Biotics in Infant or Follow-On Formulae: A Position Paper by the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Special Interest Group on Gut Microbiota & Modifications

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ABSTRACT

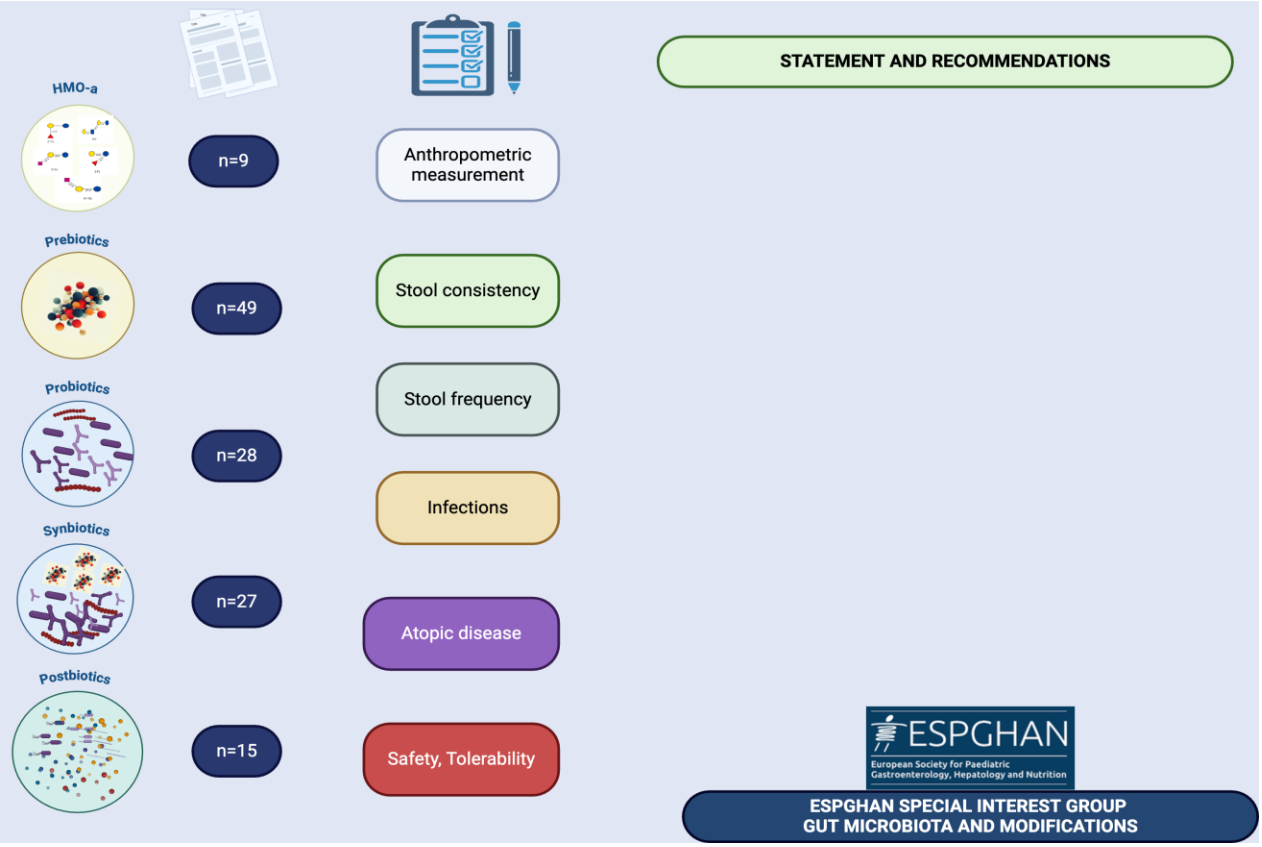
Breastfed infants generally have better health outcomes than those who are formula-fed, partly due to differences in their gut microbiota. For many years, biotics have been added to infant formula in an effort to reduce differences in gut microbiota composition and, ultimately, to enhance the health outcomes of formula-fed infants.

To review and update the evidence on biotic-supplemented infant formula, the Special Interest Group on Gut Microbiota and Modifications (SIG-GMM) of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) evaluated the clinical outcomes of infant formula supplemented with probiotics, prebiotics, synbiotics, postbiotics, and human milk oligosaccharides-analogues (HMO-analogues). Our focus was on safety, tolerability, growth and clinical health outcomes. Recommendations were formulated only when at least two well-designed randomized controlled trials (RCTs) evaluating similar biotics were available. If only one RCT was available, no recommendation was made, regardless of whether a benefit was demonstrated. A modified Delphi process was used to establish consensus on the statements. Additionally, we discuss the limitations of the current evidence and identify research gaps. This document is supported by separately published technical reviews, providing a detailed synthesis of the evidence from which these recommendations were formulated.

The ESPGHAN SIG-GMM concludes that the currently evaluated infant formulas supplemented with probiotics, prebiotics, synbiotics, postbiotics and HMO-analogues for healthy infants do not raise safety concerns regarding growth, tolerance and adverse effects. While some beneficial clinical effects are possible, there is currently no robust evidence to strongly recommend or discourage their routine use. This conclusion may reflect the limited data on specific biotics and outcomes rather than an actual lack of effect. To strengthen conclusions and formulate evidence-based recommendations, at least two independent high quality and adequately powered studies with a similar design and methodology should be performed. A major limitation is the heterogeneity of the RCTs. Due to differences in interventions (e.g., duration, amount, composition), inclusion criteria, and primary and secondary outcomes, no recommendations can be made "in favor" or "against" the biotic interventions evaluated so far, except prebiotics have been shown to soften stools by reducing stool consistency.

Key words: breastfeeding, prebiotic, probiotic, synbiotic, postbiotic, human milk oligosaccharide, HMO, human milk oligosaccharide-analogue, infant formula, health outcome

GRAPHICAL ABSTRACT



INTRODUCTION

Exclusive breastfeeding is the gold standard for optimal nutrition in all infants, especially during the first six months of life. The World Health Organization recommends that infants initiate breastfeeding within the first hour after birth and be exclusively breastfed for the first 6 months of life [1]. When breastfeeding is not possible, infant formula (IF) is the second nutritional choice for infants. It has been known for many years that the gut microbiota composition of breastfed infants differs substantially from that of infants fed formula not supplemented with biotics [1-4]. The rationale for adding biotics to IF stems from the understanding that the gut microbiota is associated with health outcomes [4]. This manuscript aims to summarize to which extent current biotic-supplemented IF interventions to research if the establishment of a beneficial microbial community in infants are of any clinically relevant benefit.

Previously, between 2004 and 2011, the Committee on Nutrition (CoN) of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) assessed the safety, health effects, and clinical outcomes associated with biotic-supplemented formulas in several position papers [5-8]. In 2004, and again in 2011, the CoN of ESPGHAN systematically reviewed published evidence on the safety and health effects of formulas supplemented with probiotics and/or prebiotics compared to non-supplemented formulas [5,6,8]. In 2007, the CoN evaluated fermented infant formulas without live bacteria and concluded that the available data did not allow for general conclusions on its use and effects for infants[7]. Regarding probiotics, the most recent ESPGHAN-statement from 2011 concluded that the administration of the evaluated probiotic-supplemented formulas to healthy infants did not raise safety concerns regarding growth and adverse effects [8]. Although some beneficial clinical effects may be possible (e.g. support normal growth in healthy term infant, reduction of diarrhea), it was concluded that there was insufficient evidence to recommend their routine use [8]. Similarly, in 2011, the Committee concluded that the administration of prebiotic-supplemented formula to healthy infants did not raise safety concerns regarding growth and adverse effects [8]. However, the Committee did not endorse the routine use of prebiotic-supplemented formulas in infants due to a lack of evidence on significant clinically relevant benefits. The conclusion similar for synbiotic-supplemented infant formulas [8].

Following the CoN documents, new evidence on the effects of the supplementation of infant formula with various biotics has been published [9-18]. In more years, prebiotic oligosaccharides with structures identical to human milk oligosaccharides (HMOs) have also been added to infant formulas [19]. Given the importance of distinguishing between HMOs naturally found in human breast milk and those produced biotechnologically, which have an identical structure to the HMOs in breast milk but are not derived from it, the SIG proposes to use the term 'HMO-analogue(s)'. Biotechnological methods, including microbial fermentation using genetically engineered microorganisms such as *Escherichia coli* and yeast, enable the production of selected HMO-analogues, such as 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) [20]. Since 2016, 2'-FL and LNnT have been added to some infant formula, and more recently other HMO-a (e.g. 3'-FL, 3'-sialyllactose (SL) and 6'-SL) have also been incorporated [19].

Since 2011, a plethora of new information on infant gut microbiota composition and factors related to the microbiota composition during the first 1000 days of life has become available [21-23]. There is increasing research on nutritional postnatal interventions using biotics to promote the establishment of a beneficial microbiota, more closely related to that in breastfed infants [4, 9].

The aim of this position paper is to evaluate the available evidence on the effects of adding biotics to infant formula on safety, tolerability, growth, and health outcomes. It also discusses the overall conclusions from these studies and presents practical recommendations. Additionally, we highlight the limitations of the current evidence and identify research gaps.

METHODS

This position paper summarizes the technical reports that evaluated the different biotics in infant formula published in studies up to December 31, 2023 [24-28]. The SIG-GMM conducted five systematic reviews [24-28] to evaluate the safety and efficacy of infant formula supplemented with probiotics, prebiotics, synbiotics, postbiotics, and HMO-analogues with one priority research question: "*Should biotics be added to infant formula? If yes, which specific biotic and for which indications?*". There still is a need to define the beneficial health

effect of an intervention resulting in a change of the gut microbiota (as an example: the effect on stool by decreasing its consistency of prebiotics might as well be explained by an osmotic effect of the prebiotics and the short chain fatty acids).

Peer-reviewed randomized controlled trials (RCTs), meta-analyses, systematic reviews, and previous ESPGHAN recommendations have been used for these analyses. The reference lists from identified studies and key review articles, including previously published meta-analyses have been also evaluated. Only RCTs including healthy term-born infants < 1 year of age receiving infant formula were included. Only studies comparing infant formula supplemented with HMO-analogues, prebiotics, probiotics, synbiotics, and/or postbiotics during the manufacturing process with formulas without these additions were considered. Studies in which HMO-analogues, prebiotics, probiotics, synbiotics, and/or postbiotics were not introduced during the manufacturing process but administered thereafter were excluded. Formulas with partially or extensively hydrolyzed protein (pHF; eHF) were excluded as well. We excluded studies that dealt with preterm infants, cow's milk allergy (CMA) or any condition or disease.

The definitions of the biotics were those proposed by the International Scientific Association of Probiotics and Prebiotics (ISAPP) (Table 1) [29-32]. The SIG used the term 'HMO analogues' for biotechnologically produced HMOs identical to those found in human milk.

Table 1. Definitions of biotics [29-32]

Probiotic	Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host[29].
Prebiotic	A substrate that is selectively utilized by host microorganisms conferring a health benefit[30].
Human Milk Oligosaccharide analogue (HMO-analogue)	Biotechnologically produced HMOs that are identical to those found in human milk

Synbiotic	A mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host[31].
Postbiotic	Preparation of inanimate microorganisms and/or their components that confers a health benefit on the host[32].

An initial screening of the title, abstract, and keywords of every record identified was performed. The next step was the retrieval of the full texts of potentially relevant publications. At least two reviewers independently assessed the eligibility of each potentially relevant trial with the use of inclusion criteria.

Probiotics: Search terms included: "probiotic" or "Lactobacillus" or "Bifidobacterium" or "Streptococcus" and "formula or infant formula for infant nutrition". We found 1185 articles with our first search, and when we included only clinical trials, RCTs, and meta-analyses, we selected 239 publications. Among them, 85 publications were appropriate for the next evaluation (excluding trials in different age groups and not related to our topic). Upon reviewing the 85 publications based on our inclusion/exclusion criteria, we eliminated 57 publications. These exclusions were attributed to factors such as pHF, eHF, CMA formula, combination with prebiotics/postbiotics/HMO-analogues or milk formula globule membrane (MFGM), or studies conducted in specific populations such as infants with allergies or prematurity. Our final analysis included 28 publications [33-60].

Prebiotics: Search terms included: "prebiotic" or "oligosaccharide" or "*fructooligosaccharide*" or "*FOS*" or "*galactooligosaccharide*" or "*GOS*" and "formula or infant formula for infant nutrition". We found 2432 articles with our first search, and when we included only clinical trials, RCTs, and meta-analyses, we selected 365 publications. Among them, 273 publications were appropriate for the next evaluation (excluding trials in different age groups and not related to our topic). Upon reviewing the 273 publications based on our inclusion/exclusion criteria, we eliminated 229 publications. These exclusions were attributed

to factors such as pHF, eHF, CMA formula, combination with probiotics/postbiotics/HMO-analogues or MFGM, or studies conducted in specific populations such as infants with allergies or prematurity. Our final analysis included 49 publications [33, 34, 35, 61-106].

HMO-analogues: Search terms included: "human milk oligosaccharide" or "milk oligosaccharide" or "2'-fucosyllactose" or "2FL" or "lacto-N-neotetraose" or "LNnT" and "formula or infant formula for infant nutrition". We found 1321 articles with our first search, and when we included only clinical trials, RCTs, and meta-analyses, we selected 118 publications. Among them, 105 publications were appropriate for the next evaluation (excluding trials in different age groups and not related to our topic). Upon reviewing the 105 publications based on our inclusion/exclusion criteria, we eliminated 96 publications. These exclusions were attributed to factors such as pHF, eHF, CMA formula, combination with probiotics/prebiotics/postbiotics or MFGM, or studies conducted in specific populations such as infants with allergies or prematurity. Our final analysis included eight publications (six RCTs and two substudies) [107-114].

Synbiotics: Search terms included: "synbiotic" or "symbiotic" and "formula or infant formula for infant nutrition". We found 376 articles with our first search, and when we included only clinical trials, RCTs, and meta-analyses, we selected 83 publications. Among them, 65 publications were appropriate for the next evaluation (excluding trials in different age groups and not related to our topic). Upon reviewing the 65 publications based on our inclusion/exclusion criteria, we eliminated 38 publications. These exclusions were attributed to factors such as pHF, eHF, CMA formula, combination with postbiotics/HMO-analogues or MFGM, or studies conducted in specific populations such as infants with allergies or prematurity. Our final analysis included 16 publications [37, 69, 115-130].

Postbiotics: Search terms included: "infant formula," "follow-on formula," "non-supplemented formula," and "postbiotic" or "fermented". We found 1885 articles with our first search, and when we included only clinical trials, RCTs, and meta-analyses, we selected 1383 publications. Among them, 73 publications were appropriate for the next evaluation (excluding trials in different age groups and not related to our topic). Upon reviewing the 73 publications based on our inclusion/exclusion criteria, we eliminated 58 publications. These exclusions were attributed to factors such as pHF, eHF, CMA formula, combination with

probiotics/prebiotics/HMO-analogues or MFGM, or studies conducted in specific populations such as infants with allergies or prematurity. Our final analysis included 14 publications [131-144].

Our review focuses on the following outcomes (if available): anthropometric measurements, safety, tolerability, stool frequency, stool consistency, infantile colic, infections and use of antibiotics, and allergic disorders.

The Cochrane Collaboration's tool for assessing risk of bias was used, which includes the following criteria: adequacy of sequence generation; allocation concealment; blinding of participants, personnel and outcome assessors, incomplete outcome data are addressed. ESPGHAN SIG-GMM is reporting evidence and recommendations related to each specific biotics. Recommendations were formulated only if at least 2 well-designed RCTs were available. If there was only one RCT, regardless of whether benefit was shown, no recommendation was formulated. The modified Delphi process was used to establish consensus on the statements. Level of agreement is presented next to every statement/recommendation. The paper has been open for public consultation and has received feedback from ESPGHAN members.

EVIDENCE SUMMARY AND RECOMMENDATIONS

Probiotics

Twenty-eight publications that evaluated the effects of probiotic-supplemented infant formula were included in the technical report [24, 33-60]. The studies varied in probiotic strains, study design, and duration of intervention. The trials were mostly conducted in Western countries. We evaluated RCTs which investigated either *Bifidobacterium animalis ssp. lactis* CNCM I-3446 (B. lactis Bb12); B. lactis Bb12*+ Streptococus (S.) thermophilus*; *B. longum* BL999 *+ Lacticaseibacillus (Lc.,* formerly known as *Lactobacillus) rhamnosus LPR*, *Lactobacillus (L.) johnsonii La1 (La1); or Limisolactobacillus (Lim.,* formerly known as *Lactobacillus) reuteri* ATCC 55730 [24]. Our evaluation demonstrated that probiotic-supplemented formulas were well tolerated, with no significant differences in growth parameters compared to non-supplemented formulas. Some evidence suggested potential benefits in reducing gastrointestinal and respiratory infections, though these findings were inconsistent and of varying quality. Certain strains were associated with a reduction in episodes of colic, the

number of days with fever and the use of antibiotics [24]. However, there was considerable heterogeneity, which reduced the level of certainty of effect.

Despite the theoretical safety concern regarding the addition of living microorganisms to formula, no severe safety concerns were reported in presumed healthy term-born infants. Overall, reports indicated that the addition of probiotics to IF was well tolerated and led to normal growth. However, due to different designs, different inclusion criteria, different amounts and compositions of probiotics, and different inclusion criteria for primary as well as secondary outcomes, no firm conclusions can be drawn regarding clinical benefits, and no recommendations can be formulated regarding relevant clinical benefits (Table 2-3).

Prebiotics

The most common prebiotic components of non-human origin added to infant formula are non-digestible carbohydrates such as fructans or glucans. The prebiotics in the considered RCTs were the following: a 9:1 mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS), acidic oligosaccharides (AOS) from hydrolyzed pectin, scGOS/lcFOS together with AOS, scGOS/scFOS; a mixture of scGOS and short-chain fructo-oligosaccharides (scFOS), oligofructose enriched inulin, polydextrose and galacto-oligosaccharides (PDX/GOS, 1:1 ratio) or PDX/GOS and lactulose (LOS) (PDX/GOS/LOS, 3:2:1 ratio), galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and bovine milk-derived oligosaccharides (BMOS) [25]. Overall, the addition of non-human prebiotic oligosaccharides to infant formula has repeatedly been concluded to be well tolerated and associated with length and weight gain within average percentiles as already shown for infant formulas. Therefore, these components are regarded to be safe. The strength of evidence and generalizability of long-term outcome data are limited.

While there are some beneficial effects on prevention of infections with GOS/FOS and GOS/PDX combinations, the effects have not been shown with two RCTs with same combination [25, 145]. The combination of scGOS/lcFOS (9:1) is the most frequently investigated prebiotic product in formulas. It was studied as a supplement to intact protein formula at concentrations from 4 to 8 g/L. Supplementation of infant formula with scGOS/lcFOS at a concentration of 8 g/L has been studied in 7 RCTs and may increase stool frequency in

presumed healthy infants [25]. Supplementation of infant formula with scGOS/lcFOS at a concentration of 4 g/L, neutral oligosaccharides (scGOS/lcFOS) together with acidic oligosaccharides (AOS), prebiotic blends of polydextrose and GOS (PDX/GOS, 1:1 ratio) or PDX/GOS and lactulose (LOS) (PDX/GOS/LOS, 3:2:1 ratio), GOS at a concentration of 4 to 5 g/L, and oligofructose enriched inulin supplemented to infant formula at a concentration of 8 g/L also reduced stool consistency in presumed healthy infants [25].

Based on the available evidence, the use of prebiotics such as scGOS/lcFOS, GOS, scFOS, oligofructose, and oligofructose-enriched inulin in infant formula primarily soften stools by reducing stool consistency in non-constipated infants, and, to a lesser extent, stool frequency in presumed healthy infants. These prebiotics have been shown to support adequate growth and are well tolerated (**Table 2-3**).

HMO-analogues

Although it has been known for more than 50 years that HMOs are the third most important component in human milk, it was only recently until it was discovered how to produce a certain number of oligosaccharides with an identical structure as those in mother's milk in sufficiently large amounts to allow commercialization [146]. Six RCTs and two mechanistic sub-studies met the inclusion criteria and investigated different combinations of HMO-analogues added to formula [26, 107-114]. The effects of HMO-analogues supplemented infant formula on growth and anthropometric outcomes were found to be adequate and comparable to control groups (breast feeding, infant formula, and infant formula supplemented with GOS) during an intervention period of four to six months. The HMO-analogues studied so far show no difference compared to control formula for outcomes such as regurgitation-related symptoms, crying, fussiness, or colic (ref technical report). A specific combination of 5 HMO-analogues (2'FL, 3'-FL, LNT, 3'-sialyllactose (SL) and 6'-SL) suggest a softer stool consistency and more frequent defecation in presumable healthy infants, but these studies also used the highest amount of HMO-analogues [26]. Regarding infection prevention, no clear conclusion can be drawn. Since HMO-analogues favors the development of a bifidogenic microbiota, which enhance the development of a balanced immune system, it was hypothesized that HMO-analogues may decrease the prevalence and severity of infections. However, up to now no study had "infection prevalence or severity" as the primary

outcome. No study reported an increase in the prevalence of infections. Some studies reported a statistically significant decrease in the prevalence of mainly respiratory tract infections [26]. There was no difference in tolerability and no safety concerns were raised with the HMO-analogues studied so far. Due to different designs, different inclusion criteria, different amounts and compositions of HMO-analogues, and inclusion criteria, in primary as well as secondary outcomes, no firm conclusions can be made regarding clinical benefits, and no recommendations can be formulated regarding relevant clinical benefits. Yet, the studies did demonstrate good tolerance and adequate, safe growth comparable to non-supplemented formula in presumed healthy infants (Table 2-3).

Synbiotics

The studies varied in terms of synbiotic composition, study design, intervention duration, and outcomes. The most commonly prebiotic component in synbiotic supplemented formula is represented by scGOS and lcFOS to infant formulas up to 0.8 g/100 mL. Formulas supplemented with synbiotics studied so far were well tolerated and showed no significant difference compared to the control formulas in growth parameters, gastrointestinal symptoms, and stool characteristics, or safety [27]. All studies reported good acceptability and tolerance, resulting in normal anthropometric data. Safety was confirmed in all trials as well. However, due to the large heterogeneity regarding synbiotics and study designs (including inclusion criteria, primary and secondary outcomes), it is not possible to formulate evidence-based recommendations. Even within the six RCTs performed with the same prebiotic mixture *of B. breve* M-16V and scGOS/lcFOS, the heterogeneity in study design and outcomes does not allow for the formulation of a recommendation [27] (Table 2-3).

Postbiotics

Postbiotics were added to infant formula, but always in combination with other formula changes, mostly the addition of other biotics as well [28]. The research primarily utilized strains like *B. breve* C50 *and Streptococcus thermophilus* 065 for fermentation, alongside various formula modifications such as the addition of prebiotics (scGOS/lcFOS in a 9:1 ratio) or several HMO-analogues, such as 2'-fucosyllactose (2'-FL) and 3’-Galactosyllactose (3’-GL). However, the latter is produced as a consequence of a fermentation process (so, the 3'-GL is

not "supplemented" to these formulas). The review found that IF-containing postbiotics are safe and well-tolerated by non-breastfed infants. Studies could not consistently demonstrate the clinical benefits of using these formulas, despite adequate growth and a number of adverse events comparable to those reported for the control formulas [28.] Despite the suggestion of some gastrointestinal benefits, the definitions of diarrhea and digestive symptoms were not uniform. Heterogeneity of formula compositions tested, differences in study designs, differences in primary outcomes, and definitions of these outcomes. Additionally, the lack of studies evaluating more than one specific postbiotic preparation makes it challenging to make firm recommendations (Table 2-3).

Table 2. Accepted statements related with biotics in infant formula [24-28]

Probiotics	In presumed healthy infants, formulas with added probiotic (*B. lactis Bb12, or B. lactis Bb12+ S. thermophilus, or B. longum BL999 + Lc. rhamnosus LPR, or L. johnsonii La1, or Lim. reuteri* ATCC 55730*)* have shown no differences in anthropometric parameters compared to non-supplemented formulas.
	Infant formula supplemented with *probiotics* (*B. lactis Bb12, or B. lactis Bb12+ + S. thermophilus,* or *B. longum* BL999 + *Lc. rhamnosus* LPR, or *L. johnsonii* La1, or *Lim. reuteri* ATCC 55730*)* was well tolerated and no difference in adverse effects was noticed during the study period with available studies.
	All studies confirmed the safety and tolerance of probiotic-supplemented formulas. However, no consistent clinical benefits were demonstrated in presumed healthy infants who received probiotic-supplemented formulas.
Prebiotics	Irrespective of the prebiotic(s) (combination) tested, the number of trials and number of infants included is limited, related to the huge variability of prebiotic combinations and dosages tested.

	Supplementation of standard infant formula with scGOS/lcFOS at a concentration of 4 g/L have been shown to soften stools by reducing stool consistency presumed healthy infants.
	No clinical health benefits have been reported for supplementation of standard infant formula with scGOS/lcFOS at a concentration of 6 g/L.
	Supplementation of infant formula with scGOS/lcFos at a concentration of 8 g/L may increase stool frequency in presumed healthy infants.
	Supplementation of standard infant formula with scGOS/lcFOS/AOS have been shown to soften stools by reducing stool consistency in non-constipated infants
	PDX/GOS at a concentration of 4 g/L have been shown to soften stools by reducing stool consistency in presumed healthy infants in 4 of 6 RCTs.
	Supplementation of infant formula with GOS at a concentration of 4 to 5 g/L have been shown to soften stools by reducing stool consistency.in softer stools in presumed healthy infants.
	Infant formula supplementation with GOS at a concentration of 2.4 to 5 g/L does not have a consistent significant effect on stool frequency in presumed healthy infants
	Infant formula supplementation with GOS at a concentration of 2.4 to 5 g/L does not prevent atopic manifestations, infections, or antibiotic use.
	Supplementation of infant formula with scFOS at a concentration of 2-5 g/L had no effect on stool frequency and stool consistency.
	Supplementation of infant formula with oligofructose at a concentration of 2-5 g/L had no effect on stool consistency have been shown to soften stools by reducing stool consistency.

	Supplementation of infant formula with scFOS or oligofructose at a concentration of 2-5 g/L did not prevent infections.
	Oligofructose enriched inulin supplemented to infant formula at a concentration of 8 g/L have been shown to soften stools by reducing stool consistency in presumed healthy infants.
	Oligofructose enriched inulin supplemented to infant formula at a concentration of 8 g/L did not affect stool frequency in presumed healthy infants.
	Oligofructose enriched inulin supplemented to infant formula at a concentration of 8 g/L does not prevent infections or infantile colic in presumed healthy infants.
	Oligofructose enriched inulin supplemented to infant formula at a concentration of 8 g/L did not significantly prevent crying in two randomized controlled trials in presumed healthy infants.
HMO-analogues	In presumed healthy infants, formulas with added HMO-analogues have shown no differences in anthropometric parameters compared to non-supplemented formulas.
	Healthy infant formulas with added HMO-analogues, studied so far, did not show a difference when compared to control formulas in regurgitation-related symptoms in healthy infants.
	The combination of 2'FL, 3-FL, LNT, 3'-SL and 6'-SL, suggests a softer stool consistency and more frequent defecation than in the non-supplemented formula control group in presumed healthy infants, although the clinical relevance for this remains uncertain. These studies used the highest amount of HMO-analogues.

	The HMO-analogues studied so far did not show a difference compared to the control group(s) in crying, fussiness or colic in presumed healthy infants.
	Considering the HMO-analogues studied so far, two studies suggest a decreased prevalence of infections and antibiotic use, while all others do not.
	Compared to non-supplemented formula, no difference in tolerability and no safety concerns were raised with the HMO-analogues studied so far.
	All studies confirmed safety and tolerance. However, no consistent clinical benefits were demonstrated in healthy infants who received HMO-analogues supplemented formulas.
Synbiotics	In presumed healthy infants, formulas with added synbiotics studied so far have shown no differences in anthropometric parameters compared to non-supplemented formulas.
	Supplementation of infant formula with synbiotics studied so far suggests softer stool consistency and more frequent defecation in presumed healthy infants compared to the non-supplemented formula control group. However, the clinical relevance for this remains uncertain and inconsistent among studies.
	The synbiotics studied so far did not show any difference compared to control group(s) in regurgitation, crying, fussiness, or colic in presumed healthy infants.
	There is insufficient data regarding a decreased prevalence of infections or reduced antibiotic use in infants receiving synbiotic-supplemented formulas studies so far.

	All studies confirmed the safety and tolerance of synbiotic-supplemented formulas. However, no consistent clinical benefits were demonstrated in healthy infants who received synbiotic-supplemented formulas.
Postbiotics	The postbiotics so far in the amounts studied so far did not result in a significant clinical benefit, but did show safety. Therefore, no recommendation for or against can be formulated.
	Infant formulas containing postbiotics, studied so far, in presumed healthy, non-exclusively breastfed infants, have not consistently demonstrated clinical benefits.

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464 **Table 3.** Recommendations of ESPGHAN Special Interest Group on Gut Microbiota &
465 Modifications about Biotics in Infant or Follow-On Formulae

Biotic	**Recommendation**	**Median / Mean**	**Votes**
Probiotic	Based on current evidence, the probiotics studied to date (*B. lactis Bb12, or B. lactis Bb12+ S. thermophilus, or B. longum BL999 + Lc. rhamnosus LPR, or L. johnsonii La1, or Lim. reuteri* ATCC 55730*)* have shown adequate growth outcomes, tolerance, and safety in healthy infants. However, at the tested doses, they have not demonstrated significant clinical benefits. Therefore, no specific recommendation for or against their routine use can be provided at this time.		

Prebiotic	Based on the available evidence, the use of prebiotics such as scGOS/lcFOS, GOS, scFOS, oligofructose, and oligofructose-enriched inulin in infant formula primarily soften stools by reducing stool consistency in non-constipated infants, and, to a lesser extent, stool frequency in presumed healthy infants. These prebiotics have been shown to support adequate growth and are well tolerated. However, no consistent clinical health benefits beyond these gastrointestinal effects have been observed, including prevention of infections, reduction of atopic manifestations, or decreased antibiotic use. Therefore, no specific recommendation for or against their routine use can be made at this time.		
HMO-analogues	Based on the current evidence, formulas supplemented with HMO-analogues, including combinations such as 2'FL, 3-FL, LNT, 3'-SL, and 6'-SL, do not show significant differences in growth (anthropometric parameters) or regurgitation-related symptoms compared to non-supplemented formulas in presumed healthy infants. These HMO-analogues may result in a softening of the stools by reducing stool consistency and increased stool frequency, particularly in higher doses; however, the clinical relevance of these effects remains uncertain. Given the limited specificity regarding the exact combinations and dosages of HMO-analogues, no specific recommendation for or against their routine use in healthy infants can be made at this time.		

Synbiotic	Based on current evidence, infant formulas supplemented with synbiotics, such as *B. breve* M-16V and scGOS/lcFOS, have shown good tolerance, safety, and acceptability in presumed healthy infants, with no significant differences in growth parameters, gastrointestinal symptoms, or stool characteristics compared to control formulas. However, due to the large variability in synbiotic composition, study design, intervention duration, and measured outcomes, there is insufficient evidence to formulate a recommendation for or against the routine use of these synbiotics for clinical benefits beyond safety and tolerance.		
Postbiotic	Infants formulas containing postbiotics that have been evaluated so far have shown to support adequate growth and are well tolerated. However, no consistent clinical health benefits have been observed. Therefore, no specific recommendation for or against their routine use can be made at this time.		

RESEARCH GAPS and CONCLUSIONS

In parallel with the innovations in the field of microbiota and biotics in the last 20 years, there have been many clinical trials published in which biotics have been added to infant formulas. None of the analyzed biotics would fulfill the ISAPP definition because of the absence of a proof of a clinically relevant "health effect". All RCTs show good acceptability, tolerance, safety, and the normal evolution of anthropometric parameters. Some studies suggested a possible effect on infection and allergy prevalence. Whenever studied, the RCTs with biotics show an effect on the gastrointestinal microbiota composition by stimulating the development of a bifidogenic microbiota and decreasing amounts of possible pathogens

(when measured) but microbiota composition was outside the scope of this position paper. Also, the methods of microbiota analysis in these studies vary [4, 10, 12, 17]. The clinical effects of "favorable" changes in microbiota composition should also be monitored. Whenever studied, all studies suggest that these microbiota changes are metabolically active by demonstrating increased levels of short-chain fatty acids such as butyrate (again outside the scope of this review).

The amount and/or composition of HMOs ingested by an exclusively breastfed baby may influence breastfeeding's clinical impact [19]. HMOs range in concentration from average 9–22 g/L in colostrum to average 8–19 g/L in mature milk, and 4–6 g/L after 6 months, the amount of HMO-analogues in formula obviously remains stable [146]. The amount added to the formula is much lower and varies between 0.2 and 1.0 g/L [19]. The minimal and/or optimal amount is not known, and dose-efficacy studies were not performed. Another difference is that HMOs are composed of both short-chain and long-chain (which are fermented in different gastrointestinal locations), while the HMO-analogues added to infant formula are only short-chain. It is not known if the absence of long-chain HMO-analogues is clinically relevant or not. While there is evidence that some specific strains of bifidobacteria only develop and grow in the presence of some HMOs, there is no data that suggests that these specific strains offer any clinically relevant benefit compared to other bifidobacteria [19, 146]. Since studies with HMO-analogues were mostly performed in Europe or other countries with a relatively low prevalence of infectious diseases [26], the question arises if the failure to find a clinically significant and consistent benefit regarding the prevalence of infections might not be related to the latter. Therefore, it would be of interest to evaluate the effect of HMO-analogues supplemented formula in developing countries with a high prevalence of infectious diseases.

According to ISAPP, a synergistic synbiotic is defined as a synbiotic in which the prebiotic substrate is designed to be selectively utilized by the co-administered microorganism(s) [31]. In contrast, a complementary synbiotic is a mixture composed of a probiotic strain combined with a prebiotic component that is designed to target autochthonous microorganisms (the resident microbiota) [31]. Regarding complementary synbiotics, the components must meet minimum criteria for the separate probiotic and prebiotic definitions. Therefore, to demonstrate the synergistic synbiotic effect, it would be

useful to include prebiotic-only and probiotic-only arms along with the synbiotic arm in the studies. Since many studies with infant formulas included more than one biotic component at the same time (HMO-analogues and/or probiotic, prebiotic OS and/or probiotic, postbiotic and/or prebiotic), it is difficult to evaluate the effect of each biotic independently.

One of the concerns is the survival of probiotics in infant formula if prepared according to the WHO recommendations to heat the water up to 70° C, which will definitively affect the survival of the probiotics [147]. However, ESPGHAN CoN did not recommend heating the water for potential effects on other components of infant formula.

Many studies on biotics in IFs are developed, designed and sponsored by industry, although this is done often in collaboration with independent clinical researchers. Industry often structures trials to maximize the likelihood of positive outcomes, focusing on primary outcomes relevant to its goals. There is frequent overreporting of (positive) secondary outcomes, even when the studies are underpowered to assess these parameters. The systematic reviews highlight the lack of independency and transparency in formula trials, with a high risk of bias. Only a small proportion of trials were conducted independent from formula companies. Regarding some prebiotic trials, the outcome of some "independent" studies were interesting and promising, but lack of blinding in these trials was a shortcoming. However, the SIG-GMM acknowledges the significant challenges in obtaining independent funding for such intervention trials [148].

Supplementation of IF with biotics has certainly brought infant formula composition and fecal microbiota composition closer to those of breastfeeding and is thus a step forward in bringing second-choice infant feeding closer to first-choice infant feeding. Studies about biotics in IF, show good acceptability, tolerance, safety, and the normal evolution of anthropometric parameters. Major limitations of the available information on biotic supplementation of infant formula are the lack of convincing clinical effects and if there were clinicla effects, the lack of replication. Therefore, further well-designed, longitudinal studies would help to address the use of biotics in infant formula.

A major limitation is the heterogeneity of RCTs evaluating the clinically relevant effects of any biotic supplemented with IF. There are differences in interventions (duration, amount, composition), inclusion criteria, and primary and secondary outcomes. Some studies reported a statistically significant decrease in the prevalence of mainly respiratory tract

infections; others reported the incidence of infection only as an adverse event. Therefore, the sample sizes were not calculated based on infections as primary outcomes. We recommend performing RCTs with the prevalence of infections, antibiotic use, atopic disease and allergy as primary outcomes. Additionally, reproducibility still is the core of recommendations. Therefore, replication of observed or reported effects by independent research teams is of fundamental importance. Formulation of robust recommendations requires at least two independent RCTs performed by different centers with identical study designs of high quality and with sufficient power. Recognizing that fully independent trials without industry sponsorship are often unfeasible due to high costs and logistical challenges, a balanced approach is necessary. This includes public-private partnerships, standardized protocols, independent monitoring, and full data transparency to mitigate potential industry znd research team bias while ensuring feasibility.

RESEARCH GAPS

- Multiple independent studies. To strengthen conclusions and formulate evidence-based recommendations, it is recommended that at least two independent RCTs of high quality with sufficient power and with the same design and biotic be conducted.
- Identification of important outcomes. Future research should prioritize scientifically and clinically relevant outcomes, ensuring they address infant health needs rather than industry-focused goals.
- Balanced reporting of secondary outcomes. Efforts are needed to ensure accurate reporting of secondary outcomes, considering the limitations of underpowered studies.
- Optimal dosing. Further research is necessary to determine the ideal dosage of biotic supplementation in infant formula, balancing efficacy and safety.
- Long-term effects. To understand the lasting impact of biotic supplementation in infant formula on infant health outcomes, including immune function, allergy development, and long-term GI health, studies with longer follow-ups are needed.
- Diverse populations. Research should include diverse geographic, socioeconomic, and genetic populations to ensure findings are generalizable and inclusive.

- Collaborative independent studies: Collaboration among regulatory bodies, academia, and industry is crucial to conduct unbiased studies. By pooling resources and expertise, these entities can ensure rigorous research that avoids conflicts of interest.
- Ethical considerations. Trials must safeguard breastfeeding practices, ensure informed parental consent, and uphold ethical standards in study design and execution.

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Supplementary Figure.