fecal samples in prebiotic group and $n = 11$ in the control group) [63]. Conversely, FOSs have induced increased intestinal production of sIgA [53–55].

GOSs/FOSs at a Ratio of 9:1

Fifteen years ago, one of the first originator studies in infants fed with a formula with GOSs/FOSs (6 g/L; GOS/FOS ratio: 9/1) ($n = 19$) showed a trend of increased rate of fecal sIgA compared to a standard formula ($n = 19$) [56]. After 1 year of intervention (4 g/L), Bruzzese et al. highlighted a reduction in digestive infections during the study period. There was a decreased number of episodes (0.12 episode per child per year vs. 0.29, $p = 0.015$), with fewer children having at least one episode of acute infectious gastroenteritis (10.4% vs. 23.9%, $p = 0.01$) and fewer children having at least two courses of antibiotics (40.0% vs. 66.2%, $p = 0.02$) ($n = 96$ in the prebiotic group; $n = 105$ in the standard formula group). Moreover, supplementation was associated with a non-significant decrease in the number of children who had at least three episodes of upper respiratory infections (28.3% vs. 44.6%, $p = 0.06$) [58].

When supplementation with GOSs/FOSs (9:1) was pursued up to 12 months of age, Shahramian et al. observed an infectious history similar to breastfed infants. The total duration of diarrhea was shorter in supplemented-formula-fed infants compared to non-supplemented (4.4 vs. 12.3 days, $p < 0.001$) and similar to that observed in breastfed infants (4.4 vs. 6.8) ($n = 60$ in each group). Additionally, GOS/FOS-supplemented infants had fewer occurrences of fever episodes and respiratory tract infections compared to regular-formula-fed infants but the same as that of breastfed infants [59].

The European Multicentric Infection Prevention Study (MIPS) demonstrated that a formula with a specific mixture of short-chain GOSs (scGOSs) plus long-chain FOSs (lcFOSs) (6.8 g/L, ratio 9:1) and pectin-derived acidic oligosaccharides (1.2 g/L) decreased the rate of atopic dermatitis by 44% in infants not considered to be at risk in their first year of life. This significant effect was not sustained at preschool age after oligosaccharide supplementation was stopped ($n = 172$ in the probiotic group) [60,61].

Holscher et al. studied the effect of a partially hydrolyzed whey formula supplemented with GOSs and FOSs (4 g/L, 9:1) on intestinal microbiota composition. After 6 weeks of GOS/FOS supplementation ($n = 36$), the absolute and relative quantities of *Bifidobacteria* were similar to those observed in breastfed infants ($n = 33$) and higher than those in non-supplemented infants ($n = 33$). The SCFAs (mainly acetate, propionate, and butyrate) were higher in the supplemented group than in the breastfed group. As a result, fecal pH was more acid in prebiotic and breastfed groups [62].

Another partially hydrolyzed whey protein infant formula containing scGOSs, lcFOSs (6.8 g/L; GOS/FOS ratio: 9:1), and pectin-derived acidic oligosaccharides (1.2 g/L) ($n = 57$) showed similar results in terms of bacterial taxonomic and metabolite compositions of gut microbiota close to those of breastfed infants ($n = 30$) [63,64]. However, this formula failed to prevent eczema by 12 and 18 months in high-risk infants ($n = 341$) compared to a standard cow's milk formula [$n = 360$] [65,66].

Several randomized controlled double-blind studies have focused on the use of a combination of GOSs/FOSs (8 g/L; GOS/FOS ratio = 9:1) added to an extensive whey hydrolyzate formula provided during the first 6 months of life. The aim of this formula was to prevent atopic disease in at-risk infants

(at least one of the two parents having atopy). At 6 months, the cumulative incidence of atopic dermatitis was lower in the supplemented group (9.8% vs. 23.1%, $p = 0.014$, total $n = 206$) [71]. In a subgroup of 84 children, the supplemented infants had significantly lower totals of IgE, IgG1, IgG2, and IgG3 antibody concentrations in serum than non-supplemented infants [72]. A gut microbiota analysis revealed an increase in the number of Bifidobacterial at 6 months under GOS/FOS supplementation (subgroup of 98 children) [71]. At 2 years of age ($n = 134$), a significant reduction in the cumulative incidence of allergic manifestations was observed (atopic dermatitis: 13.6% vs. 27.9%; recurrent wheezing: 7.6% vs. 20.6%; urticaria: 1.5% vs. 10.3%) [73]. Supplemented infants also had fewer episodes of upper respiratory infections and fevers and fewer courses of antibiotics [67,68]. At 5 years ($n = 92$), i.e., 4.5 years after stopping the prebiotics, a lower cumulative incidence of allergic manifestations was still observed in the supplemented group (30.9% vs. 66.0%, $p < 0.01$), with notably less atopic dermatitis [69].

GOSs and/or FOSs

After 4 months of supplementation with GOSs (0.6 g/100 g), FOSs (0.8 g/100 g), and 1,3-olein-2-palmitin (OPO) (4 g/100 g), the most abundant triacylglycerol in breastmilk, ($n = 22$ in the supplemented formula group), the alpha diversity and richness of gut microbiota decreased compared to infants fed with regular formula ($n = 13$), approximating that of breastfed children ($n = 48$). GOS/FOS/OPO supplementation was associated with a beta diversity (meaning the phylogenetic distance between samples) closer to that of breastfed infants, with a higher relative abundance of *Enhydrobacter* and *Akkermansia* [70]. In terms of microbiota metabolism functions, supplemented children and breastfed children had similar proportions of intestinal bacteria related to septicemia and ureolysis [71].

In an ELFE cohort, no association was observed between the consumption of GOSs/FOSs or GOSs only at 2 months and the occurrence of respiratory disease up to 5.5 years. Nevertheless, early use of GOSs was associated with a lower risk of upper respiratory tract infections compared to infants never supplemented with GOSs (OR: 0.87; 95% CI:

Symbiotic

Definition

A symbiotic is a “mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host” [73]. The synergistic and the complementary effects of the substrate, which is not necessarily a prebiotic, make it possible to gain the effects of the probiotic and the substrate as a nonstimulant on the microbiota and immune functions. Yogurt is the archetype symbiotic food in lactose intolerance, with a health claim recognized by the EFSA.

Postbiotics

Definition

Postbiotics are a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”. They are “deliberately inactivated microbial cells with or without metabolites or cell components that contribute to demonstrated health benefits” [74,75].

Postbiotics Produced by *Lactobacillus paracasei* (CBA L74): Several Italian teams have taken an interest in postbiotics re-

sulting from the fermentation of skimmed milk with *Lactobacillus paracasei* (CBA L74), a strain isolated from the feces of healthy infants [76,77]. In brief, the fermented milk was prepared from skimmed milk fermented with 106 CFU of *L. paracasei* CBA L74/g. The bacterial growth was stopped after 15 h of incubation at 37 °C when the bacteria reached 5.9×10^9 CFU/g, and the bacteria was inactivated with a quick heating. An initial study in mice showed a protective effect of milk fermented using *Lactobacillus paracasei* (CBA L74) in induced colitis, protection against pathogens (*Salmonella*), and inhibition of pro-inflammatory cytokines in favor of anti-inflammatory cytokines [78]. The active components were the metabolites from the fermentation and not the live or killed bacteria [78]. Then, thanks to a skim cow's milk fermented with *L. paracasei* L74 (not infant formula), the authors reported fewer common infections (in particular, acute gastroenteritis, pharyngitis, laryngitis, and tracheitis) and less use of drugs (antipyretics, antibiotics, and corticosteroids) in children from 12 to 48 months of age supplemented over a period of 3 months. Immuno-stimulation has been demonstrated, with increases in concentrations of fecal peptides and proteins (α -defensin, β -defensin, sIgA, and cathelicidin LL-37) resulting from the activation of the innate and acquired immune system [78]. Finally, this principle of fermentation (fermented spray-dried milk for infant milk tins) was applied to an infant formula administered to newborns up to 3 months of age (three groups: intervention, control, and breastfed; $n = 26$ in each group) [78]. Infants receiving the fermented formula had a similar microbiota to that of breastfed infants, namely a reduction in fecal bacterial diversity, an intermediate level of sIgA, and a metabolomic profile close to that of breastfed infants. However, over the period studied, unlike the studies by Corsello et al. [79] and Nocerino et al. [79], no difference in antimicrobial peptides was observed [80].

Postbiotics Produced by *Bifidobacterium breve* C50 and *Streptococcus thermophilus* ST065:

Bifidobacterium breve C50 and *Streptococcus thermophilus* ST065 are lactic-acid-producing bacteria with anti-inflammatory properties on intestinal cells in vitro [80]. A fermented infant formula based on these two strains with no living bacteria in the final product was tested in healthy infants ($n = 464$) and compared to infants receiving non-supplemented formula ($n = 449$). Fermented and control formula were provided for 5 months after the age of 4 months. While the incidence of acute diarrhea was the same in both groups, the severity of acute gastroenteritis was less in the fermented milk group, with fewer hospitalizations, fewer cases of acute dehydration, fewer medical consultations, and fewer prescriptions for oral rehydration solutions [81]. Between 6 and 24 months of age, the incidence of cow's milk protein allergy was the same in both groups ($n = 66$ and 63 in the fermented milk group and standard formula group, respectively), but sensitization to milk assessed by skin prick tests and digestive or respiratory symptoms of suspected allergy were lower in infants at high risk of atopy receiving the postbiotic-supplemented formula [82]. The fecal pH was similar from day 3 of life to 4 months in newborns for infants fed the fermented milk ($n = 30$) and breastfed ($n = 30$) and was more acidic than in infants fed the standard formula ($n = 30$) [83].

When this fermented formula (containing 0.25 g of 3'-galactosyllactose/L) was combined with GOSs/FOSs (8 g/L, ratio of 9:1) ($n = 30$), fecal sIgA concentrations and the compo-

sitions of the fecal microbiota were similar to those of breastfed infants ($n = 30$) [84,85]. Nevertheless, untargeted metabolomic profiles remained distinct, even if stable over time, between infants fed with pre- and postbiotic-supplemented formula and breastfed infants, with 261 different metabolites at the end of the study (vs. 404 different metabolites between the control formula and the breastfed group) [86].

Postbiotics produced by *Bifidobacterium animalis* sp. lactis CECT 8145 BPL1TM:

According to secondary outcomes of the INNOVA 2020 study, infants randomized to be fed with an intervention formula (containing a thermally inactivated postbiotic, BPL1TM, and a lower amount of protein, a lower casein-to-whey protein ratio, and a double amount of docosahexaenoic acid/arachidonic acid compared to a standard formula) ($n = 70$) exhibited less atopic dermatitis and fewer bronchitis and bronchiolitis episodes than infants in the standard group ($n = 70$) ($p = 0.03$). These rates were similar as in breastfed children ($n = 70$) ($p = 1.0$). Other morbidities, such as infections, were not different among the three groups during the timeframe of the study

Atopic Diseases

Prevention of Atopic Disease

As previously mentioned, the sequence of bacterial intestinal colonization of neonates and young infants is probably important in the development of the immune response [87]. Recognition by the immune system of self and nonself, as well as the type of inflammatory responses generated later in life, are likely affected by the infant's diet and acquisition of the commensal intestinal bacterial population superimposed on genetic predisposition. During pregnancy, the cytokine inflammatory-response profile of the fetus is diverted away from cell-mediated immunity (T-helper 1 [Th1] type) toward humoral immunity (Th2 type). Hence, the Th2 type typically is the general immune response in early infancy. The risk of allergic disease could well be the result of a lack or delay in the eventual shift of the predominant Th2 type of response to more of a balance between Th1- and Th2-type responses [88]. Administration of probiotic bacteria during a time period in which a natural population of lactic-acid-producing indigenous intestinal bacteria is developing could theoretically influence immune development toward more balance of Th1 and Th2 inflammatory responses [89]. The intestinal bacterial flora of atopic children has been demonstrated to differ from that of nonatopic children. Specifically, atopic children have more *Clostridium* organisms and fewer *Bifidobacterium* organisms than do nonatopic study subjects [89,90], which has served as the rationale for the administration of probiotics to infants at risk of atopic diseases, particularly for those who are formula fed.

In a double-blinded RCT, LGG or a placebo was given initially to 159 women during the final 4 weeks of pregnancy. If the infant was at high risk of atopic disease (atopic eczema, allergic rhinitis, or asthma), the treatment was continued for 6 months after birth in both the lactating woman and her infant [90]. A total of 132 mother-infant pairs were randomly assigned to receive either placebo or LGG and treated for 6 months while breastfeeding. The primary study end point was chronic recurrent atopic eczema in the infant. Atopic eczema was diagnosed in 46 of 132 (35%) of these study children by 2 years of age. The frequency of atopic eczema in the LGG-treated group was 15 of 64 (23%) versus 31 of 68 (46%) in the placebo group (RR: 0.51 [95% CI: 0.32–0.84]; $P < .01$). The

number of mother-infant pairs required to be treated with LGG to prevent 1 case of chronic recurrent atopic eczema was 4.5. By 4 years of age, eczema occurred in 26% of the infants in the group treated with LGG, compared with 46% in the placebo group (RR: 0.57 [95% CI: 0.33–0.97]; $P < .01$). However, only 67% of the original study group was analyzed at the 4-year follow-up. These results support a preventive effect for giving a probiotic to mothers late in pregnancy and to both mothers and infants during the first 6 months of lactation for the prevention of atopic eczema in infants who are at risk of atopic disease. However, these results have not been confirmed in subsequent clinical trials, as summarized in a recent review by Kopp and Saalfeld [91]. Conversely, found that probiotic supplementation did not reduce the risk of atopic dermatitis in children at high risk with the report of some increased risk of subsequent allergen sensitization. As concluded in a review by Prescott and Björkstén [92] and in a 2007 Cochrane review, [93] despite the encouraging results of some studies, there is insufficient evidence to warrant the routine supplementation of probiotics to either pregnant women or infants to prevent allergic diseases in childhood. Explanations for varied study results include host factors such as genetic susceptibility, environmental factors such as geographic region and diet, and study variables including probiotic strains and doses used [94,95].

Treatment of Atopic Disease

In an RCT, 53 Australian infants with moderate-to-severe atopic dermatitis were given either *Lactobacillus fermentum* or placebo for 8 weeks. At final assessment at 16 weeks, significantly more children who received the probiotic had improved extent and severity of atopic dermatitis as measured by the Severity of Scoring of Atopic Dermatitis (SCORAD) index over time compared with those who received placebo ($P = .01$) [94,95]. These results are encouraging, but as summarized in a 2008 Cochrane review [96] probiotics have not yet been proven to be effective in the treatment of eczema.

Prevention of Necrotizing Enterocolitis in Low-Birth-Weight Neonates

A new born's gut is sterile at birth, with bacterial colonization beginning shortly after birth.(97) Preterm infants frequently have delayed and aberrant acquisition of the "normal" digestive microflora, possibly because of restricted enteral feedings and frequent use of antibiotic therapy.(98,99) Delayed enteral feeding, frequent use of antibiotic therapy, and altered acquisition of normal digestive microflora are believed to be primary contributing factors for the increased risk of necrotizing enterocolitis (NEC) in preterm infants(99) and is the rationale for probiotic supplements.

In a 2008 Cochrane review based on 9 RCTs [99], enteral probiotic supplementation significantly reduced both the incidence of NEC (stage II or more) (RR: 0.32 [95% CI: 0.17–0.60]) and mortality (RR: 0.43 [95% CI: 0.25–0.75]) [100]. Nosocomial sepsis was not reduced significantly (RR: 0.93 [95% CI: 0.73–1.19]). A total of 1425 infants who were born at less than 37 weeks' gestational age and/or less than 2500 g birth weight were included in this meta-analysis. No systemic infections or serious adverse events that were directly attributed to the administered probiotic organism were reported for these RCTs. The authors concluded that the results of their analysis supported a change in clinical practice to supplement preterm infants who weighed more than 1000 g at birth with a probiotic. Data regarding the outcome of preterm extremely low birth

weight infants who weighed less than 1000 g at birth could not be used by the authors to reliably estimate the efficacy and safety of probiotic supplementation to this high-risk group. A large RCT was recommended to investigate the potential benefit and safety of probiotic supplementation to extremely low birth weight infants.

However, because of the large heterogeneity of the studies included in the Cochrane review [101], caution is urged in interpreting the results, which are somewhat problematic. The studies all used different probiotics, including LGG, *Bifidobacterium breve*, *Saccharomyces* species, and mixtures of *Bacteroides bifidus*, *S thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium infantis*. Doses of individual probiotics varied and were administered with human milk feedings, formula feedings, or both human milk and formula feedings in some studies. Not all of the studies had the same end points, including the primary outcome of NEC. A second and larger study by Lin et al [102], the results of which were published after the Cochrane review, repeated the 2005 study [103] by using a different mixture of probiotics: *L acidophilus* and *B bifidus*. The overall incidence of NEC and death was less in the second study [103] compared with that in the first [104] in the controls, and the second study revealed that probiotics did not reduce the incidence of sepsis compared with that in the first, and the intervention group actually had a higher incidence of sepsis. The number needed to treat to prevent 1 case of NEC was 27 in the first study by Lin et al and 21 in the second study [105]. Another point that makes the data problematic is that the combinations of probiotics used in the Lin et al studies, which are the most convincing for NEC prevention, are not available in the United States. Not all probiotics have been studied; therefore, all probiotics cannot be generally recommended.

Infantile Colic

Prevention of Colic

To date, no RCTs have been conducted with colic as a primary end point.

Treatment of Colic

Colic is a common condition that typically affects infants in the first 4 months of life. The primary mechanism remains unknown. Available evidence suggests that colic potentially has a number of independent causes, including dietary protein hypersensitivity [105]. A recent unblinded RCT examined the effect of the administration of *L reuteri* versus simethicone in the treatment of colic in 90 exclusively breastfed infants in Italy [106]. The administration of *L reuteri* improved the symptoms of colic (minutes of crying per day) within 1 week of treatment, compared with simethicone therapy. The breastfeeding mothers were instructed to eliminate dairy products from their diets during the study period to minimize potentially confounding adverse effects of dietary protein hypersensitivity. The authors of the study proposed several theories for a positive therapeutic benefit, including probiotic modulation of proinflammatory responses. Further confirmatory RCTs are required to recommend routine use of probiotics in the treatment of infantile colic in both breastfed and formula-fed infants. On the basis of limited information, probiotics may be of benefit in treatment of colic in exclusively breastfed infants, but more studies are needed before they can be recommended.

Combined Prebiotics and Probiotics to Prevent Allergy

Clinical benefit in preventing allergic diseases by co-therapy

with probiotics and prebiotics in pregnant women and their infants was demonstrated in an RCT in Finland [107]. A total of 1223 pregnant women who had been identified to deliver infants who would be at high risk of atopic disease because of parental atopic disease history were randomly assigned to be given a mixture of 4 probiotic strains plus GOS or placebo daily for 2 to 4 weeks before delivery. After delivery, their infants then either received the same probiotic mixture plus GOS or the same placebo as the mother. Probiotic/prebiotic treatment showed no effect on the cumulative occurrence of allergic diseases but tended to reduce immunoglobulin E-associated (atopic) diseases (OR: 0.71 [95% CI: 0.50–1.00]; $P = .052$). Probiotic and prebiotic treatment reduced the occurrence of eczema (OR: 0.74 [95% CI: 0.55–0.98]; $P = .035$) and atopic eczema (OR: 0.66 [95% CI: 0.46–0.95]; $P = .025$). Confirmatory studies are necessary.

Prebiotics and Probiotics in Infant Formula

Prebiotics

As mentioned earlier in this review, human milk contains a number of substances that are prebiotic, the most plentiful of which are oligosaccharides [107,108]. Oligosaccharide prebiotics are also added to many commercially available dietary food supplements. Regarding their addition to infant formula, the European Commission's Scientific Committee on Food concluded in 2003 that they had no major concerns regarding the addition of oligosaccharides to infant formulas, including follow-up infant formulas (formulas modified especially for 6- to 12-month-old infants), up to a total concentration of 0.8 g/dL in ready-to-feed formula products.

Few RCTs have examined the effects of adding prebiotic oligosaccharides to infant formula [107,108]. Boehm et al [109], studied the effect of the addition of oligosaccharides at a concentration of 1 g/dL to preterm infant formula for 1 month (90% GOSs and 10% FOSs). Stool bifidobacteria counts in the oligosaccharide-supplemented group increased significantly compared with the nonsupplemented group, and the bifidobacteria counts reached the range of a breastfed reference group. In a separate study, Moro et al fed term infants the same oligosaccharide-supplemented formula. These infants had higher counts of bifidobacteria as well as lactobacilli in their stools. Schmelzle et al conducted a multicenter trial that also examined the efficacy of the addition of prebiotics to infant formula. They reported good overall tolerance and no adverse effects during the 12-week study period. A large multicenter trial to evaluate the safety of FOS-supplemented infant formula was conducted in the United States in 2004 [110]. The study demonstrated that infant growth was maintained during the 12-week study period for the FOS-supplemented infant-formula group without any adverse effects. After weaning infants from formula, the addition of prebiotics to solid food seems to have a bifidogenic effect, as shown by the results of a recently published RCT by Scholtens et al. Infant formulas that contain either GOS or FOS are now marketed in the United States. However, more information, including data from RCTs, is needed before the efficacy of adding prebiotics to infant formulas can be determined.

Probiotics

Two infant formulas currently contain a probiotic. One contains *B lactis*, and the other contains LGG. These probiotics are only added to powdered formulas at present. The rationale for adding probiotic organisms to infant formula was discussed

in the introduction of this clinical report. The overall health-benefit efficacy of adding probiotics to infant formula remains to be demonstrated in large RCTs.

Safety of Probiotics and Prebiotics in Infants and Children

Concerns exist about the overall safety of administering probiotic products to high-risk patient groups, including adults, children, and term and preterm infants. Cases of serious infection have occurred and been reported in the literature [111,112]. Patients at risk would be those who are immunocompromised, including ill preterm neonates, and/or children who have intravenous catheters or other indwelling medical devices. In most cases, the offending organism that caused the sepsis seems to have stemmed from bacteria from the individual's own endogenous flora. Sepsis has also been reported in adults, children, and infants who received probiotic supplements [113,114]. Land et al recently reported LGG probiotic sepsis occurring in immunocompromised infants and children. A medically fragile infant 6 weeks of age became septic with a strain of LGG that was being provided as a supplement. Molecular DNA-fingerprinting confirmed that the LGG probiotic supplement was the bacterial isolate from the infant. Neonatal sepsis and meningitis that were apparently associated with the administration of a probiotic supplement were also reported.

A recent report focused on probiotic tolerance and safety in healthy term infants who were randomly assigned to be given a high-dose probiotic formula, a low-dose probiotic formula, or control formula for 18 months [115]. There were no apparent reported adverse events. All infants demonstrated normal growth. Reports of colic were significantly fewer in the 2 probiotic-formula-fed groups, and the frequency of health care visits and antibiotic use was less ($P < .001$) compared with those in the control formula group. In a separate study, Petschow et al reported that healthy term infants given varying amounts of LGG in infant formula for 2 weeks resulted in good overall feeding tolerance with successful intestinal tract colonization, without adverse events.

Summary on Safety

The Committee on Nutrition of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition previously concluded that more studies are required to establish the safety and efficacy of probiotic and prebiotic products in children [116]. To date, these products seem to be safe for healthy infants and children. The committee also stated that it would be optimal to have a centralized mechanism of oversight to ensure probiotic microorganism safety, identity, and genetic stability [116]. Centralized oversight and probiotic product monitoring were also recommended in a report from the Food and Agriculture Organization of the United Nations World Health Organization. This organization supports the addition of prebiotic products to infant formulas designed as follow-up formulas meant for infants aged 5 months and older. It was reasoned that these infants are more likely to have a more mature immune response and established intestinal colonization. In terms of oversight and product safety in the United States, products marketed as dietary supplements, such as probiotics, do not require premarket review and approval by the US Food and Drug Administration (FDA). However, probiotics or prebiotics that are marketed specifically for the treatment or prevention of a disease are classified as biological products and do require FDA review and approval. Infant formulas must be made with

compliance with what are considered good manufacturing practices under the Infant Formula Act of 1980 and are under the regulatory auspices of the FDA because these products are often used as the sole source of nutrition by infants during a critical period of growth and development. Additional statutory and regulatory requirements address appropriate infant formula manufacture, composition, and nutrient content. All ingredients used in infant formula must be safe and lawful—that is, food ingredients that are, to date, generally regarded as safe (GRAS) for use in infant formula and those that are used in accordance with the food-additive regulations of the FDA. Prebiotics and probiotics now being added to commercial infant formulas are classified as GRAS. Information on FDA regulations for infant formula and food ingredients and packaging may be found at www.fda.gov/Food/FoodSafety/Product-SpecificInformation/InfantFormula/default.htm and www.fda.gov/Food/FoodIngredientsPackaging/default.htm.

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