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Scientific evidence for health benefits of infant formula and follow-on formula with human-identical milk oligosaccharides and/or galactooligosaccharides is insufficient

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Manufacturers of infant formula and drinks for young children sometimes promote their products as containing human milk oligosaccharides (HMO). They claim, for example, that feeding babies with these products has a positive effect on their immune system and that infections are less likely to occur.

HMOs are polysaccharides with various structures that occur naturally in breastmilk. Whether HMO in breastmilk are of significance for the growth and development of the infant, and whether some of the positive health effects of breastfeeding can be explained by the presence of HMO in breastmilk is currently the subject of debate.

The German Federal Institute for Risk Assessment (BfR) has assessed the suitability of infant formula, follow-on formula and drinks for young children with oligosaccharides that are chemically and structurally identical to oligosaccharides occurring in human milk (HiMOs) used in products on the German market.

Result: To date, only few studies have investigated the effects of individual HiMOs and/or galactooligosaccharides (GOS) in healthy infants and young children. The data currently available do not provide any indications of adverse health effects. On the other hand, the data also offer no proof that infant formula or drinks for young children with added HiMO and/or GOS are more suitable than conventional products without such additions.

According to the opinion of the BfR, more data from controlled intervention studies are needed to derive reliable conclusions on the suitability and, in particular, the health benefits of infant formula or drinks for young children with HiMO and/or GOS that are currently offered on the market.

1 Subject of the assessment

The BfR has assessed the suitability of infant formula and follow-on formula with added oligosaccharides that are chemically and structurally identical to oligosaccharides occurring in human milk (human-identical milk oligosaccharides (HiMO) and/or galactooligosaccharides (GOS). According to a search in the internet conducted by the BfR, products with the following HiMOs were marketed in Germany as of December 2021 (see also table 1):

- 2'-fucosyllactose (2'-FL)
- Difucosyllactose (DFL)
- Lacto-N-tetraose (LNT)
- Lacto-N-neotetraose (LNnT)
- 6'-sialyllactose (6'-SL)
- 3'-sialyllactose (3'-SL)

In addition, the BfR has assessed the suitability of infant formula and follow-on formula with GOS, either alone or combined with 2'-FL, as well as of one follow-on formula containing 3'-

galactosyllactose (3'-GL) in combination with GOS and fructooligosaccharides (FOS). According to the manufacturer, 3'-GL is not added to that formula intentionally but is produced by fermentation with bacteria of the species *Bifidobacterium breve* and *Staphylococcus thermophilus*.

This assessment takes into account the study results and references of publications submitted by three manufacturers. Since for some of the HiMO only few studies were available as full-length-manuscripts, summaries of poster or conference abstracts have also been taken into account here. In addition, the BfR has also performed an extensive literature search (of German- and English-language articles up to December 2021). For the assessment of the biological plausibility of potential prebiotic and immunological or general health effects, results have been consulted both from *in vitro* and *ex vivo* studies, in addition to findings from animal studies and human studies in infants. Opinions published by the European Food Safety Authority (EFSA), which have assessed HiMO as novel food ingredients for use in infant formula, follow-on formula and drinks for young children, have also been taken into account (EFSA, 2015; EFSA, 2019 and EFSA, 2020).

As proof of the efficacy or expected benefits of products with HiMO and/or GOS, only positive results from placebo-controlled intervention studies in healthy, full-term infants (no premature infants or infants with health problems) or young children have been accepted.

Definitions

Prebiotics are defined as substances that are selectively fermented by microorganisms in the gut and lead to specific changes in the composition and/or activity of the gastrointestinal microbiota. As a result, they contribute to a health benefit for the individual (Gibson et al., 2004; Roberfroid et al., 2010; Hutkins et al., 2016).

Infant formula is a food intended for the specific nutrition of infants during the first months of life and, on its own, meets the nutritional requirements of these infants until the introduction of appropriate complementary foods. Infant formula is also suitable for infants after the introduction of appropriate complementary foods (from the fifth to the seventh month of life) as a liquid component of an increasingly diversified diet.

Follow-on formula refers to foods intended for the special nutrition of infants after the introduction of appropriate complementary foods and constitutes the largest, liquid element in a progressively diversified diet for these infants.

In the following opinion, the terms ‘infant formula’ and ‘follow-on formula’ are used depending on the study formula used in the studies assessed. Since some studies did not distinguish clearly between these terms, and since in practice there is no need to switch from infant formula to follow-on formula in the course of the first year of life, the general term ‘infant formula’ is used in all cases where a clear differentiation was not possible or necessary.

2 Results

The BfR has assessed the suitability of human-identical milk oligosaccharides (HiMO) in infant formula and follow-on formula (as defined in Article 3 of Delegated Regulation (EU) 2016/127), and in drinks for young children in relation to the expected benefits and potential adverse health effects.

In terms of the **expected benefits**, it can be concluded that the results from experimental

studies and human observational studies indicate that HiMO, as prebiotics, have positive effects on the composition of the intestinal microbiota, particularly in the case of infants delivered by Caesarean section. In addition, they also appear to contribute to the positive health effects observed in association with breastfeeding, via a variety of mechanisms. Available data also permit the conclusion that Galactooligosaccharides (GOS) stimulate the growth of bifidobacteria.

From the few intervention studies that have investigated effects of infant formula supplemented with 2'-FL and LNnT, however, no reliable inferences can be drawn about any beneficial effects on health that may be provided by these two HiMO in infant formula or drinks for young children marketed in Germany. The BfR is also unaware of any data from studies in humans that have investigated the effects on infant gut microbiota of an infant formula with a mixture of 2'-FL and GOS. To the best of the BfR's knowledge, there are also no human study data available on health effects of the other HiMO currently used in infant formula (DFL, LNT, 6'-SL and 3'-SL).

The study results indicate that fermented infant formula may have positive effects on the intestinal microbiota during the first four months of life and can stimulate the growth of bifidobacteria in infants. That having been said, the BfR is unaware of any studies that have investigated specific effects of fermented follow-on formula supplemented with 3'-GL and GOS/FOS – as available on the German market – on the gut microbiome of infants aged between six and 12 months.

Notwithstanding the above, the BfR does not consider a positive effect on bifidobacteria populations as satisfying the criteria for a health benefit. A health benefit could result from a situation where the number of pathogenic microorganisms in the intestinal tract is reduced and infectious diseases in particular and/or atopic disorders are proven to occur at a lower rate. However, there is insufficient evidence that individual HiMO and/or GOS in infant formula or drinks for young children are associated with clinically relevant health benefits.

In terms of immunomodulatory effects, it can be asserted as indisputable that breastfed infants are provided with vital immune functions via breastmilk. Since human milk oligosaccharides (HMOs) from breastmilk are able to bind to several immune receptors or to increase their expression, effects of HiMO on the infant immune system do seem plausible. According to published studies, however, the potential for benefits of HiMO is limited to their potential increase – or inhibition – of inflammatory cells and cytokine formation. Since the immune effects reported in the literature should be interpreted as indications of a potential, rather minor, beneficial effect on the immune response, any effect of individual HiMO should be considered to have limited modulatory impact. Also, no direct immune effect of 3'-GL alone or in combination with GOS/FOS can be derived from the available data.

To summarise, positive effects from individual HiMO on the development of the immune system of the infant appear plausible but have not been conclusively demonstrated. Accordingly, further controlled intervention studies are necessary, not only to assess the importance of HMO/HiMO and GOS, whether alone or in mixtures, for the gut microbiota and the development of the immune system, and to understand their impact on health, but also to demonstrate the suitability of infant formula or drinks for young children with HiMO with regard to the expected benefits.

With respect to safety considerations, the few intervention studies available on this matter lead to the conclusion that the use of 2'-FL and LNnT in infant formula is not associated with impairments to health and that infant growth is promoted in a manner comparable to that of

breastfed infants. Yet, there are no specific study data that also demonstrate that this is the case for infant formula and follow-on formula with 2'-FL and/or GOS.

To the best of the BfR's knowledge, only a single intervention study has been conducted with infants in the first four months of life, in which the effects of two infant formula products with differing concentrations of 2'-FL and GOS were investigated, compared with a third formula supplemented only with GOS. In the opinion of the BfR, the fact that the products utilised for this study were well tolerated and enabled normal growth cannot be used in support of an argument that this is therefore equally true for other infant formula or follow-on formula products on the market which have differing concentrations of 2'-FL and GOS.

In addition, although infant formula with GOS/FOS is generally well-tolerated, this fact cannot be used to assume that hydrolysed infant formula with GOS, as currently offered on the German market, will indeed be similarly well-tolerated.

Finally, study data on the effects of fermented follow-on formula containing 3'-GL and GOS/FOS are sparse. Accordingly, no reliable statement can therefore be made on health risks arising from the use of such products. On the other hand, available data do not suggest any real likelihood of impairments to health, not least because the technique of fermenting infant formula with L(+)-lactic acid-producing cultures has been accepted international practice for many years.

To date, the BfR has not become aware of any negative effects in relation to (fermented) infant formula or drinks for young children containing HiMO and/or GOS, which may be taken as an indication that health impairments as a result of the consumption of these products are indeed unlikely. On the other hand, there has been no systematic collection of data in Germany on potential adverse events connected with the use of these products. Accordingly, the BfR recommends performing clinical application studies and additional controlled intervention studies as necessary, to confirm that the products are well-tolerated by the target group of healthy infants and young children, and that impairments to health are not to be expected from their consumption.

3 Rationale

3.1 Properties and sources of HMOs and GOS

3.1.1 HMO

HMOs are formed in the mammary glands of breastfeeding women. To date, 200 different HMOs have been characterised: it is estimated that 50 of these account for 99 % of the oligosaccharides most commonly found in breastmilk (Austin et al., 2019; Smilowitz et al., 2014).

Common to all HMO is the fact that they contain lactose (milk sugar) at the reducing end (Bode and Jantscher-Krenn, 2012), which can be extended enzymatically to lacto-N-biose (galβ1-3GlcNAc) or n-acetyllactosamine (galβ1-4GlcNAc) by other disaccharides from galactose and n-acetylglucosamine. In addition, sialic acid and/or fucose may be present at the terminal positions of the HMO base structures (Bode, 2015). Accordingly, we may distinguish between three groups of HMOs: fucosylated (30–55 %), sialylated (12–14 %) and non-fucosylated neutral (42–55 %) oligosaccharides.

While HMO secretion patterns differ from one woman to another, they remain relatively constant during the entire breastfeeding period (Samuel et al., 2019). In contrast, the concentrations of individual HMO change during the course of breastfeeding and are subject to significant overall fluctuations. Colostrum contains an average of 0.9–2.2 g/100 ml HMO and therefore the highest overall concentration, followed by transitional milk, with an average of 0.8–1.9 g/100 ml and mature human milk, which contains 0.6–1.5 g/100 ml in the first month and up to 0.4–0.6 g/100 ml after six months of breastfeeding (Thum et al., 2021). Also, various HMO predominate at different phases of breastfeeding: for example, while the concentration of 3'-FL increases over the course of lactation, concentrations of most other HMO decline as breastfeeding progresses (Thum et al., 2021).

HMO concentrations and patterns differ depending on genetic, geographical and other environmental factors (Milani et al., 2017; McGuire et al., 2017). Some women, for example, lack the gene for expressing the FUT-2 enzyme and are therefore unable to form 2'-FL, being referred to in the literature as ‘secretor-negative’. Breastmilk from these women contains 35–45 % less HMOs (especially due to a lack of 2'-FL) (Azad et al., 2018). Roughly 75 % of European and American women are secretor-positive (Azad et al., 2018; McGuire et al., 2017). In this context, 2'-FL, with contents of 0.006 to 0.47 g/100 ml (Chaturvedi et al., 2001), accounts for the highest proportion of total HMO concentrations in most women, at around 30 % (Urashima et al., 2013).

Maternal obesity also appears to be associated with changes in HMO concentrations and these in turn with infant growth. In a study by Saben et al. (2021), for example, a positive correlation was observed between maternal BMI and the concentrations of LNnT, 3'-FL, 3'-SL and 6'-SL in breastmilk, as well as a negative correlation between BMI and disialyllacto-N-tetraose, disialyllacto-N-hexaose, fucodisialyllacto-N-hexaose and total acidic HMO concentrations in breastmilk. Also, intake of 3'-FL, 3'-SL and 6'-SL, as well as disialyllacto-N-tetraose, disialyllacto-N-hexaose and acidic HMO was positively associated with infant growth in the first six months of life (Saben et al., 2021).

Mank et al. (2020) note that there may be exceptions to the general HMO structure described, such as the presence of galactosyllactoses (GL), which may be present in breastmilk as 6'-GL, 3'-GL and 4'-GL.

To date, there is little information on the concentrations of 3'-GL in breastmilk. Data from 24 Japanese women (Sumiyoshi et al., 2004), whose milk was analysed for GL in the first 100 days (on days 4, 10, 30 and 100) *post partum* (pp) indicate that 6'-GL reaches a maximum concentration as early as day 4 pp, while concentrations of 3'-GL (the predominant GL in cow’s milk) and 4'-GL remained relatively stable over the breastfeeding period. Concentrations of 3'-GL were approximately 5 mg/l.

In a recent study, Eussen et al. (2021) conducted comprehensive analytical investigations on concentrations and the stability of GL over the lactation period and in relation to maternal secretor and Lewis phenotypes. For this purpose, in a subgroup of the PreventCD study population, samples of breastmilk from 24 Dutch women were collected monthly and for a period of up to one year. The samples were homogenised and the absolute concentrations of 6'-GL and 3'-GL were measured. The highest concentrations of 3'-GL, with mean values of 3.2 mg/l and 5.5 mg/l, were measured in the milk from women of the secretor and Lewis phenotype groups II and IV (in groups I and III, mean 3'-GL concentrations were 2.1 and 2.2 mg/l, respectively). Absolute concentrations of 3'-GL remained stable over the study period of 12 months. In months 8 and 9, however, values above the detection limit were measurable for only 2 of 171 breastmilk samples (1.2 %) of a single donor. Furthermore, in the overall group

of the PreventCD study, in which a total of 715 milk samples from 371 test subjects were analysed in the first four months of breastfeeding, it was shown that 3'-GL was detectable in more than 75 % of the milk samples analysed.

The results of Eussen et al. (2021) are considered reliable, even though only very few milk samples were available for analysis for months eight to 12. Also, for the stratification of the data according to secretor and Lewis phenotype groups in the individual test subject groups, in some cases, only very few samples from individual women were available. For example, in groups II and IV, in which the highest concentrations of 3'-GL were measured: samples from three women (21 samples) and one woman (9 samples), respectively.

The concentrations of 3'-GL in breastmilk determined by Eussen et al. (2021) and their stability over the breastfeeding period are comparable to findings by Sumiyoshi et al. (2004).

All in all, on the basis of the currently available data, relatively stable concentrations of 3'-GL in breastmilk between 2 and 6 mg/l can be assumed.

3.1.2 GOS

GOS are produced by transgalactosylation of lactose using a yeast- or bacteria-derived β-galactosidase, which produces linear β-1-4 and β-1-6 isomers, but also β-1-2 or β-1-3 isomers, depending on the enzyme source. Important components of HMO such as fucose, sialic acid (N-acetylneuraminic acid) or amino sugars (e. g. N-acetylglucosamine) are missing in GOS (Bode, 2012).

Although GOS – similar to human milk – contain galactose and lactose, they are structurally not comparable to the HMOs contained in breastmilk.

3.2 HiMO and/or GOS in infant formula and drinks for young children

The HiMOs produced for the purpose of being added to foodstuffs do not differ structurally from HMOs naturally occurring in breastmilk. However, when assessing the health benefits, it should be noted that breastfed infants receive a very individual and over time changing mix of HMOs through breastmilk.

HMOs differ structurally from the oligosaccharides contained in cow’s milk, among other things in that they are more fucosylated. HiMOs are also structurally not comparable with GOS and FOS, which are at times used as additives to infant formula.

The table below provides an overview of the infant formula and follow-on formula currently (as of December 2021) available in Germany, as well as drinks for young children with (mixtures of) HiMO and/or GOS. Details provided are taken from the list of ingredients and nutritional values provided online (please note that this summary does not claim to be complete) (table 1).

As can be seen from the table, the concentration of HiMO in infant formula products is only about 10 % of the average HMO concentrations measured in mature human breastmilk. In follow-on formula and drinks for young children, concentrations are as low as 2 to 5 % of those measured in breastmilk after six months of breastfeeding. In contrast, the levels of 3'-GL in infant formula are magnitudes higher than those detected in breastmilk (0.015 g/100 ml versus 0.0002 to 0.0006 g/100 ml).

Table 1: HiMO and GOS in infant/follow-on formula and drinks for young children on the German market.

Products	2'-FL	LNnT	LNT	DFL	3'-SL	6'-SL	3'-GL	GOS
	g/100 ml ready-to-consume product							
Infant formula [a)]	0.1	0.05						
Infant formula [a)]	0.1	0.03		0.01	0.004	0.02		
Infant formula [a)]	0.1		0.03	0.01	0.003	0.02		
Infant formula [a)]	0.1		0.04	0.01	0.01	0.02		
Infant formula	0.1							
Infant formula	0.1							0.3
Infant formula	0.11							0.4
Infant formula								0.5
Follow-on formula	0.03		0.01	0.004	0.001	0.005		
Follow-on formula	0.03		0.009	0.004	0.001	0.004		
Follow-on formula	0.03							
Follow-on formula	0.02							
Follow-on formula	0.025							
Follow-on formula	0.024							
Follow-on formula	0.04							0.4
Follow-on formula							0.015	0.72 [c)]
Drinks for young children [a)]	0.02		0.007	0.003	0.005	0.005		
Drinks for young children [b)]	0.025							
Drinks for young children	0.026							
Drinks for young children	0.03							

[a)] Based on hydrolysed whey protein

[b)] with *Lactobacillus reuteri* DSM17938 added in addition

[c)] In combination with 0.08 g/100 ml FOS

3.3 Suitability of infant formula supplemented with HiMO and GOS

According to Recital 6 of Commission Delegated Regulation (EU) 2016/127, ensuring innovation and product development should be possible by "the voluntary addition to infant formula and follow-on formula [during manufacture] of ingredients not covered by specific requirements of this Regulation. All ingredients used in the manufacture of infant formula and follow-on formula should be suitable for infants and their suitability should have been demonstrated, when necessary, by appropriate studies. It is the responsibility of food business operators to demonstrate such suitability and of national competent authorities to consider, on a case-by-case basis, whether this is the case. […]."

Article 3, para. 3 of the Commission Delegated Regulation specifies that the suitability of ingredients "shall be demonstrated by the food business operator through a systematic review of the available data relating to the expected benefits and to safety considerations as well as, where necessary, appropriate studies, performed following generally accepted expert guidance on the design and conduct of such studies."

3.3.1 Suitability of infant formula containing HiMO and/or GOS in relation to safety considerations

3.3.1.1 Infant and follow-on formula containing HiMO (2'-FL, LNnT, LNT, DFL, 6'-SL and/or 3'-SL, individually or in combination)

EFSA has evaluated several HiMOs (2'-FL, LNnT, LNT, DFL, 6'-SL and 3'-SL, individually and partly in combination) as novel ingredients intended for use in infant formula and follow-on formula. According to this assessment, there are no health concerns in relation to the use of a mixture of LNnT and 2'-FL – up to 0.06 g/100 ml LNnT and up to 0.12 g/100 ml 2'-FL, in a 1:2 ratio – in these products or in relation to their use, individually or in combination, in drinks for young children (EFSA, 2015 a and b; table 2). EFSA has also classified a mixture of up to 0.16 g/100 ml 2'-FL and DFL in infant formula and up to 0.12 g/100 ml each in follow-on formula and drinks for young children as safe for health (EFSA, 2019). On this basis, the addition of 2'-FL together with LNnT was authorised in the EU by Commission Implementing Decision (EU) 2016/376 and the addition of 2'-FL together with DFL was authorised by Commission Implementing Decision (EU) 2019/1979, in each case with the conditions of use indicated in table 2, for infant formula, follow-on formula and drinks for young children.

In addition, EFSA has also assessed the use of LNT as well as 6'-SL and 3'-SL for use in infant formula, follow-on formula and drinks for young children, and has not expressed any health concerns up to the concentrations indicated in table 2. These HiMOs are now also approved in the EU for use in infant formula, follow-on formula and drinks for young children (CID (EU) 2021/82; CID 2021/96; CID 2020/484).

Table 2: HiMOs assessed by EFSA (NDA Panel) as safe for addition to infant formula, follow-on formula and in drinks for young children over one year of age

HiMO	Infant formula	Follow-on formula	Drinks for young children	References
	g/100 ml ready-to-consume product			
LNnT and 2'-FL	0.06 and 0.12	0.06 and 0.12	0.06 and 0.12	EFSA, 2015a/2015b
LNT	0.08	0.06	0.06	EFSA, 2019a
2'-FL and DFL (as mixture)	0.16 (of mixture)	0.12 (of mixture)	0.12 (of mixture)	EFSA, 2019b
6'-SL	0.04	0.03	0.03	EFSA, 2020a
3'-SL	0.02	0.015	0.015	EFSA, 2020b

3.3.1.2 Infant formula with GOS, alone or in combination with 2'-FL

In the early 2000s, the former Scientific Committee on Food (SCF) of the EU Commission assessed the safety of infant formula and follow-on formula containing a mixture of GOS and FOS in a 9:1 ratio and up to a total concentration of 0.8 g/100 ml and observed no indications of any negative health effects, but also no evidence for any potential positive effects (SCF, 2001; SCF, 2003). Since this date, this additive has been permitted in the EU (Delegated Regulation (EU) 2016/127). As a result, there is a basic, accepted maximum concentration of GOS in combination with FOS in infant formula and follow-on formula of 0.72 g/100 ml.

An EFSA opinion published in 2014, looking at the potential use of prebiotics in infant formula, advised that, according to an assessment from the SCF (2001), GOS and FOS used as additives in infant formula or follow-on formula are harmless to health. In contrast, the safety of products with other non-digestible oligosaccharides or novel mixtures of non-digestible oligosaccharides must be proven in clinical studies for each specific product (EFSA,

2014). It follows that, in the absence of further evidence, the generally good tolerability of infant formula and follow-on formula with GOS/FOS cannot be used to conclude that an infant formula with the addition of GOS alone or with GOS/FOS and 3'-GL is comparably well-tolerated and poses no risk to health.

a) Infant formula with 100 % GOS

Three clinical studies were identified in which infants were given infant formula or follow-on formula with added GOS:

Ashley et al. (2012) report on a double-blind, placebo-controlled multi-centre study involving a total of 426 healthy, full-term infants. In the first four months of life, these infants received either:

a) Study formula 1: Infant formula containing 0.4 g/100 ml GOS;
b) Study formula 2: Infant formula containing a mixture of 0.4 g/100 ml GOS and polydextrose (PXD);
c) Standard infant formula without such additives (control group).

The primary objective of the study was to determine anthropometric parameters (body weight, length and head circumference) of the infants at baseline (14 days pp) and after 30, 60 and 120 days. As secondary parameters, further anthropometric measures and food tolerance as well as any adverse events that occurred were recorded. For this purpose, parents kept a diary of food intake, food tolerance (measured by signs of restlessness and flatulence) and stool characteristics (frequency and consistency) during the first 14 days and 24 hours before each of the three follow-up check-ups. In both the control and GOS/PXD groups, about 30 % dropouts (participants who left the study prematurely) were recorded by the end of the intervention; in the GOS group this figure was as high as 35 %.

The evaluation of the anthropometric data showed no significant differences between the three study groups. Moreover, in both intervention groups, higher stool frequencies in the first 60 days and consistently more frequent very soft, shapeless or even watery stools were observed. Severe adverse effects occurred in 21 infants (seven of them in the control group, four in the GOS/PXD group and ten in the GOS group), which, however, were assessed by Ashley et al. (2012) as not being associated with the study formula. The study authors concluded that the infant formula products containing GOS/PXD or GOS resulted in adequate growth of the infants in the first months and that the stool characteristics of infants fed on this were closer to those of breastfed infants (Ashley et al., 2012).

From the BfR's point of view, this conclusion is not justified for the following reasons:

1. The drop-out rate was high in all three groups, but especially high in the GOS group (30 % and 35 %, respectively). This limits the validity of the data analysis conducted according to the study protocol.
2. It is unclear to what extent the study formula was consumed by the infants. (Ashley et al. (2012) considered a single instance of consumption as sufficient.)
3. The study did not include a reference group of breastfed infants with which the findings could have been compared. Regardless of this, from the BfR's point of view, the very soft or even watery stools observed more frequently in both intervention groups should rather be seen as undesirable effects and not as an approximation to characteristics of breastfed infants.

A further placebo-controlled, multi-centre study investigated the effects of infant formula with

0.44 g/100 ml GOS and follow-on formula with 0.5 g/100 ml GOS on the microbiota and the frequency of infections and allergies during the first year of life. Again, stool frequency was significantly higher in the GOS group, and soft as well as watery stools were observed more often (Sierra et al., 2015).

In the third study, Fanaro et al. (2009) conducted a randomised, placebo-controlled, multicentre study with 159 healthy infants aged between four and six months who were not (or no longer) breastfed. Over a period of 18 weeks, the infants received either a standard follow-on formula with 0.5 g/100 ml maltodextrin (control group; n = 82) or a follow-on formula with 0.5 g/100 ml GOS (intervention group; n = 77). The parents were asked to document the following aspects in a diary:

- Food intake (at least 230 ml/day or 1.15 g GOS/day in the intervention group)
- Point in time of introduction of solid food
- Stool frequency and consistency
- Signs of regurgitation (reflux of milk from the stomach into the oral cavity), vomiting, bloating and other health problems.

Infants were examined at the beginning of the study and after six and 18 weeks. Anthropometric measurements (weight and length) were taken and urine osmolarity (to assess water and electrolyte balance) was determined in the first follow-up examination. In addition, microbiological stool analyses were carried out in some of the children. A total of 115 children (GOS: n = 56, control: n = 59) completed the study (drop-out: 28 %).

The analysis of the anthropometric data showed no significant differences between the study groups, neither at baseline nor after six weeks (data from 144 children) or 18 weeks (data from 130 children). While no differences were identified in mean stool frequency (determined within a subgroup of 88 infants), stool consistency was significantly softer in the GOS group. However, none of the infants had watery stools. Finally, no differences were observed in urine osmolarity or in the frequency of symptoms such as crying, regurgitation, vomiting and bloating.

In summary, it can be stated that, to the best of the BfR's knowledge, there are only a few intervention studies that have investigated the effects of infant formula and/or follow-on formula with 100 % GOS. In most studies, the infant formula investigated contained GOS and FOS in a ratio of 9:1, as currently approved in the EU.

The results of Fanaro et al. (2009) can be taken as evidence that no adverse health effects are to be expected when consuming a follow-on formula with GOS alone. In contrast, the results of Ashley et al. (2012) do not seem suitable to prove that no negative health effects can occur with the use of infant formula with the addition of GOS alone, in view of the study's methodological deficits, the high dropout rate and the findings of the stool characteristics, some of which can be considered as undesirable. Nor can any conclusions be drawn from these data for an infant formula based on hydrolysed whey protein and the addition of 0.5 g/100 ml GOS.

The study results are not transferable to infant formula and follow-on formula containing GOS as available on the German market. Accordingly, there are knowledge gaps regarding the effects of infant formula with added GOS on growth and other health parameters (body composition, nutrient bioavailability, water balance, urine production and urine osmolarity) in infants.

The BfR is not aware of the date on which infant formula with addition of GOS was first placed on the market in Germany. To date, no negative effects have been reported in association with these products. This can be interpreted as an indication that negative health effects from the consumption of these products are unlikely. On the other hand, there is no systematic collection of data on potential adverse events associated with the consumption of foodstuffs in this country. Overall, no reliable statements can be made on the tolerability and health risks of infant formula with GOS.

b) Infant formula with 2'-FL and GOS

The BfR is aware of a controlled, multi-centre study in which the effects on infant growth as well as the absorption of 2'-FL in healthy infants were investigated by administering infant formula with 2'-FL and GOS in comparison to formula with GOS alone (Marriage et al., 2015). A total of 420 infants were recruited for the study, of which 106 were breastfed (reference group) and the others split into three groups, each fed with one of the following formulae from the fifth day of life:

a) Infant formula based on cow's milk with 0.24 g/100 ml GOS (n = 101; GOS group)
b) Study formula 1, with 0.22 g/100 ml GOS and 0.02 g/100 ml 2'-FL (n = 104)
c) Study formula 2, with 0.14 g/100 ml GOS and 0.1 g/100 ml 2'-FL (n = 109)

In each case, the total oligosaccharides content was 0.24 g/100 ml. The products were all adjusted to an energy density comparable to breastmilk, namely 64 kcal/100 ml.

The primary endpoint was weight gain from day 14 to 119. Further anthropometric parameters and data on the tolerability of the formulae were collected as secondary endpoints. The authors also intended to investigate immunological and prebiotic effects associated with 2'-FL. The infants were examined at baseline and at five further time points (at an age of 14, 28, 42, 84 and 119 days). In a subgroup, blood, urine and stool samples were taken at 42 and 119 days of age.

Of the infants initially enrolled in the study, 338 participated until the end, of which 304 had been fed exclusively one of the study formulas or breastmilk (drop-out rate in the three infant formula groups: 30 to 34 %). The data were analysed as *per-protocol*:
Marriage et al. (2015) report that the study formula with 2'-FL were well accepted. The relative intakes and excretions of 2'-FL in infants fed the study formula were comparable to those of breastfed infants, especially in the case of study formula 2. No significant differences in growth (weight, length and head circumference) were observed between the four groups over the entire intervention period. Mean stool frequency was significantly higher in breastfed infants and spitting up or vomiting within an hour of being fed was significantly less frequent than in the three formula groups. In addition, stools of breastfed infants were softer on average than those of non-breastfed infants, although the three formula groups did not differ significantly on this point either.

Adverse or severe adverse effects were equally observed in the three formula groups, with a significantly higher incidence of 'infections and other diseases' such as upper respiratory tract diseases, otitis media, viral infections and oral thrush (mouth fungus) in the GOS group and intervention group 2 (with 0.14 g GOS and 0.1 g 2'-FL per 100 ml). Furthermore, cases of eczema were reported significantly more often in the GOS group. Nevertheless, the study formulae were well tolerated overall, according to Marriage et al. (2015).

Deficits of the study include the high drop-out rate of over 30 % and the lack of a ‘real’ control group receiving a standard infant formula. Regardless of this, the results of Marriage et al. (2015) are not transferable to the infant formula products marketed in Germany. Although the 2'-FL contents in the infant formulas on the German market are comparable to those in the products of Marriage et al. (2015), the study products each contained only a total of 0.24 g oligosaccharides per 100 ml per ready-to-consume product, while the total contents in the products on the German market range from 0.4 to 0.5 g/100 ml. In view of this, the effects observed by Marriage et al. (2015) are not transferable to products marketed in this country.

In summary, there are gaps in our knowledge in terms of the effects of the infant formulas and follow-on formulas with 2'-FL and/or GOS available on the German market on growth and other health parameters (body composition, nutrient bioavailability, water balance, urine production and urine osmolarity) in infants. Therefore, as of this writing, from the BfR’s point of view, no reliable statements can be made about the risk of undesirable health effects from such foods.

The BfR does not know how long the products have been on the market in Germany. To date, no negative effects have been reported in association with these products. This can be interpreted as an indication that adverse health effects from the consumption of the foodstuffs are unlikely. However, there has been no systematic collection of data in this country on potential adverse events occurring in this context.

c) Fermented infant formula with 3'-GL and GOS/FOS

The use of GOS/FOS in a 9:1 ratio up to a total concentration of 0.8 g/100 ml in infant formula and follow-on formula has been permitted in the EU for many years and is regulated by Delegated Regulation (EU) 2016/127.

While infant formula products with GOS/FOS are generally known to be well tolerated, this cannot simply be transferred to products containing GOS/FOS and 3'-GL. Also, in contrast to other (human) oligosaccharides, no scientific opinion on the safety of 3'-GL has been issued by EFSA as of this writing.

According to statements from manufacturers, 3'-GL is not actively added to infant formula, but is produced as a result of a special fermentation process (Lactofidus) that utilises bacteria of the species *Bifidobacterium breve* and *Staphylococcus thermophilus*.

The practice of fermenting infant formula with L(+)-lactic acid-producing cultures has been standard for infant formula in the Codex Alimentarius (Codex Alimentarius, 2007) for many years and is therefore accepted internationally.

Data from human studies indicate that no adverse health effects are to be expected from (partially) fermented infant formula with added GOS/FOS in the concentrations typically used to date. In intervention studies by Huet et al. (2016), Rodiguez-Herrera et al. (2019), Vandenplas et al. (2017; 2020) and Béghin et al. (2021), in which infants were fed a formula with fermented infant formula with added GOS/FOS during the first 17 weeks of life, compared to standard infant formula, good tolerability of the study formulae, adequate growth of the infants and in some cases softer stools were observed in the intervention groups. Similar findings were obtained in other intervention studies conducted by Vandenplas et al. (2014a), Herrera et al. (2015) and Rodriguez-Herrera et al. (2016 a and b), which were, however, only published as conference abstracts and therefore cannot be evaluated in detail.

It should be noted that the study formula used in the study by Vandenplas et al. (2020) was an infant formula, whereas the infant formulas with GOS/FOS and 3'-GL available on the German market are follow-on formulas. Furthermore, the study formula used by Vandenplas et al. (2020) also contained 2'-FL in addition to GOS/FOS and 3'-GL, and is therefore not comparable with the products on the German market.

To the knowledge of the BfR, there is only a single intervention study to date (Thibault et al., 2004), in which infants in the second half of life were fed a fermented follow-on formula in a placebo-controlled manner over a period of up to five months. Adequate infant growth was also observed in this study.

In summary, no reliable statements on the safety of fermented follow-on formula with GOS/FOS and 3'-GL can be made at present from the few data available. On the other hand, no specific safety concerns can be derived from the data either.

3.3.2 Suitability of infant formula with HiMO and/or GOS in terms of expected benefits

HMO are resistant to gastric acid and enzymatic hydrolysis in the gastrointestinal tract (Gnoth et al., 2000; Rudloff et al., 2006; Rudloff et al., 2012). As a result, they reach the distal small intestine and colon largely intact and influence the development of the intestinal microbiota. Alongside these prebiotic effects, HMOs are discussed in the context of antimicrobial, anti-adhesive and immunomodulatory properties (Bode, 2012; Davis et al., 2020; Milani et al., 2017; Rudloff und Kunz, 2015).

3.3.2.1 Influence of HMO and/or GOS as well as fermented infant formula with 3'-GL and GOS/FOS on the intestinal microbiota

a) HMO

Initial bacterial colonisation of the gastrointestinal tract occurs during and shortly after birth and is influenced by a number of perinatal factors such as mode of delivery and antibiotic administration. It is assumed that the composition of the intestinal microbiota influences the development of the immune system and the gastrointestinal tract as well as related physiological processes. A maldevelopment of the intestinal microbiota (dysbiosis) is discussed with an increased risk for immune-mediated disorders such as allergies and asthma, as well as for gastrointestinal and metabolic disorders (Dogra et al., 2020; Milani et al., 2017; Relman, 2012).

The composition of the microbiota is very diverse, however, and varies depending on both intrinsic and extrinsic factors. A large proportion of the variations between individuals cannot be explained by a single specific factor (Dogra et al., 2020). Nevertheless, in early infancy, a dominance of bifidobacteria or a change in the microbiota towards an increased number of bifidobacteria, resulting in the production of short-chain fatty acids, is generally considered to be beneficial to health (Fukuda et al., 2011; Gibson und Wang, 1994; Isolauri, 2001). One traditional assumption made is that breastfed infants exhibit lower phylogenetic diversity and a higher proportion of bifidobacteria in their stool than non-breastfed infants in the first few months of life.

However, studies conducted since the 1980s suggest that breastfed and non-breastfed infants differ less in terms of their relative proportions of bifidobacteria than in the distribution

and variability of individual bifidobacterial species (Adlerberth und Wold, 2009; Davis et al., 2017; Hao et al., 2019).

Moreover, differences in the distribution of other genera and species have been observed, such as the occasionally lower prevalence of *Clostridium difficile* and *Streptococci* in breastfed infants (review in: Davis et al., 2017). It is discussed that these differences, among other things, may be attributable to the HMO contained in breastmilk. For example, in one study conducted by Wang et al. (2015), comparable relative proportions of bifidobacteria were observed in breastfed and non-breastfed infants at the age of three months. Breastfed infants, on the other hand, had lower proportions of bacteria of the genus *Firmicutes* (especially species of *Clostridium* and *Streptococcus*) as well as higher proportions of *Bacteroides*. In addition, an association between certain bacterial genera/species and the prevalence of HMO in the breastmilk has been shown (Wang et al., 2015; De Leoz et al., 2015; Davis et al., 2016).

These and many other studies show differences in the bacterial colonisation of the intestine between breastfed and non-breastfed infants. The data suggest that the HMOs in breastmilk are of particular importance in this respect. In principle, it seems plausible and advantageous that

- a HMO-induced dominance of certain bifidobacteria species can displace other potentially harmful bacteria and that
- the short-chain fatty acids produced by bifidobacteria in the gut can create an environment that promotes the growth of commensal bacteria (Bode, 2012; Gibson and Wang, 1994).

HMO can promote the growth not only of bifidobacteria, but also of other bacteria genera such as *Bacteroides*, *Lactobacillaceae* and *Staphylococci* (Hunt et al., 2012; Salli et al., 2021; Yu et al., 2013).

The available study data suggest that 2'-FL can have very different effects on the microbiota from infant to infant. This should also be kept in mind when investigating and assessing infant formula with 2'-FL.

Lewis et al. (2015) found in the stool of infants breastfed by secretor-positive mothers compared to secretor-negative mothers:

- higher proportions of bifidobacteria, especially *B. longum ssp. longum and ssp. infantis,* and *Bacteroides*; and
- lower proportions of enterobacteria, clostridia and streptococci.

Lewis et al. (2015) explained this finding by higher concentrations of fucosylated HMOs, which include 2'-FL, in the milk of secretor-positive women. Furthermore, selection in favour of bifidobacteria was associated with increased concentrations of short-chain fatty acids in faeces (Lewis et al., 2015). Despite the relatively high concentration of 2'-FL in breastmilk, only a few bifidobacteria species/strains (especially *B. longum subsp. infantis* and *bifidum*) appear to be ablet to use 2'-FL as a substrate (Nogacka et al., 2021; Salli et al., 2019; Salli et al., 2021).

Puccio et al. (2017) investigated the faecal microbiota of infants aged three and 12 months (64 and 49 infants, respectively, in the intervention group, and 58 and 47 infants, respectively, in the control group compared to 35 and 30 breastfed infants of the same age) and observed significant differences in alpha diversity between the three groups. While variability in

bifidobacteria concentration was greater in the group of breastfed infants than in the HiMO group, the two groups did not differ in their median concentrations of bifidobacteria and *Escherichia*. Also, supplementation with HiMO had no significant effect on the relative abundances of individual bifidobacterial species and subspecies (Berger et al., 2020).

An analysis of the data stratified by delivery mode revealed that, compared to the other two groups, infants delivered by Caesarean section in the control group (infant formula without HiMO) exhibited the lowest median concentrations of bifidobacteria and the highest median concentrations of *Escherichia* at three months of age. The bifidobacteria concentration of Caesarean section-delivered infants in the HiMO group was comparable to that of infants delivered vaginally in the control group (Berger et al., 2020). This last finding can be interpreted as an indication of a positive influence of HiMO supplementation on the intestinal microbiota of infants delivered by Caesarean section. The study therefore offers initial evidence that an infant formula with 1 g/l 2'-FL and 0.5 g/l LNnT could have a favourable influence on the microbiota, especially in the case of infants delivered by Caesarean section. However, given serious methodological flaws and ambiguities in the recruitment and randomisation of the study group, these findings cannot serve as sufficient evidence for such effects. It is unclear, for example, how and from which of the two study centres – in Belgium and/or Italy – the control group of breastfed infants was recruited. Also, the ethnicity of the infants, alongside other potential confounders (sex, familial environment, etc.) was not taken into account in the evaluation of the microbiota data. The fact that other potential confounders were discussed neither by Puccio et al. (2017) nor Berger et al. (2020) weakens the quality and validity of the findings in both studies.

b) GOS

In the past, the BfR has repeatedly published opinions on the effects of GOS (with or without FOS) and acknowledged that GOS stimulates the growth of bifidobacteria. This has been demonstrated both *in vitro* (van Loo et al., 1999; Gibson and Collins, 1999; Sotoya et al., 2017) and in human studies in which infants were fed infant formula or follow-on formula with GOS at concentrations ranging from 0.2 to 0.44 g/100 ml and 0.5 g/100 ml, respectively (Ben et al., 2004; Ben et al., 2008; Fanaro et al., 2009; Giovanni et al., 2014; Sierra et al., 2015; Matsuki et al., 2016).

In some cases, higher concentrations of lactobacilli (Ben et al., 2008), lower concentrations of clostridia (Giovanni et al., 2014) and lower pH values were also measured in the faeces of the GOS-supplemented groups (Ben et al., 2004; Sierra et al., 2015).

The available data suggest that infant formula with added GOS is capable of stimulating the growth of bifidobacteria. However, the intake of bifidobacteria alone is not in itself beneficial for infants. Furthermore, it is unclear whether the effects are permanent or disappear after discontinuation of feeding.

From the trends observed concerning a bifidogenic effect of infant formula with GOS or from the predominantly *in vitro* or *ex vivo* observed effects of (individual) HiMOs on the intestinal microbiota, no conclusions can be drawn about potential effects of 2'-FL and GOS in infant formula or follow-on formula in the concentrations currently used on the intestinal microbiota of infants. It is known that HMO may have very different inter-individual effects on microbial colonisation. This should be taken into account in particular for infant formulas to which only individual HiMOs have been added.

Further studies are necessary in order to understand the importance of formula with individual HiMO such as 2'-FL in combination with other non-digestible oligosaccharides such as GOS for the development of gut microbiota and their effects on human health.

c) Fermented infant formula containing 3'-GL and GOS/FOS

In intervention studies, it has been observed that an infant formula fermented with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 had a positive effect on the composition of the intestinal flora and increased the bifidobacteria concentration in the infants' intestine (Tims et al., 2018 a and b[1]; Béghin et al., 2021). On the other hand, the BfR is unaware of any studies that have investigated specific effects of fermented follow-on formula with 3'-GL and GOS/FOS on the intestinal microbiome of infants in the second half year of life.

In summary, the prebiotic effects of HMOs have been substantiated by a large number of studies. In principle, it seems plausible and beneficial that

- a dominance of certain bifidobacteria species, as promoted by HMO, may displace other potentially harmful bacteria; and that
- the short-chain fatty acids produced by bifidobacteria in the gut can create an environment that promotes the growth of commensal bacteria (Bode, 2012; Gibson and Wang, 1994).

The effects are structure-specific, however, and not exclusively 'bifidogenic'. As a result, HMOs can have different effects on microbiota from one infant to another. This has to be considered in particular for infant formulas to which only individual HiMOs are added. Further studies are therefore necessary to understand the importance of individual HMO/HiMOs for the development of the gut microbiota and their effects on human health.

Furthermore, a reliable assessment of the effect of HMO/HiMOs and/or GOS on microbiota composition is complicated by the fact that not only the diet, but also to a large extent other factors have an impact on intestinal bacterial colonisation (see e.g. Blekhman et al., 2015; Bokulich et al., 2016; Davis et al., 2017; De Leoz et al., 2015; Hill et al., 2017; Ma et al., 2020; Milani et al., 2017; Penders et al., 2006; Samuel et al., 2019; Tanaka et al., 2009; Thum et al., 2012; Yassour et al., 2016; Zimmermann and Curtis, 2020). These include:

- Mode of delivery (vaginal or Caesarean section)
- Gestational age (premature or full-term infants)
- Sex
- Administration of antibiotics
- Geographical origin
- Other maternal and genetic factors.

In view of the high instability and variability of the intestinal microbiome in the first year of life and due to the large number of possible factors influencing bacterial colonisation, it is currently not possible to reliably assess what can be described as 'normal' or 'healthy' intestinal flora, despite the large number of studies on this subject. Furthermore, it is not clear what significance factors such as the intake of individual HiMOs have in this regard – the latter particularly in light of the fact that infants receive an individual and highly diverse mixture of HMOs in breastmilk. In general, infant formulas contain only individual HiMOs in lower concentrations. Finally, the predominantly *in vitro* or *ex vivo* observations made on the intestinal

[1] The study data of Tims et al. (2018 a and b) were only published in conference abstracts.

microbiota to date do not allow any reliable conclusions to be drawn on the health benefits of an infant formula with individual HiMOs in comparison with a standard infant formula.

3.3.2.2 Anti-adhesive effects of the HiMOs under evaluation, including 3'-GL and/or GOS

Some HMOs are structurally similar to surface glycans of the intestinal epithelium and can, as receptor mimics, apparently prevent the attachment of viruses or pathogenic bacteria to intestinal epithelial cells (Gustafsson et al., 2006; Kunz et al., 2000; Newburg et al., 2005).

Thus, it has been observed in *in vitro* and *ex vivo* studies that 2'-FL is capable of preventing the adhesion of *Campylobacter jejuni* to epithelial cells (Ruiz-Palacios et al., 2003). An observational study conducted in Mexico City in more than 90 mother-child pairs found that breastfed infants who received breastmilk with higher concentrations of 2'-FL were less likely to develop diarrhoea caused by *Campylobacter jejuni* (Morrow et al., 2004).

On the basis of experimental studies, positive effects for individual HiMOs such as 2'-FL have also been postulated in the context of defence against other pathogenic (potentially disease-causing) bacteria (e.g. *Pseudomonas aeruginosa*, *Streptococcus (S.) pneumoniae* and *S. mutans*), viruses (e.g. HIV, rotavirus and norovirus) and unicellular parasites (e.g. Entamoeba histolytica) or fungi (*Candida albicans*), as well as for organs other than the gut (Bode, 2012; Salli et al., 2020; Tong et al., 1999; Weichert et al., 2013; Zhang et al., 2021).

Despite these promising data, the available studies do not permit conclusions to be drawn concerning the direct benefits to be expected from the consumption of infant formula with HiMOs, whether individually or in mixtures, and with or without GOS.

3.3.2.3 Influence of the HiMO and/or GOS to be assessed and of fermented infant formula with 3'-GL and GOS/FOS on the immune system

a) Development of the immune system

The infant immune system does not fully develop until after birth. Cell populations with functions vital for survival, such as B cells in the germinal centres and marginal zone, single-positive $CD4^+$ and $CD8^+$ T cells, and cytotoxic T lymphocytes, do not emerge until perinatally (from 28 weeks of gestation to 7 days of life) or thereafter (Zhang et al., 2017).

In terms of HMO effects on the immune system, many publications describe an influence on the *homing* of lymphocytes, the extravasation of leucocytes and receptor-mediated pathogen-host interactions (Donovan and Comstock, 2016). Yet when it comes to discussing the specific immunomodulatory effects of individual HMOs, the first, critical question concerns the actual diversity of the immune system at the point of birth in terms of the presence of specific immune cell subpopulations and how the immune system develops over the following months.
Until recently, scientific data on this was limited, since complex phenotyping requires blood samples taken from the infant at intervals of only a few days. However, new insights have recently been provided as a result of innovative and less cumbersome analytical methods. Thus, complete phenotyping of cell populations is now possible with mass cytometry, even with small volumes of 100 µl of whole blood. Thanks to these developments, a longitudinal study at the Karolinska University Hospital in Sweden was able to systematically investigate the developing immune systems of 100 neonates (Olin et al., 2018). Between April 2014 and

July 2017, blood samples were analysed from neonates at birth (here: umbilical cord blood) and after one week as well as after four and 12 weeks (here: peripheral blood). The results reveal considerable differences between individual immune systems, even immediately after birth. In the 58 cell populations and 267 plasma proteins examined from premature infants, counts of memory B cells (unswitched), $CD8^+$ central and effector memory cells, $CD4^+$ terminally differentiated effector memory cells (TEMRA) and mature dendritic cells were elevated in particular, as were the chemokines CXCL11 and interleukin-8 (IL-8). In contrast, certain effector memory T cells of the $CD8^+$ TEMRA type were elevated in full-term infants. The important inflammatory markers IL-12, IL-17A and IL-27, as well as IL-10, were also lower in premature infants and in full-term infants directly after birth, when compared with infants more than five months old.

Accordingly, significant differences in the development of the immune system are found at the time of birth, both inter-individually and depending on the gestational age. Another key finding from the study by Olin et al. (2018) is that individual immune systems converge along a stereotypical development pathway: after about 100 days of life, the immune cells and cytokines released differ considerably less from one another than directly after birth. Surprisingly, this adaptation was also discovered in the few premature infants who needed to remain in hospital for treatment for weeks or months after birth, and thus had a different microbial exposure due to the environment and had received antibiotics and antipyretics (fever-reducing drugs) more frequently than the comparison groups.

The publication by Olin et al. (2018) also provides clear indications that the conversion of the immune system is essentially influenced by the interaction of immune cells with intestinal bacteria. In particular, genes of the major histocompatibility complex (MHC)-II were found to be upregulated and induced by bacteria in an interferon-γ-dependent manner. By means of stool investigations using RNA profiling (16S), a progressive change in the microbiome could be observed over the period of postnatal colonisation of the gut. The microbiota colonisation is obviously important for the training and conversion of the immune system, since activated inflammatory T cells and other markers continue to be found in infants with exceptionally low bacterial diversity in the gut microbiome (early dysbiosis) up to the age of three months. In infants with disorders in the intestinal colonisation, immune conversion is therefore significantly less well-developed.

All in all, the publication by Olin et al. (2018) demonstrates for the first time that the immune systems of neonates differ significantly and then converge over the course of three months depending on intestinal colonisation with different bacterial populations. These insights into the stereotypical development of the immune system also have consequences for the assessment of potential immunomodulatory effects of HMOs, which, as discussed in section 3.3.2.1, may be prebiotic in nature.

b) Effects of HMO on immune receptors and the immune system

Immune regulation is well-documented as an immunomodulatory effect resulting from breastfeeding. In the first few months of life, during which the infant's acquired immune system is still developing, breastmilk or breastfeeding form the main resource of active and passive immunity. This correlation is also supported by evidence that breastfeeding is regarded worldwide as the most effective preventive measure for reducing mortality in children under five years of age (Labbok et al., 2004). Alongside secretory IgA, lactoferrin, lysozyme, interferon-γ and nucleotides, oligosaccharides from breastmilk can indirectly compensate for delays in the development of the infants' immune system. Breastfed infants also have a lower risk than

non-breastfed infants for inflammatory disorders such as sepsis and enterocolitis (Klement et al., 2004).

Specifically, breastfeeding can reduce the risk of intestinal infections with gram-negative bacteria, which display the lipopolysaccharide (LPS) endotoxin in their bacterial cell wall. LPS can be recognised by epithelial and immune cells via the cell surface molecules toll-like receptor (TLR) 4 and also via CD14. Breastmilk contains not only lactadherin and lactoferrin, but also CD14 in a soluble form (He et al., 2016a). These receptors are known to mediate anti-inflammatory effects via the TLR4 signalling pathway. While the specific function of soluble CD14 in breastmilk for homeostasis in neonates has yet to be described in detail, the receptor can counter inflammation in tissues distant from a site of infection. Although these receptors contained in breastmilk do not themselves belong to the HMO group, this context is relevant for the assessment of HMOs, since effects on membrane-bound CD14 have also been published for HMOs.

For example, an experimental study investigated the individual effect of six separate HiMOs on the ability to inhibit LPS-induced release of IL-8 in human mature intestinal epithelial cells – as occurs in *E. coli* infection (He et al., 2016b). IL-8 inhibition by 2'-FL alone was equivalent to the inhibition achieved by a mixture of all six HiMOs together, at approximately 50 % compared to the control. Inhibition by 2'-FL was dose-dependent, reaching a plateau of 80 % IL-8 inhibition at an *in vitro* concentration of 4 mg/ml 2'-FL. The HiMOs as a whole were able to suppress the transcriptional expression of CD14, CD14 translation and also the translocation of this receptor. Marked effects were observed in the upregulation of a cytokine suppressor, SOCS2, and in the activation of the signal transduction molecule STAT3 in T cells, with simultaneous inhibition of CD14 and IL-8.

Apart from binding to TLR4 and CD14, binding to other functional immune receptors – such as DC-SIGN, specific siglecs and galectins (table 3) – has been shown for several HiMOs, including 2'-FL.

Table 3: Overview of HiMOs that bind to immune receptors and are potentially involved in immunomodulation (according to Zuurveld et al., 2020)

HMO as ligands	Receptor	Receptor-bearing cells	Receptor function
2'-FL, 3'-FL	DC-SIGN	Antigen-presenting cells	Antigen presentation
3'-SL, 6'-SL	Siglec 5, 9	Neutrophils, monocytes, dendritic cells	Immune signalling
LNnT, LNT	Galectin 1, 2, 3, 7, 8, 9	Intestinal cells, lymphocytes, antigen-presenting cells	Immune signalling
2'-FL, 3'-SL	TLR4	Various cell types, primarily immune cells	Pathogen detection

In an experimental animal study, in which neonate piglets were fed an HiMO-containing formula with 40 % 2'-FL, 35 % LNnT, 10 % 6'-SL and 5 % 3'-SL over a period of 15 days, and infected with a rotavirus after 10 days, it was shown that virus-induced diarrhoea cleared up more quickly in the HiMO-supplemented animals (1.3 days on average) than in the animals fed with standard formula (Li et al., 2014). The animals had higher rotavirus-specific IgG and IgM titres in serum and increased transcript level of IL-6, IL-8, IL10 and IFN-γ in ileal tissue (ileum: lower part of the small intestine) compared with the controls. Unlike the other cytokines, IL-10 is an anti-inflammatory factor that generally counters inflammation. Overall, the

results suggest a possible influence of the overall formulation on mucosal immunity in the animal model. Nevertheless, the clinical relevance of these findings is unclear.

However, in a study with mice in which the genes for IL-10 and 3'-SL biosynthesis had been switched off, it was observed that oral supplementation with 3'-SL directly influenced the mucosal immune system and increased the susceptibility of these animals to colitis through pro-inflammatory effects (Kurakevich et al., 2013). The influence of IL-10 deficiency for these findings is difficult to assess, since an effect of external 3'-SL in mice without 3'-SL biosynthesis but intact IL-10 was not investigated.

In a clinical study by Newburg et al. (2016), HMO were purified from 'pooled' breastmilk samples taken from 40 donors at all phases of lactation as well as seven colostrum samples. Subsequent use of these HMOs in a cell stimulation model in which cells of the H4 cell line (*human normal foetal intestinal epithelial cells*) were treated with the pro-inflammatory cytokines TNF-α and IL-1β, resulted in up to 51 % lower expression of IL-8, MIP-3α and MCP-1 in these cells compared to cells not treated with HMO. In a second pathogen stimulation model with the T84 cell line (*human metastatic colonic epithelial cells*), mRNA levels of IL-8 and MCP-1 were reduced in real-time PCR under HMO to up to 30 % of the corresponding positive controls (without HMO).
Following induction with TNF-α, the p65 subunit of the inflammatory-associated transcription factor NF-kB tended to remain in the cytoplasm and did not translocate into the cell nucleus in studies with different cell lines under HMO. This fact is interpreted as an indication that HMO could prevent inflammation caused by intestinal pathogens.

Additional *ex vivo* findings also suggest anti-inflammatory effects of HMO. These show that HMOs in organ cultures of human foetal intestinal mucosa were able to inhibit the protein concentrations of IL-8 and MCP-1 induced by TNF-α or by *Salmonella enterica* to 78 % and 52 %, respectively, and to 26 % and 25 %, respectively, of the corresponding positive controls. Overall, the *in vitro* and *ex vivo* experiments conducted by Newburg et al. (2016) demonstrated significant anti-inflammatory effects of HMOs derived from breastmilk.

In a similarly designed study, the immune effects of HMOs were investigated in *ex vivo* organ cultures prepared from human foetal intestinal mucosa, also using pathogen stimuli (He et al. 2014). Here, the total fraction of HMO from human colostrum was purified in comparison to mature breastmilk from pooled samples taken from 20–50 donors using an identical method. Since Toll-like receptors 3 and 5 are expressed primarily in the intestinal mucosa, specific ligands were used for stimulation: double-stranded RNA (PolyI:C), flagellin and IL-1β, a cytokine that plays a crucial role in the body's response to microbes. In qRT-PCR analyses and antibody membrane arrays, the cytokine transcriptome and cytokines were analysed at the protein level, respectively. In their findings, He et al. (2014) highlight in particular the protein levels of IL-8, IL-6, MCP-1/2 and IL-1β being downregulated by HMO – following prior Toll-like receptor (TLR) stimulation. They conclude that the HMO contained in colostrum can attenuate mucosal responses to inflammatory surface stimuli in early child development. It should be critically noted that the study by He et al. (2014) only analysed data from three experiments at the protein level. However, the significant reduction of the two anti-inflammatory cytokines IL-10 and TGF-β, which was also shown, does not fit into the interpretation of a purely anti-inflammatory effect of colostral HMO. In the case of an anti-inflammatory effect, IL-10 and TGF-β, which mediate tolerance in the human body, should rather be found to be strengthened by HMO. In He et al. (2014), physiological concentrations of 3'-GL inhibited the Poly(I:C)-induced IL-8 response, but not the response induced by flagellin or IL-1β, in stimu-

lated H4 cells. Since the reduction of the IL-8 response by the colostrum HMO was significantly more pronounced than by individual GLs, other oligosaccharide components of the HMO mixture investigated appear to be responsible for the immunomodulatory effects.

Lastly, with regard to 3'-GL, immunomodulatory effects of trans-galactosyl oligosaccharides, whose primary component is 3'-GL, were investigated in mice in an animal feeding study. The rodents were vaccinated with an influenza virus-based vaccine and additionally treated/not treated with the mycotoxin deoxynivalenol, given in their feed (Toutounchi et al., 2021). It was observed that the administration of dietary trans-galactosyl oligosaccharides enhanced the immune responses in the mice treated with deoxynivalenol, in terms of the following parameters: ear swelling (delayed-type reaction), specific IgG1 and IgG2a in serum. In addition, the release of IFN-γ and IL-10 was also elevated in re-stimulated splenocytes. The mice not treated with deoxynivalenol exhibited an increased frequency of Th1-cells with the activation marker CD69. In relation to villus height and the expression of junction proteins in the ileum, the oligosaccharides showed a protective effect. No significant effects were observed on the occurrence of short-chain fatty acids in the appendix, as an indicator of metabolic activity of the microbiota.
A number of *in vitro* experiments were also conducted in the same study, investigating the effects of supplementation with 3'-GL alone on Caco-2 cells following treatment with the mycotoxin deoxynivalenol. At a concentration of 0.5 % 3'-GL, the mycotoxin-induced release of IL-8 was reduced and the integrity of the epithelial barrier was improved. Only three experiments were performed, however, and the IL-8 reduction was in the range of 50 pg/ml, which can be considered a small effect even for *in vitro* experiments and this prominent inflammatory marker. On the other hand, the IL-8 repressing effect and the barrier-maintenance effect observed by Toutounchi et al. (2021) count as some of the few indications of immunomodulatory effects of 3'-GL found at all in the scientific literature. Since some inflammatory markers such as IL-8, but also anti-inflammatory markers like IL-10 were found to be increased, the significance of these findings for an assessment of the suitability of infant formula containing 3'-GL is unclear.

Certain molecules in human breastmilk – such as the soluble Toll-like receptors (TLR) 2, 4 and 5, and CD14, lactadherin, lactoferrin and β-defensin 2 – can display anti-inflammatory effects. Thus, they can help suppress inflammation in tissues distant from a pathogen entry point (He et al., 2016a). In terms of specific immune effects of 3'-GL in context of TLRs, a review by He et al. (2016) referred to a study conducted by Chen et al. (2014) in which no evidence supporting these effects is shown, however. The immunosuppressive effect of 3'-GL described by He et al. (2016) must therefore be considered as unproven at this time.

c) Effects of GOS on immune receptors and the immune system

The potential influence of GOS on inflammatory bowel disease (IBD) was investigated in an animal model of intestinal inflammation using knockout mice for a receptor-regulated Smad protein (Gopalakrishnan et al., 2012). These rodents lack the protein Smad3, which plays a key role in TGF-β signalling by translocating into the nucleus within a signalling complex. In addition, the mice were infected with *Helicobacter hepaticus* via their feed. The results observed showed that daily oral administration of 5 g GOS/kg body weight significantly reduced the inflammatory symptoms of colitis. Compared to control mice receiving sterile water, GOS supplementation resulted in fewer infiltrations and a lower reduction of Goblet cells in the appendix and intestinal crypts. This effect was associated with a 1.5-fold increase in faecal bifidobacteria. Furthermore, the proportion of natural killer cells (NK cells) increased in the spleen and mesenteric lymph nodes, as well as the expression of the chemokine receptor CCR9 on these cells, as did the release of IL-15 in the intestinal tissue in the short term.

These findings could be interpreted mechanistically as attenuation of the induced intestinal inflammation through activation of NK cells, which, however, – in contrast to their conventional role as Th1 promoters – function here rather as immunoregulators. No evidence for this was presented by this study, however, such as by immunosuppressive IL-10 or identification of other, anti-inflammatory immune cell subpopulations in the intestines of these mice. Overall, GOS was reported to significantly attenuate colitis with a simultaneous increase in specific inflammatory cells. Owing to the absence of relevant human studies, no assessment can be made at this time concerning the extent to which these findings, from a genetically modified mouse model in the hepatitis infection model, are transferable to the situation in human infants.

In another animal study in colostrum-deprived piglets, which were fed different formulae[2] and partially infected with rotavirus, higher counts of NK cells, effector T memory cells and basophils were found in the GOS/FOS group in comparison to the control group (Comstock et al. 2017). However, the immunomodulatory effects were less pronounced in the GOS/FOS group than in the HiMO group. Alongside the observations made in the infection model, IFN-γ-producing cells and plasmacytoid dendritic cells were also elevated by GOS/FOS in the non-infected animals. A study group receiving formula with either exclusively GOS or FOS was not included in this study, however. Thus, the immunomodulatory effects can only be attributed to both sugars together. A combination effect of the tested GOS/FOS formulation on the enhancement of a viral response in the animal model can be considered as proven by the Comstock study.

The effects of infant formula with GOS on immune parameters have also been investigated in studies in humans. However, almost all of these studies also utilised a mixture of GOS and FOS in a 9:1 ratio, with the exception of the study conducted by Marriage et al. (2015) described earlier.

Goehring et al. (2016) report on the effects of 2'-FL and/or GOS on the infants' immune system observed in the Marriage study. For this, peripheral blood mononuclear cells were enriched from blood samples taken after six weeks and some inflammatory cytokines in plasma were analysed. In the case of plasma concentrations of IL-1 α/β and IL-1 receptor α as well as IL-6 and TNF-α, infants who had received one of the study formulas containing 2'-FL and GOS exhibited similarly low concentrations as those of breastfed infants. Infants who had received the GOS formula without 2'-FL showed higher cytokine concentrations overall. This tendency was also seen in the cytokine analyses in an *ex vivo* stimulation model of blood cells with phytohaemagglutinin. Marriage et al. (2015) consequently conclude that infant formula with 2'-FL leads to the achievement of inflammatory cytokine profiles that are comparable with those found in breastfed infants.
However, there were no differences between the three formula groups in other inflammatory plasma cytokines also investigated in this study, such as IFN-α2, IL-1α, IP-10 and RANTES. Since each of the three study formulae contained GOS, however, this study lacked a 'true' control group that received a standard cow's milk-based infant formula without added GOS or 2'-FL. Also, all study products were normalised for their carbohydrate content: as a result, formulae with 2'-FL contained correspondingly lower amounts of GOS. This makes it impossible to exclude a causal effect of GOS content on cytokine concentrations. Accordingly, these study results cannot be used to unambiguously assign GOS or 2'-FL an immunomodulatory role for the Th1/Th2 balance, as postulated for HMO by Bode (2012). Moreover, no

[2] Intervention groups:
- Formula with 4 g/l GOS/FOS prepared from 3.6 g short-chain GOS and 0.4 g long-chain FOS
- Formula with HiMO: 40 % 2'FL, 35 % LNnT, 10% 6'-SL, 5 % 3'-SL and 10 % free sialic acid

Control group: standard formula with lactose

conclusions can be drawn about the effects of the formulae investigated in comparison to standard cow's milk-based infant formula.

Further placebo-controlled intervention studies (Scholtens et al., 2008; Bakker-Zierikzee et al., 2006) have investigated the effect of infant formula containing GOS and FOS, as currently approved within the EU, on the faecal secretory IgA (sIgA) response, in comparison with standard infant formula and breastmilk. Study results show that the GOS/FOS groups exhibited significantly higher concentrations of sIgA in faeces after roughly four to six months. The concentrations were partly comparable or even higher than those in breastfed infants. However, the numbers of test subjects were too low to reliably determine statistically significant differences. Furthermore, it is questionable whether the observed sIgA concentrations – some of which were significantly higher than those in breastfed infants – represent an added health benefit in the non-infected state. Also, no group was included in the studies that received infant formula containing solely GOS or FOS. Accordingly, the observed effects are not transferable to infant formula containing 100 % GOS.

Further effects on basal immune parameters in the blood were investigated by Raes et al. (2010) in a randomised, double-blind, placebo-controlled study. This involved the recruitment of 215 infants, either breastfed (n = 159) or given a standard infant formula (n = 29) and compared to an identical formula with 0.6 g/100 ml GOS/FOS in a ratio of 9:1 (n = 27). Blood tests were performed at weeks 8 and 26, although only 21 and 22 infants respectively had been continuously fed the GOS/FOS formula up to these points in time. The tests showed no significant differences between the two formula groups in terms of leucocyte count in the blood. At the 26th week, this count was significantly higher in breastfed infants than in the GOS/FOS group. For the immunoglobulins IgE, IgG, IgA and IgM, no significant differences were observed between the groups – neither compared to standard infant formula nor in comparison with breastfed infants. In the same study population, Scholtens et al. (2008) had evaluated higher concentrations of sIgA in the faeces of the GOS/FOS group as a prebiotic immune effect.

In terms of lymphocyte populations, Raes et al. (2010) also found no significant differences between the intervention and control group, although differences were found in comparison with breastfed infants. The proportion of activated CD23-positive B cells and $CD8^+$ T cells with activation marker CD25 was significantly higher in the latter group than in the GOS/FOS group. Conversely, for the activation marker CD38, the proportion of $CD8^+$ T cells was significantly lower in breastfed infants than in the GOS/FOS group. There were no significant differences in the case of cytokines. For TNF-α and IL-5, a tendency towards higher concentrations was observed in the GOS/FOS group compared to breastfed infants. Raes et al. (2010) concluded that feeding infant formula with added GOS/FOS to healthy infants in the first six months has no influence on basal immune parameters and therefore on the immune status of the developing immune system – and therefore infant formula with GOS/FOS offers no benefit over standard infant formula when compared with breastmilk. Once again, this study did not include any groups who received infant formula containing solely GOS or FOS. As a result, no conclusions can be drawn about a potential effect of GOS alone on immune parameters.

Lastly, the effects of GOS (and FOS) on the neonatal immune system were investigated in a randomised, double-blind study including 48 pregnant women (Shadid et al., 2007), who supplemented their diet three times a day with 3 g short-chain GOS and long-chain FOS in a 9:1 ratio. In addition to the primary investigation of the potential transferability of maternal microbiota to newborns, the expression of lineage markers, chemokine receptors and cytokines in cord blood was also analysed. No significant differences in the proportions of specific $CD4^+$

helper and $CD8^+$ cytotoxic T cell subpopulations and chemokine receptors CCR1-9 and CXCR3-5 were identifiable between the GOS/FOS group and the placebo group with maltodextrin. There were also no differences in the inflammatory markers TNF-α, IL-1, IL-6, IL-8, MIP-1β, the Th1 cytokines IFN-γ and IL-2 and the Th2 cytokine IL-4, as well as in the anti-inflammatory factor IL-10. Overall, therefore, even the combined administration of the GOS/FOS formulation revealed no immunomodulatory effects in this study design by Shadid et al. (2007).

d) Influence of infant formula with HiMO and/or GOS on the emergence of allergies

To date, few studies have investigated the influence of HMO on the emergence of allergies. In one clinical trial, no correlation was observed between the concentrations of HMO 3'-fucosyllactose (3'-FL), 2'-FL, LNT and LNnT in colostrum and the development of allergies in infants up to 18 months of age (Sjögren et al., 2007). There was a tendency for children who had developed allergic symptoms by this age to have received colostrum with higher concentrations of HMO. However, this difference did not reach statistical significance and could not be attributed to any single HMO.

In another prospective cohort study, HMO concentrations were investigated in breastmilk given to infants diagnosed as allergic to cow's milk or as having no allergies (Seppo et al., 2017). While concentrations of 6'-SL, lacto-N-fucopentaose (LNFP) I and III, and disialyllacto-N-tetraose were lower in the breastmilk received by infants with cow's milk allergies, the differences were not significant except in the case of LNFP III after correction for multiple comparisons. Seppo et al. (2017) speculated that it might be the Lewis X antigen in LNFP III that binds to the DC-SIGN receptor for pathogen molecular structures (PAMPs) on dendritic cells. In this way, the antigen competes with glycoproteins for binding to this receptor and therefore counters allergies in the gastro-intestinal tract by mediating tolerance.

Sprenger et al. (2017) observed a significant inverse association between 2'-FL and allergic disorders in general, as well as with IgE-associated diseases, eczema and IgE-associated eczema in Caesarean section-delivered infants. In this study, FUT-2-dependent oligosaccharides were investigated in breastmilk overall and the association with the occurrence of allergies after two and five years. The study showed that after two, but not after five years, the risk for IgE-associated eczema was lower when FUT-2-dependent oligosaccharides had been present in breastmilk.

In an experimental model for food allergies, experiments in ovalbumin-sensitised mice showed that a diet supplemented with 2'-FL or 6'-FL was able to attenuate the passive cutaneous anaphylactic response, measured via IgE-induced colour deposition in the mouse ear, as well as the levels of mast cell protease-1 in serum (Castillo-Courtade et al., 2015). A simultaneous increase in tolerance-mediating regulatory T cells was observed in Peyer's patches and mesenteric lymph nodes. In cell culture, 6'-SL was also able to inhibit the degranulation of mast cells. Although these mice were already eight to nine weeks old at the start of the study and the results are not transferable to human infants, the experiments indicate that 2'-FL (and 6'-SL) could be suitable for having an immunomodulatory effect and attenuating existing allergies.

However, the published scientific literature has yet to provide unambiguous evidence that a diet with individual HiMOs can prevent or positively influence food allergies in rodents or human infants.

With regard to the immunomodulatory effects of GOS on the development of allergic diseases, there are published studies in animal models and also in humans.

Animal studies
In ovalbumin-immunised two-week-old female BALB/c mice, supplementation with GOS (from alpha-galactosidic bound galactose) prevented an allergic inflammation of the diaphragm (allergic peritonitis) (Pirapatdit et al., 2008). In the *ex vivo* model, significantly fewer extravasated blood leucocytes were found in the peritoneal lavage fluid of mice supplemented with 5 % GOS for seven days. In addition, lower levels of the proteins MCP-1 and eotaxin were measured than in control mice fed a synthetic diet without GOS (American Institute of Nutrition, AIN-93G). In other mouse studies, formulations containing oligosaccharides stimulated hypersensitivity reactions in the influenza vaccination model and were able to attenuate parameters of allergic asthma (Vos et al., 2006; Vos et al., 2007). Also, in the mouse model of allergic sensitisation, a perinatally administered combination of GOS and inulin could enhance tolerance-associated biomarkers IgA and TGF-β (Gourbeyre et al., 2013). In addition, the development of atopic dermatitis-like skin lesions in a specific strain of mice (NC/Nga) could be prevented by supplementation with GOS (Tanabe and Hochi, 2010).

Human studies
In infants with an elevated risk of allergies due to parental atopic disorders, multiple intervention studies were conducted in order to investigate whether a (hydrolysed) infant formula with 0.8 g/100 ml GOS and FOS, in a 9:1 ratio, was able to reduce the risk of atopic disorders (Moro et al., 2006; Arslanoglu et al., 2008; Schouten et al., 2011). It was observed that up to the age of six months or at the two-year follow-up, children in the intervention groups had significantly less often atopic dermatitis than those in the control groups; in Arslanoglu et al. (2008), the GOS/FOS-supplemented children also suffered less frequently from respiratory problems and allergic urticaria (hives).

Van Hoffen et al. (2009) were able to show that plasma concentrations of total IgE, IgG1, IgG2 and IgG3 as well as cow's milk protein-specific IgG1, but not IgG4, were significantly reduced by administration of infant formula with GOS/FOS for six months. In these infants, who had been vaccinated against diphtheria, tetanus and polio (DTP) with *Hexavac* at the age of three months, the vaccine-induced DTP-specific Ig levels remained unchanged. These findings from 41 children in the treatment group may indicate that GOS/FOS provide a beneficial immunoglobulin profile with reduced antibodies against food allergens while simultaneously maintaining vaccine protection.

Lastly, Grüber et al. (2010) conducted an intervention study in which 414 infants with a low risk of atopy received a formula containing a mixture of neutral oligosaccharides and acidic pectin oligosaccharides for the first eight weeks of life. In this study, a 44 % lower rate of atopic dermatitis was observed in the intervention group by the end of the first year of life (Grüber et al., 2010).

In contrast to these positive results, more recent intervention studies have observed no clear association between supplementation of 0.8 g/100 ml GOS/FOS in a 9:1 ratio and the prevalence of atopic disorders (Boyle et al., 2016; Ranucci et al., 2018, Wopereis et al., 2018). In comparison with standard cow's milk formula, Boyle et al. (2016) also observed no effect on most immune markers and total/specific IgE in particular, at the age of 12 and 18 months. However, reduced levels of cow's milk-specific IgG1 and increased proportions of regulatory T cells and plasmacytoid dendritic cells were observed in association with the consumption of a GOS/FOS-formula.

To the best of the BfR's knowledge, the only study in which the effects of infant formula containing solely GOS were investigated in infants in the first year of life is a double-blind, multicentre study conducted by Sierra et al. (2015). This study recruited 365 healthy infants in the first eight weeks after birth, using a placebo-controlled study design to administer an infant formula with 0.44 g/100 ml GOS in the first four months and then follow-on formula with 0.5 g/100 ml GOS until the end of the first year of life. In addition to stool characteristics, the occurrence of infections and allergic manifestations as well as the use of antibiotics were documented over the first full year of life. At the start of the study and at the fourth month of life, stool samples were collected in order to analyse IgA, short-chain fatty acids and changes in microbiota. The number of episodes of upper respiratory tract infections diagnosed by paediatricians was slightly higher on average per child in the GOS group. The allergic manifestations investigated here, such as atopic dermatitis, asthmatic wheezing and food allergies, occurred in 28 of 132 infants in the control group and in 39 of 132 infants in the GOS group. Accordingly, no positive effect from GOS was observable in relation to these diseases. The BfR considers these findings to be relevant, since in this study – unlike in many other studies – an infant formula was administered that contained solely GOS and not a combination (Sierra et al., 2015).

In summary, the BfR does not see a clear and scientifically validated argument for the positive effects of infant formula with added GOS on the prevention or attenuation of allergic diseases. As a further problem, most studies have utilised a combined GOS/FOS product or a mixture of neutral oligosaccharides and acidic pectin oligosaccharides, with sometimes contradictory results. No conclusions on the effects of GOS or FOS administered separately can be drawn from the data supplied by these studies. The only study known to the BfR in which infant formula containing solely GOS did not show any allergy-preventive effect. As of this writing, a health-promoting effect of infant formula with GOS on the development of the immune system and the risk of allergies therefore does not appear to have been demonstrated.

3.3.2.4 Other health effects of infant formula with HiMO and fermented infant formula with 3'-GL and GOS/FOS

a) HiMO

In a randomised, double-blind, multi-centre study, 175 infants (95 from Belgium and 80 from Italy) were studied from the age of 14 days. They were split into two groups, each receiving a separate infant formula (Puccio et al., 2017). The study formula (n = 88) contained 1.0 g/l 2'-FL and 0.5 g/l LNnT. The control formula (n = 87) was a standard cow's milk-based infant formula without HMO. The formulae were given for the first four to six months; thereafter, all infants received a standard follow-on formula without HMO. The primary goal of this study was to investigate the safety of infant formula with an addition of 2'-FL and LNnT. The primary endpoint selected for this purpose was weight gain in the first four months. Data on other parameters were also collected as secondary endpoints, some of which indirectly allowed conclusions to be drawn about the development of the immune system:

- Length, BMI, head circumference and tolerance (formation of gas, spitting, vomiting)
- Stool characteristics (consistency and frequency)
- Behaviour (agitation, colic, waking up at night)
- Infant food intake
- Symptoms of illness
- Use of medicines in the first 12 months

Parents were requested to keep a diary, documenting infectious diseases contracted by their children as well as prescriptions of antibiotics and antipyretics. The results showed that cases of bronchitis were significantly lower in infants in the intervention group at four (2.3 % versus 12.6 %), six (6.8 % versus 21.8 %) and 12 months (10.2 % versus 27.6 %). In addition, these infants contracted fewer infections of the lower respiratory tract over the study period of 12 months (19.3 % versus 34.5 %), exhibited less use of antipyretics over four months (15.9 % versus 29.9 %) as well as less use of antibiotics over six (34.1 % versus 49.4 %) and over 12 months (42.0 % versus 60.9 %). In all cases, however, the results were not significant. The groups showed no differences in terms of weight development and other anthropometric parameters, nor in food intake, the frequency and consistency of stools, nor in cases of agitation and colic as observed by their parents. In a subgroup analysis, parents of Caesarean section delivered infants in the intervention group reported significantly fewer cases of colic at the age of four months as well as fewer cases of waking at night at the age of two months (Puccio et al., 2017).

The lower incidence of bronchitis and lower respiratory tract infections can be interpreted as indication of a potential benefit of infant formula with added 2'-FL and LNnT in terms of immune system competence. However, the absolute number of cases e. g. for bronchitis was very low with two cases in the intervention group and 11 cases in the control group after four months. Also, no confirmation of the parental diagnosis by a study doctor is documented. Furthermore, neither the different geographical origin of the infants nor sociodemographic parameters were considered as potential confounders of the endpoints investigated in this study. Moreover, the interpretation of the findings is complicated by the fact that the study does not include a comparison group of breastfed infants. Finally, the statistical power of the study was not designed to determine differences in the secondary endpoints and can therefore only provide indications in this regard that need to be confirmed in further studies.

In a further uncontrolled intervention study by Riechmann et al. (2020), healthy infants were recruited at the age of seven days in six separate study centres in Spain. From birth, the infants had either received exclusively infant formula (n = 82), been exclusively breastfed (n = 63) or had received breastmilk and infant formula (n = 62). After entering the study, all infants who were not (exclusively) breastfed were fed an infant formula based on partially hydrolysed whey protein with added 1.0 g/l 2'-FL and 0.5 g/l LNnT plus *Lactobacillus reuteri* DSM 17938 for a period of about eight weeks. The other infants continued to be breastfed. Riechmann et al. (2020) reported comparable anthropometric measures in all three groups and good tolerance of the study formula.

Lastly, Kajzer et al. (2016) conducted a prospective, randomised, double-blind multi-centre study involving non-breastfed infants in the first eight days after birth who received one of the following formulae over a period of 35 days:

- Standard infant formula without oligosaccharides (n = 42)
- Infant formula with 2 g/l scFOS and 0.2 g/l 2'-FL (n = 46)

A third group of breastfed infants served as a reference group (n = 43) The primary objective was to collect data on the infants' stool consistency, food intake and anthropometric measures. A total of 86 % of infants completed the study; when considering this group, a significantly higher average number of stools per day was observed in the breastfed infants. No further differences were observed between the three groups (Kajzer et al., 2016; Reverri et al., 2018).

In summary, there are very few intervention studies to date that have investigated the effects of infant formula with added HiMO on the growth and health of infants. In the available studies, infant formula containing 2'-FL, either alone or in combination with LNnT (as well as being with *Lactobacillus reuteri* DSM 17938 in addition) were used.

The findings suggest that no adverse health effects are to be expected from the use of such an infant formula and that growth is promoted comparably to that of breastfed infants or infants fed with standard infant formula. However, the study results neither offer adequate proof of health benefits from such a formula in comparison with standard infant formula, nor are these results transferable to partially hydrolysed infant formula (as it is partly offered on the German market) or to infant formula products with other HiMO (combinations) and concentrations.

The BfR is not aware of any intervention studies that have utilised infant formula, follow-on formula or drinks for young children with added LNT, 3'-SL and/or 6'-SL as study formula.

b) Fermented infant or follow-on formulae with 3'-GL and GOS/FOS

In the study by Rodriguez-Herrera et al. (2019) mentioned above, a fermented infant formula with 3'-GL and GOS/FOS had no influence on gastrointestinal or other health symptoms in infants as reported by their parents. Furthermore, an intervention study conducted by Vandenplas et al. (2014b[3]; 2015[3] and 2017) offers indications that (partially) fermented infant formula with GOS/FOS may positively influence the frequency of colic in infants during the first four months of life.

In the placebo-controlled intervention study conducted by Thibault et al. (2004), in which infants were fed fermented follow-on formula, no differences in the frequency of diarrhoea and hospitalisations resulting from such events were reported. On the basis of a per-protocol analysis, it was only shown that diarrhoea in infants in the intervention group tended to have slightly milder progressions, were associated with fewer medical consultations, fewer cases of dehydration and prescriptions of oral rehydration solutions. These infants were also less likely to switch to different infant formulas.

In summary, intervention studies that have been conducted to date do not provide sufficient evidence that the use of fermented infant formula is associated with clinically relevant health benefits in infants. This is especially true for fermented follow-on formula with GOS/FOS as currently offered on the market in Germany.

Further information on the subject of infant formula is available on the BfR website:

A-Z index: Infant formula
https://www.bfr.bund.de/en/a-z_index/baby_food-130273.html

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