FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk assessment – Application A1265

A1265 – 2′-FL/DFL, LNT, 6′-SL sodium salt and 3′-SL sodium salt for use as nutritive substances in infant formula products

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Executive summary

FSANZ has assessed an Application from Glycom A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit the addition of four human-identical milk oligosaccharide (HiMO) products for use as nutritive substances in infant formula products (IFP). The four HiMO products are produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* K-12. The HiMOs and proposed maximum permitted amounts are:

- Mixture of 2′-fucosyllactose (2′-FL) and difucosyllactose (DFL), referred to as 2′-FL/DFL (96 mg/100 kJ);
- lacto-*N*-tetraose (LNT; 32 mg/100 kJ);
- 6′-sialyllactose sodium salt (6′-SL; 16 mg/100 kJ); and
- 3′-siallyllactose sodium salt (3′-SL; 8 mg/100 kJ).

FSANZ has undertaken an assessment of the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of 2′-FL/DFL, LNT, 6′-SL and 3′-SL to IFP.

Information reviewed in the food technology assessment demonstrates 2′-FL/DFL, LNT, 6′-SL and 3′-SL are chemically and structurally identical to the naturally occurring forms of these substances in human milk. The substances were shown to be stable in IFP with an adequate shelf-life. Multi-batch analyses showed the oligosaccharides can be consistently produced to meet their proposed specifications.

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and poses no risks to humans. Analyses of the gene donors also confirmed there were no safety concerns. The GM *E. coli* K-12 production strain used to produce 2′-FL/DFL conforms to the permitted source organism listed in Schedule 26 of the Code and was therefore not further assessed. The GM *E. coli* K-12 production strains used to manufacture LNT, 6′-SL and 3′-SL were characterised to confirm the presence of the introduced genes and to demonstrate that each production strain was genetically and phenotypically stable.

The safety assessment concluded there are no public health and safety concerns associated with the addition of 2′-FL/DFL, LNT, 6′-SL and 3′-SL to IFP at the proposed use levels.

Intestinal absorption of human milk oligosaccharides (HMOs) is limited and a significant proportion reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces. As the Applicant's HiMOs are identical to naturally occurring HMOs it is not anticipated that there will be any significant differences in pharmacokinetics between naturally occurring and manufactured forms of these substances.

2′-FL/DFL, LNT, 6′-SL and 3′-SL were not mutagenic, clastogenic or aneugenic *in vitro*. No adverse effects were observed in sub-chronic oral toxicity studies in neonatal rats with 2′-FL/DFL, LNT, 6′-SL or 3′-SL at doses up to 5000 mg/kg bw/day (2′-FL/DFL, 6′-SL or 3′-SL) or 4000 mg/kg bw/day (LNT), the highest doses tested.

In a human clinical study, consumption of infant formula containing 1.5 or 2.5 g/L of a mixture of the five HiMOs was safe, well tolerated and did not affect growth. Two human clinical studies with infants fed formula containing 5.75 g/L of an alternative mixture of five HMOs, including 2′-FL, LNT, 6′-SL and 3′-SL, also found it was well tolerated and did not affect growth.

Post-marketing surveillance data have also found no safety concerns following consumption of infant formula containing the mixture of 2′-FL/DFL, LNT, 6′-SL and 3′-SL.

No microbiological safety concerns were identified with the HiMOs.

The dietary intake assessment of 2′-FL, DFL, LNT, 6′-SL and 3′-SL for 3- and 9- month old infants was conducted using a model diet approach. For 2′-FL and DFL, minimum, mean and maximum concentration values were derived from compositional information and the proposed maximum permitted amount of the 2′-FL/DFL mixture provided by the Applicant. For LNT, 6′-SL and 3′-SL, proposed maximum permitted amounts were used to estimate dietary intake. Mean estimated dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from infant formula products are comparable to mean estimated dietary intakes from mature human milk. High (90th percentile) estimated dietary intakes from infant formula products do not exceed estimated dietary intakes from mature human milk at high consumption and high concentration levels, except for DFL when assuming a representative maximum composition of 25% in the proposed 2′-FL/DFL mixture rather than the mean composition provided by the Applicant (12%).

Given the absence of any identifiable hazard in toxicological and clinical studies, and noting that estimated dietary intakes are generally within the range of intakes based on human milk concentrations of these substances, there are no safety concerns from the addition of 2′-FL/DFL, LNT, 6′-SL or 3′-SL to IFP at the proposed maximum permitted amounts.

The weight of evidence supports health effects of HiMOs added to IFP through an increase in the abundance of *Bifidobacterium* spp. in the infant gut microbiota. Clinical trial data confirmed anti-pathogenic effects of the HiMO blend through a significant reduction in toxigenic *Clostridioides difficile* in infant gut. There is evidence to support a role for some HiMOs, particularly 2′-FL, in inflammatory suppression and facilitating appropriate immune responses and antigenic memory. The inclusion of a wider range of HiMOs to IFP enables the microbiota profile to more closely resemble that of breast-fed infants.

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1 Introduction

FSANZ received an Application from Glycom A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit the addition of four human-identical milk oligosaccharide (HiMO) products for use as nutritive substances in infant formula products (IFP). The four HiMO products are produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* K-12. The HiMOs requested are:

- Mixture of 2′-fucosyllactose (2′-FL) and difucosyllactose (DFL), referred to as 2′-FL/DFL;
- lacto-*N*-tetraose (LNT);
- 6′-sialyllactose sodium salt (6′-SL) ; and
- 3′-siallyllactose sodium salt (3′-SL).

The products are proposed to be added to IFP alone or in combination at levels up to 96 mg/100 kJ (2′-FL/DFL), 32 mg/100 kJ (LNT), 16 mg/100 kJ (6′-SL) and 8 mg/100 kJ (3′-SL). The stated purpose for adding 2′-FL/DFL, LNT, 6′-SL and 3′-SL to IFP is to better reflect the compositional profile of oligosaccharides in human milk.

The primary risk assessment question to be addressed is whether the addition of these HiMO products to IFP poses a risk to public health and safety, and whether there is evidence for beneficial health effects.

2 Food technology assessment

The food technology assessment provides information on chemical identification, physicochemical properties and specifications for the oligosaccharides proposed to be added to infant formula products: a blend of 2′-FL and DFL; LNT; 6′-SL and 3′-SL. The assessment primarily aimed to address whether the microbiologically-synthesised oligosaccharides proposed to be added to infant formula products are identical to those present in human milk. The assessment also considered the manufacturing process and the validity of analytical methods used to quantify and characterise the oligosaccharides during production and as a component of infant formula products.

FSANZ has assessed a number of recent Applications requesting permissions for HiMO in food. The information in this section has built on the reports written for the assessment of those Applications. 2′-FL has been assessed in Applications A1155, A1190 and A1233 (FSANZ 2019; FSANZ 2021a; FSANZ 2022a). Application A1155 assessed permitting both 2′-FL and LNnT in infant formulas and other products.

2.1 Chemical and physical properties

The HiMOs 2′-FL/DFL; LNT; 6′-SL and 3′-SL are components of the human milk oligosaccharide (HMO) fraction of human milk. The Applicant produces these oligosaccharides via a microbial fermentation process to produce what are claimed as HiMO. The fermentation is performed using a GM strain of *Escherichia coli* (*E. coli*) K-12, which is detailed in section 3.1.

The chemical names and properties of the oligosaccharides that are requested to be permitted is provided in Table 1 with information as provided in the Application.

2′-FL and DFL are oligosaccharides that contain the sugar fucose (a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$) and so are called ‘fucosylated’ HMOs. 2′-FL is a

trisaccharide consisting of the monosaccharides L-fucose, D-galactose and D-glucose. It can also be described as a monosaccharide L-fucose and the disaccharide D-lactose, connected by an alpha (1→2) glycosidic linkage. DFL is a tetrasaccharide derived from 2′-FL by the addition of a second fucose sugar to the 3-glucose position of the 2′-FL.

2'-FL and DFL are two distinct fucosylated HMOs that are structurally and biologically closely related since DFL is metabolically obtained from 2'-FL by the addition of a second fucose unit ("fucosylation"). Thus, 2'-FL and DFL are always found together in human milk. In this Application, 2'-FL and DFL are produced in the same fermentation and are isolated together, to produce the 2'-FL/DFL mixture.

LNT is a constitutional isomer[1] of the permitted oligosaccharide LNnT (A1155). It is a tetrasaccharide consisting of D-galactose, N-acetyl-D-glucosamine, D-galactose and D-glucose.

6′-SL and its constitutional isomer 3′-SL are both trisaccharides derived from lactose with the subsequent addition of sialic acid (N-acetylneuraminic acid). 6′-SL contains sialic acid at the 6 position of the D-glucose unit, while 3′-SL contains sialic acid at the 3 position. It is noted that both 6′-SL and 3′-SL are provided as sodium salts.

Molecular structures of the different oligosaccharides are provided within the Application.

The substances 2′-FL, DFL, LNT, 6′-SL and 3′-SL are white to off-white amorphous powders or agglomerates that are all readily soluble in aqueous solutions (between 400 to 500 mg/mL at 25°C). They are poorly soluble in organic solvents.

The Application included analytical data (Confidential Commercial Information) to indicate that 2′-FL/DFL; LNT; 6′-SL and 3′-SL are produced using microbial fermentation processes and are chemically and structurally identical to the substances naturally present in human milk. The analytical methods provided used ^{1}H and 2D (2 dimensional, Nuclear Overhauser Effect Spectroscopy (NOESY)) nuclear magnetic resonance (NMR) spectroscopy. FSANZ assessed the information provided and agreed with the Applicant's conclusions that the spectral analysis confirms that the microbially produced substances have the same stereochemical configuration and three-dimensional structure as those naturally occurring in human milk.

[1] Constitutional (structural) isomers have the same molecular formula but a different bonding arrangement among the atoms.

Table 1: Nomenclature and chemical properties of 2'-FL, DFL; LNT; 6'-SL and 3'-SL

Property	**2′-FL**	**DFL**	**LNT**	**6′-SL sodium salt**	**3′-SL sodium salt**
Common name	2′-Fucocsyllactose	Difucosyllactose	Lacto-N-tetraose	6'-Sialyllactose sodium salt	3'-Sialyllactose sodium salt
IUBMB Chemical name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucose	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose	N-Acetyl-α-D-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt	N-Acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt
Alternative names	Fucosyl-α-1,2-galactosyl-β-1,4-glucose 2′-O-L-Fucosyl-D-lactose	Lacto-difuco-tetraose (LDFT)	None located	6'-O-(N-Acetylneuraminyl)-lactose sodium salt, 6'-O-Sialyllactose sodium salt, 6'-Lactaminyllactose sodium salt	3'-O-(N-Acetylneuraminyl)-lactose sodium salt, 3'-O-Sialyllactose sodium salt, 3'-Lactaminyllactose sodium salt
IUPAC abbreviation	Fuc-(α1→2)-Gal-(β1→4)-Glc	Fuc-(α1→2)-Gal-(β1→4)-[Fuc-(α1→3)]-Glc	Gal-(β1-3)-GlcNAc-(β1→3)-Gal-(β1→4)-Glc	Neu5Ac-(α2→6)-Gal-(β1→4)-Glc, sodium salt	Neu5Ac-(α2→3)-Gal-(β1→4)-Glc, sodium salt
CAS name	O-6-Deoxy-α-L-galactopyranosyl-(1→2)-O-β-D-galactopyranosyl-(1→4)-D-glucose	O-6-Deoxy-α-L-galactopyranosyl-(1→3)-O-[6-deoxy-α-L-galactopyranosyl-(1→2)-O-β-D-galactopyranosyl-(1→4)]-D-glucose	O-β-D-Galactopyranosyl-(1→3)-O-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-glucose	O-(N-acetyl-α-neuraminosyl)-(2→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt (1:1)	O-(N-acetyl-α-neuraminosyl)-(2→3)-O-β-D-galactopyranosyl-(1→4)-D-Glucose, sodium salt (1:1)
CAS registry number	41263-94-9	20768-11-0	14116-68-8	157574-76-0	128596-80-5
Chemical formula	$C_{18}H_{32}O_{15}$	$C_{24}H_{42}O_{19}$	$C_{26}H_{45}NO_{21}$	$C_{23}H_{38}NO_{19}Na$	$C_{23}H_{38}NO_{19}Na$
Molecular mass g/mol	488.44	634.58	707.63	655.53	655.53

Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose; GlcNAc = N-Acetylglucosamine; Neu5Ac = sialic acid or N-acetylneuraminic acid

2.1.1 Stability under conditions of use

Data was provided on the stability of the individual substances as bulk powders stored under ambient conditions (25°C at 60% relative humidity (RH)) and under accelerated ageing conditions (40°C at 75% RH). The current results confirmed the 2′-FL/DFL, LNT, 6′-SL and 3′-SL are stable for 4 years at ambient room temperature. The accelerated test concluded the substances were stable for 2 years under such more extreme storage conditions.

Of more direct relevance to the end use of the substances were stability trials performed when the substances were added to powdered infant formula and stored for 30 months at different temperatures (4, 20, 30 and 37°C). The substances were added and produced using the usual method of production to replicate real commercial powdered infant formula product. The results concluded that there was good stability of each of the substances over 30 months of storage at the different storage temperatures.

The stability of the individual substances when made up in aqueous solutions were also studied. Stability tests were performed at 60°C for 8 weeks, and 80°C for 4 weeks at different pHs for 2′-FL/DFL and LNT. For 6′-SL and 3′-SL stability tests were performed at different pH levels at 35°C for 28 days, at acidic and basic solutions at 35°C for 24 hours and with the addition of oxidation agents at 25°C over 24 hours.

The stability studies were performed to identify the likely degradation products formed under the harsh stability conditions.

2′-FL and DFL at neutral pH underwent only minor isomerisation. At acidic solutions at pH<5.0 they underwent hydrolysis, and isomerisation at pH >6.0. These chemical degradations also occurred for LNT, being hydrolysis at pH <5.0 and isomerisation at pH >6.0. None of these degradation products are of safety concern as they are all components of milk and milk products.

For 6′-SL and 3′-SL the pH degradation pathways were also pH dependent, being hydrolysis at pH<3.0 and isomerisation at pH>9.0. In neutral aqueous solution both 6′-SL and 3′-SL were stable for 1 month at 35°C. This was not the situation for acidic or basic solutions though such degradation products are not relevant for commercial products.

2.2 Manufacturing processes

The method of production for 2′-FL/DFL; LNT; 6′-SL and 3′-SL is the same as that of the Application A1155 for the production of 2′-FL and LNnT as summarised within SD1 of the 2nd CFS document (FSANZ 2019). Therefore this detail has not been reported in the report but only summarised. The substances are produced by a microbial fermentation process using a modified strain of *E. coli* K-12. The production process is conducted in two stages: upstream processing (USP) and downstream processing (DSP). The USP can be considered the fermentation steps while the DSP captures the purification, isolation and concentrations steps.

2.3 Impurities

The Application contained information relating to possible impurities in the final purified oligosaccharides. In particular the Applicant reported on analyses for the likely impurities including the residual starting materials, D-lactose and L-fucose as well as manufacturing by-products such as difucosyllactose and 2'-fucosyl-D-lactulose for 2′-FL and DFL (which have limits in the Applicant's specifications). Lactose, fucose and difucosyllactose are natural

components of human milk. For LNT the likely impurities are D-lactose and manufacturing by-products lacto-N-triose II, para-lacto-N-hexose-2 (both naturally present in human milk) and LNT fructose isomer. For 6′-SL and 3′-SL the starting material is D-lactose and sialic acid and manufacturing by-products of 6'-sialyl-lactulose and 3'-sialyl-lactulose respectively. Again the Applicant's specifications have limits on these substances. Toxicity studies submitted in the Application conformed to these specifications.

The production microorganism is removed during the processing and purifications steps during production of the oligosaccharides. Qualitative polymerase chain reaction (qPCR) methods are used to confirm no residual DNA from the production microorganism remains in the final purified substances. As well no detectable levels of biogenic amines, amino acids and their metabolites have been identified in the final purified substances.

Information provided confirms that analyses of production batches of the oligosaccharides meet the contamination limits for heavy metals (lead, arsenic, cadmium and mercury) as required by section S3—4 of the Code.

2.4 Specifications

There are no specifications for the substances 2′-FL/DFL, LNT, 6′-SL and 3′-SL within the primary (S3—2) and secondary (S3—3) sources of specifications in Schedule 3. Specifications for previously approved HiMOs - 2'-FL and LNnT - are written into S3—2(2).

FSANZ assessed the manufacturing specifications and analytical results and has proposed the specifications in Tables 2-4 for inclusion in Schedule 3 of the Code. Limits for Enterobacteriacea, yeasts, moulds, and residual endotoxins are process hygiene parameters which are consistent with specifications for similar HiMO substances permitted in the Code. Schedule 27 food safety microbiological limits for *Cronobacter* (absent in 10 g) and *Salmonella* (absent in 25 g) in IFP apply to the substances and therefore are not listed in the specification. For heavy metals lead, cadmium, mercury and arsenic, the default values listed in S3—4 apply. For all substances 2′-FL/DFL, LNT, 6′-SL and 3′-SL, the Applicant has specified a limit for lead (0.1 mg/kg) which being lower (i.e. more restrictive) than the default value for lead (2 mg/kg), does not pose a risk to infant health.

Table 2 Specification for 2'-FL/DFL – compositional parameters

Parameter	**2'FL/DFL**
2′-FL	Not less than 75.0%
DFL	Not less than 5.0%
Sum of 2′-FL and DFL	Not less than 85.0%
Sum of human identical milk saccharides: 2′-FL, DFL, D-lactose, L-fucose, 3-FL[2]	Not less than 92.0%
D-lactose	Not more than 10%
L-fucose	Not more than 1.0%
2'-fucosyl-D-lactulose	Not more than 2.0%
pH (20°C, 5% solution)	4.0-6.0
Water	Not more than 6.0%
Ash, sulphated	Not more than 0.8%
Residual protein	Not more than 0.01%
Lead	0.1 mg/kg
Microbiological	
Aerobic mesophilic bacteria total count	Not more than 1,000 cfu/g
Enterobacteriaceae	Absent in 10 g
Yeasts	Not more than 100 cfu/g
Moulds	Not more than 100 cfu/g
Residual endotoxins	Not more than 10 E.U./mg

Table 3 Specification for LNT – compositional parameters

Parameter	**LNT**
LNT	Not less than 70.0%
Sum of saccharides (human identical milk saccharides (HiMS)	LNT, D-lactose, lacto-N-triose II: not less than 90.0%
D-lactose	Not more than 12.0%
Lacto-N-triose II	Not more than 10.0%
para-lacto-N-hexaose	Not more than 3.5%
LNT fructose isomer[1]	Not more than 1.0%
pH (20°C, 5% solution)	4.0-6.0
Water	Not more than 6.0%
Residual protein	Not more than 0.01%
Ash, sulphated	Not more than 0.5%
Lead	0.1 mg/kg
Microbiological:	
Aerobic mesophilic bacteria total count	Not more than 1,000 cfu/g
Enterobacteriaceae	Absent in 10 g
Yeasts	Not more than 100 cfu/g
Moulds	Not more than 100 cfu/g

[1] Chemical name: β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-fructose

[2] 3-FL refers to 3-fucosyllactose

Table 4 Specifications for 6′-SL and 3′-SL sodium salts – compositional parameters

Parameter	6′-SL sodium salt	3′-SL sodium salt
The HiMO purity	Not less than 90.0%	Not less than 88.0%
Sum of saccharides (human identical milk saccharides (HiMS)	6′-SL sodium salt, D-lactose, sialic acid: not less than 94.0%	3′-SL sodium salt, D-lactose, sialic acid: not less than 90.0%
D-lactose	Not more than 5.0%	Not more than 5.0%
Sialic acid	Not more than 2.0%	Not more than 1.5%
Sialyl-lactulose	6'- isomer: not more than 3.0%	3'- isomer: not more than 5.0%
Sodium	2.5-4.5%	2.5-4.5%
Chloride	Not more than 1.0%	Not more than 1.0%
pH (20°C, 5% solution)	4.5-6.0	4.5-6.0
Water	Not more than 6.0%	Not more than 8.0%
Residual protein	Not more than 0.01%	Not more than 0.01%
Lead	Not more than 0.1 mg/kg	Not more than 0.1 mg/kg
Microbiological:		
Aerobic mesophilic total plate count	Not more than 1,000 cfu/g	Not more than 1,000 cfu/g
Enterobacteriaceae	Absent in 10 g	Absent in 10 g
Yeasts	Not more than 100 cfu/g	Not more than 100 cfu/g
Moulds	Not more than 100 cfu/g	Not more than 100 cfu/g
Residual endotoxins	Not more than 10 EU/mg	Not more than 10 EU/mg

2.5 Analytical methods for detection

The analytical methods of analyses to detect and quantify the presence of the 2′-FL/DFL, LNT, 6′-SL and 3′-SL in milk products is based on high performance liquid chromatography (HPLC) (Austin and Benet 2018). The Application also provided analytical methods used to quantify the parameters listed in their specifications for each of the oligosaccharides. Data provided in the Application as CCI Certificates of Analysis for five non-consecutive batches confirm each substance 2′-FL/DFL, LNT, 6′-SL and 3′-SL is able to meet the specification provided.

2.6 Food technology conclusion

FSANZ concludes from its assessment of the data provided in the Application that 2′-FL/DFL, LNT, 6′-SL and 3′-SL produced by a microbial fermentation method of production are chemically and structurally identical to naturally occurring substances in human milk.

Stability studies of 2′-FL/DFL, LNT, 6′-SL and 3′-SL incorporated in powdered infant formula products conclude they are stable up to 30 months even when stored at 37°C. Such storage times exceed the generally understood shelf like of two years for powdered infant formulas. When the powders are made up in aqueous solutions to be fed to infants the substances are also stable for at least 24 hours. This is the maximum recommended storage time for made up infant formula stored at refrigerated temperatures.

The Application provided specifications for each substance. Multi-batch analyses showed that 2′-FL/DFL, LNT, 6′-SL and 3′-SL are able to be produced consistently to meet their

proposed specifications. Therefore, the food technology assessment concludes that the substances 2′-FL/DFL, LNT, 6′-SL and 3′-SL are sufficiently characterised and pose no risk to infant health from a food technology perspective. Tables 1-4 provide information for inclusion in Schedule 3 as specifications for the substances.

3 Safety assessment

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1 Genetically modified (GM) production strain assessment

The 2′-FL, DFL, LNT, 6′-SL and 3′-SL oligosaccharides assessed in this Application are all produced via fermentation using GM strains of *E. coli* K-12.

Notably, the GM *E. coli* K-12 used to produce 2′-FL/DFL conforms to the permitted source organism listed in Schedule 26 of the Code. Therefore, based on the FSANZ previous assessment as part of A1155 and the data provided by the Applicant, a biotechnology assessment of this production strain was not required.

3.1.1 Host organism

E. coli K-12 has a long history of use in the human biopharmaceutical industry, with ~30% of currently approved recombinant therapeutic proteins in the United States (US) being produced in *E. coli* K-12, starting with the US FDA approval of biosynthetic human insulin in 1983 (Huang et al. 2012; Jozala et al. 2016). The use of this bacterium as a source for the production of food enzymes began in the 1980s (WHO 1991). *E. coli* K-12 is permitted in the Food Standards Code as a source microorganism for the production of chymosin. *E. coli* K-12 is considered a model organism and has been thoroughly characterised for use in research and industry, it is therefore considered a safe organism.

3.1.2 Gene donor organisms

Four different gene donor organisms were assessed: *Helicobacter pylori*, *Neisseria meningitides*, *Photobacterium damsela* and *Campylobacter jejuni*. *H. pylori*, *N. meningitides and C. jejuni* have been categorised in the NIH Guidelines as Risk Group 2 agents for organisms that are associated with human disease, where the disease is rarely serious and there is likely availability of preventative or therapeutic interventions (NIH 2019). *Photobacterium damsela*, previously known a *Vibrio damsela*, is a marine bacteria and is a known pathogen for marine life including fish and dolphins. Similar to other *Vibrio* spp. it can cause opportunistic wound infections in humans through exposure to seawater (Rivas et al. 2013).

The identity of donor gene sequences were confirmed using standard bioinformatic tools. As described in Section 3.1.3 the required gene sequences were synthesised and no antimicrobial resistance or virulence genes were inserted into the *E. coli* K-12 host. No safety concerns were identified.

3.1.3 Characterisation of the genetic modification(s)

To create the LNT, 6′-SL, and 3′-SL production strains, multiple expression cassettes were generated containing the genes of interest, flanked by well characterised promoter and terminator sequences. Transformation of DNA constructs was performed by electroporation

and positive transformants were identified using standard selection techniques.

To produce LNT, *E. coli* K-12 was modified to produce beta-1,3-N-acetylglucosaminyltransferase from *N. meningitides* and beta-1,3-galactosyltransferase from *H. pylori*.

To produce 6′-SL, *E. coli* K-12 was modified to produce alpha-2,6-sialyltransferase from *P. damsela* and CMP-Neu5Ac synthetase, Neu5Ac synthase and N-acetylglucosamine-6-phosphatase epimerase from *C. jejuni*.

Finally, to produce 3′-SL, *E. coli* K-12 was modified to produce alpha-2,3-sialyltransferase from *N. meningitides* and CMP-Neu5Ac synthetase, Neu5Ac synthase and N-acetylglucosamine-6-phosphatase epimerase from *C. jejuni*.

No antibiotic resistance markers are present in any of the final production strains for LNT, 6′-SL or 3′-SL.

3.1.4 Characterisation of the genetically modified production organisms

Characterisation of all genetic modifications made to create the GM *E. coli* oligosaccharide production strains for LNT, 6′-SL, and 3′-SL were verified using standard molecular biology techniques and genomic DNA sequencing methods. No safety concerns were identified.

3.1.5 Genetic stability and inheritance of the introduced DNA

The Applicant supplied data showing that there were no changes in the gene stability or phenotypic performance over 50+ generations, for each oligosaccharide production strain under review. FSANZ was satisfied that the introduced DNA is inherited and genetically stable.

3.1.6 Key findings of the GM assessment

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and poses no risks to humans. Analyses of the gene donors also confirmed there were no safety concerns. Characterisation of the production strains confirmed integration of the introduced genes into the genome and that the inserted DNA was both genetically and phenotypically stable.

No potential safety concerns were identified in the biotechnology assessment of the LNT, 6′-SL or 3′-SL production strains.

3.2 Toxicological assessment

3.2.1 Toxicokinetics

The Applicant's 2′-FL, DFL, LNT, 6′-SL and 3′-SL are structurally and chemically identical to the forms of these substances naturally present in human milk (Section 2.1). It is not anticipated that there will be any significant differences in pharmacokinetics between naturally occurring and manufactured forms of these human milk oligosaccharides (HMOs).

FSANZ has previously reviewed data on the pharmacokinetics of HMOs including 2′-FL, DFL, LNT, 6′-SL and 3′-SL. These data included *in vitro* and *in vivo* studies as well as studies in human infants (FSANZ 2019; FSANZ 2020). Overall, the available data indicate that

intestinal absorption is limited, and a significant proportion of HMOs reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces.

3.2.2 Toxicological studies with 2′-FL/DFL

As part of Application A1155, FSANZ reviewed a 90-day oral toxicity study in rats, as well as *in vitro* genotoxicity studies, of the Applicant's 2′-FL/DFL product (92.2% 2′-FL/DFL w/w; Batch No. CPN6317 1000517 FD) (Phipps et al. 2018a). 2′-FL/DFL was not mutagenic, clastogenic or aneugenic *in vitro* and no treatment-related adverse effects were observed in the 90-day toxicity study. The no observed adverse effect level (NOAEL) was 5000 mg/kg bw/day 2′-FL/DFL, the highest dose tested.

3.2.3 Toxicological studies with LNT

The same test item was used in all toxicity and genotoxicity studies of LNT (Batch No. CPN4215 1000416 FD; 77.0% w/w LNT). Doses/concentrations used in these studies were adjusted to account for the presence of other carbohydrates in the test item, and are expressed in terms of pure LNT.

In vivo toxicity studies with LNT

14-day dose-range finding study in neonatal rats (Stannard 2019b) Regulatory status: non-GLP; non-guideline

In a dose-range finding study, groups of neonatal Crl:CD(SD) rats (8/sex/group) were administered 0, 3250 or 4000 mg/kg bw/day LNT by oral gavage from Day 7 of age for 14 days. Water was used as the vehicle control. The high dose in this study was the maximum feasible dose taking into account LNT's solubility in water and viscosity of the prepared LNT formulation. The study was conducted in a facility operating in accordance with GLP principles, although GLP compliance was not claimed for this study. All animals were weighed and observed daily for changes in clinical condition. At the end of the study animals were subjected to a gross necropsy.

No deaths, adverse clinical signs or differences in body weight were observed. No treatment-related macroscopic abnormalities were observed at necropsy.

It was concluded that LNT was well tolerated and that 4000 mg/kg bw/day LNT would be an acceptable high dose for a 90-day toxicity study in neonatal rats.

90-day toxicity study in neonatal rats (Phipps et al. 2018b; Stannard 2018) Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

Groups of neonatal Crl:CD(SD) rats (10/sex/group) were administered 0, 1000, 2500 or 4000 mg/kg bw/day LNT by oral gavage from postnatal day (PND) 7 for at least 90 days. The vehicle control was water. An additional reference control group (10/sex/group) was administered 5000 mg/kg bw/day oligofructose. A further 5 males and 5 females in each group were dosed daily for at least 90 days and then kept without dosing for 4 weeks to assess reversibility of any observed effects.

Clinical observations were performed daily, with detailed physical examinations performed daily from PND 7 – 20 and weekly thereafter. Body weights were recorded daily until weaning on PND 21 and twice weekly thereafter. Food intake was recorded twice weekly from weaning. Ophthalmological examinations were performed in the control, high-dose and reference control groups during Week 13. Pre-weaning reflex development (eye opening, air

righting, startle response and pupil closure response), ulna length and sexual maturation (balano-preputial separation and vaginal opening) were recorded for all animals. All animals were subjected to a functional observational battery in Week 11, comprising observations in-hand and in a standard arena, in addition to assessments of grip strength and learning and memory (using the Morris water maze). Blood samples were collected for haematology, coagulation and clinical chemistry analysis during Week 13 and at the end of the treatment-free period. Urine samples were collected for urinalysis during Week 13 and from recovery animals at the end of the treatment-free period. All surviving animals were subjected to gross necropsy at the end of the treatment or recovery periods, and organ weights were recorded. Tissues from animals that were killed or died prematurely, animals in the vehicle control and high-dose main phase groups, as well as any tissues with macroscopic abnormalities from other groups, were examined microscopically.

There were no test item-related deaths. Six animals died or were killed for welfare reasons during the study: one male and one female from the vehicle control group, one reference control male, one male and one female given 1000 mg/kg bw/day LNT and one female given 4000 mg/kg bw/day LNT. The females in the vehicle control, reference control and low dose LNT groups had findings indicative of an intubation error (perforated oesophagus). The cause of death for the other three animals was undetermined but all were considered to be incidental and unrelated to treatment.

There were no test-item related clinical signs or differences in body weight, food consumption, ophthalmic findings, pre-weaning development, ulna growth and sexual maturation. No test item-related differences in the functional observational battery were observed, with clear evidence of learning and memory. No treatment-related adverse effects on haematology, coagulation, clinical chemistry or urinalysis parameters were observed. Organ weights were unaffected by treatment with the test item. There were no treatment-related macroscopic abnormalities or histopathological findings.

The NOAEL in this study was 4000 mg/kg bw/day LNT, the highest dose tested.

Genotoxicity studies with LNT

Bacterial reverse mutation assay (Phipps et al. 2018b; Šoltésová 2018b) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

Test systems for this assay were *Salmonella enterica* ser. Typhimurium strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli* strain WP2 *uvr*A. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method, and both assays were performed in the presence and absence of metabolic activation (S9 mix). Concentrations of up to 5106 µg/plate LNT were tested. Water was used as the solvent and negative control. Positive controls in the absence of metabolic activation were sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide. In the presence of metabolic activation positive controls were 2-aminoanthracene and benzo[a]pyrene.

No increases in the number of revertant colonies compared with negative controls were observed following treatment with LNT in either test, in the presence or absence of metabolic activation. The positive controls produced the expected increases in revertant colonies, confirming the validity of the test system.

It was concluded that LNT was not mutagenic at concentrations up to 5106 µg/plate.

In vitro micronucleus assay in human lymphocytes (Gilby 2018b; Phipps et al. 2018b)

Regulatory status: GLP; conducted in accordance with OECD TG 487 (2016)

LNT was assessed for its potential to induce micronuclei in cultured human peripheral lymphocytes. Cells were exposed to LNT for 3 hours in the absence or presence of metabolic activation (S9 mix), and for 20 hours in the absence of S9. At the end of the 3-hour exposures the medium was replaced and cells were treated with the cytokinesis inhibitor cytochalasin B for an additional 17 hours until harvest. For cell cultures under long-term exposure, cytochalasin B was added at the beginning of treatment. The vehicle control was water. Positive controls were mitomycin C and colchicine in the absence of S9 and cyclophosphamide in the presence of S9. Based on the results of a preliminary cytotoxicity test, LNT concentrations up to 2042 µg/mL were evaluated for the induction of micronuclei.

No evidence of cytotoxicity was observed following exposure to LNT. Following short-term exposure in the absence of S9, a statistically significant increase in the percentage of micronucleated binucleate cells (MNBC) was observed at the highest LNT concentration compared with vehicle controls, which was also associated with a statistically significant linear trend. However, as the mean value was below the laboratory historical vehicle control upper limit, the increase at this concentration was not considered to be biologically relevant and the results were considered negative overall. LNT did not cause any significant increases in the number of MNBC compared with vehicle controls following 3-hour treatment in the presence of S9 or after 20-hour treatment in the absence of S9. The positive controls produced substantial increases in the number of MNBC compared with the vehicle controls and the historical vehicle control range, confirming the sensitivity of the assay and the metabolic activity of the S9 preparations.

It was concluded that LNT was not clastogenic or aneugenic under the conditions of this study.

3.2.4 Toxicological studies with 6′-SL

The same test item was used in all toxicity and genotoxicity studies of 6′-SL produced by the Applicant (Batch No. CPN5615 1000317 FD; 96.8% w/w 6′-SL). Doses/concentrations used in these studies were adjusted to account for the presence of other carbohydrates in the test item, and are expressed in terms of pure 6′-SL.

In addition, studies with 6′-SL produced by GeneChem Inc (Republic of Korea) are available in the scientific literature. These studies have also been reviewed.

In vivo toxicity studies with 6′-SL produced by the Applicant

14-day dose-range finding study in neonatal rats (Flaxmer 2018a; Phipps et al. 2019b)
Regulatory status: non-GLP; non-guideline

In a dose-range finding study, groups of neonatal Crl:CD(SD) rats (8/sex/group) were administered 0, 4000 or 5000 mg/kg bw/day 6′-SL by oral gavage from Day 7 of age for 14 days. Water was used as the vehicle control. The total dose of the test item (purity 95.8% w/w 6′-SL) was adjusted to account for the presence of other carbohydrates (i.e. total doses of 0, 4176 or 5220 mg/kg bw/day test item were administered). The study was conducted in a facility operating in accordance with GLP principles, although GLP compliance was not claimed for this study. All animals were weighed and observed daily for changes in clinical condition. At the end of the study animals were subjected to a gross necropsy.

No deaths, adverse clinical signs or treatment-related differences in body weight were observed. No treatment-related macroscopic abnormalities were observed at necropsy.

It was concluded that 6′-SL was well tolerated and that 5000 mg/kg bw/day 6′-SL would be an acceptable high dose for a 90-day toxicity study in neonatal rats.

90-day oral gavage toxicity study in neonatal rats (Flaxmer 2018b; Phipps et al. 2019b); Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

Groups of neonatal Crl:CD(SD) rats (10/sex/group) were administered 0, 1000, 3000 or 5000 mg/kg bw/day 6′-SL by oral gavage from postnatal day (PND) 7 for 90 days. The vehicle control was water. An additional reference control group (10/sex/group) was administered 5000 mg/kg bw/day oligofructose, as a non-digestible oligosaccharide already permitted in infant nutrition. Additional groups of 5 males and 5 females were administered 0 or 5000 mg/kg bw/day 6′-SL, or 5000 mg/kg bw/day oligofructose, for 90 days and then kept without dosing for 4 weeks to assess the reversibility of any observed effects.

Clinical observations were performed daily, with detailed physical examinations performed daily from PND 7 – 20 and weekly thereafter. Body weights were recorded daily until weaning on PND 21 and twice weekly thereafter. Food intake was recorded twice weekly from weaning. Ophthalmological examinations were performed in the control, high-dose and reference control groups during Week 13. Pre-weaning reflex development (eye opening, air righting, startle response and pupil closure response), ulna length and sexual maturation (balano-preputial separation and vaginal opening) were recorded for all animals. All animals were subjected to a functional observational battery in Weeks 11 and 12, comprising observations in-hand and in a standard arena, in addition to assessments of grip strength and learning and memory (using the Morris water maze). Blood and urine samples were collected for haematology, coagulation, clinical chemistry and urinalysis during Week 13 and from recovery animals at the end of the treatment-free period. All surviving animals were subjected to gross necropsy at the end of the treatment or recovery periods. At the end of the treatment period organ weights were recorded for all surviving animals, and tissues from the vehicle control and high-dose groups were examined microscopically. Microscopic examinations of the testes and epididymes were also performed on mid- and low-dose males, and on control, high-dose and reference control males at the end of the treatment-free period.

No treatment-related deaths or clinical signs were recorded. One male in the reference control group was killed for welfare reasons on PND 94 (day 88 of dosing) with clinical signs including gasping and unresponsiveness. The only notable macroscopic findings in this animal were depressions on the kidneys, which correlated with a minimal severity infiltrate of mononuclear inflammatory cells in the renal cortex seen microscopically. The cause of the animal's poor clinical condition could not be identified after macroscopic and microscopic examination, but as it was an isolated instance it was considered unrelated to administration of the reference control. One male in the high-dose group was found dead on PND 20 with no notable macroscopic or microscopic findings reported. As this was an isolated instance it was considered unrelated to the administration of the test item.

There were no treatment-related differences in body weight or food consumption compared with controls. No treatment-related differences in ophthalmic findings, pre-weaning development, ulna growth and sexual maturation were observed. There were no test item-related differences in the functional observational battery, with clear evidence of learning and memory observed. There were no treatment-related adverse effects on haematology, coagulation, clinical chemistry or urinalysis parameters. Organ weights were unaffected by treatment with the test item.

No treatment-related adverse macroscopic or microscopic changes were observed. At the

end of the dosing period, four males in the high-dose group had a small, soft testis and small epididymis. This change was unilateral, with contralateral organs unchanged. Microscopic examination found unilateral tubular atrophy in the testis and absence of sperm in the epididymis, in the same tissue as was affected macroscopically. Unilateral tubular atrophy (minimal) was also observed in the testis of one male in the reference control group. The study pathologist concluded these changes were incidental and unlikely to be related to treatment based on the following considerations: Complete unilateral testicular tubular atrophy is occasionally seen in young male rats as an incidental background change (Creasy 2012; Sahota PS 2013), and given it was seen unilaterally it is highly unlikely there was any direct effect on spermatogenesis, where a bilateral change would be expected. The severity of atrophy and absence of sperm in the epididymis indicate a level of chronicity rather than an ongoing degenerative change, possibly even of a condition pre-existing before start of study. Significantly the absence of these findings in the recovery animals substantiates this, as complete recovery of testicular and epididymal findings would not be expected in just 4 weeks. Finally, there were no test-item related findings reported in a recently published 90-day study also conducted on 6′-SL (Gurung et al. 2018).

The study author considered it was not possible to conclusively rule out a test item-related effect, as the incidence was outside the historical control range for studies of this type performed at the laboratory. On this basis the study author concluded the mid-dose, 3000 mg/kg bw/day, was the NOAEL. FSANZ considers the available evidence indicates these changes were highly unlikely to be treatment-related, however, and concluded the NOAEL was 5000 mg/kg bw/day 6′-SL, the highest dose tested.

Genotoxicity studies with 6'-SL produced by the Applicant

Bacterial reverse mutation assay (Šoltésová 2018a; Phipps et al. 2019b) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

Test systems for this assay were *Salmonella enterica* ser. Typhimurium strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli* strain WP2 *uvr*A. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method, and both assays were performed in the presence and absence of metabolic activation (S9 mix). Concentrations of up to 5000 µg/plate 6′-SL were tested. Water was used as the solvent and negative control. Positive controls in the absence of metabolic activation were sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide. In the presence of metabolic activation positive controls were 2-aminoanthracene and benzo[a]pyrene.

No increases in the number of revertant colonies compared with negative controls were observed following treatment with 6′-SL in either test, in the presence or absence of metabolic activation. The positive controls produced the expected increases in revertant colonies, confirming the validity of the test system.

It was concluded that 6′-SL was not mutagenic at concentrations up to 5000 µg/plate.

In vitro micronucleus assay in human lymphocytes (Gilby 2018a; Phipps et al. 2019b) Regulatory status: GLP; conducted in accordance with OECD TG 487 (2016)

6′-SL was assessed for its potential to induce micronuclei in cultured human peripheral lymphocytes. Cells were exposed to 6′-SL for 3 hours in the absence or presence of metabolic activation (S9 mix), and for 20 hours in the absence of S9. At the end of the 3-hour exposures the medium was replaced and cells were treated with the cytokinesis inhibitor cytochalasin B for an additional 17 hours until harvest. For cell cultures under long-term

exposure, cytochalasin B was added at the beginning of treatment. The vehicle control was water. Positive controls were mitomycin C and colchicine in the absence of S9 and cyclophosphamide in the presence of S9. Based on the results of a preliminary cytotoxicity test, 6′-SL concentrations up to 2000 µg/mL were evaluated for the induction of micronuclei.

No evidence of cytotoxicity was observed following exposure to 6′-SL. The test item did not induce any increases in the numbers of micronucleated binucleate cells (MNBC) compared with vehicle controls in any of the tests. The positive controls produced substantial increases in the number of MNBC compared with vehicle controls, confirming the sensitivity of the assay and the metabolic activity of the S9 preparations.

It was concluded that 6′-SL was not clastogenic or aneugenic under the conditions of this study.

Toxicity studies with 6'-SL produced by other manufacturers

Several toxicity studies were conducted with 6′-SL (>98% purity, 0.09% lactose, 1.13% N-acetylneuraminic acid) produced by GeneChem Inc., Republic of Korea. The test item was produced by enzymatic synthesis using a strain of beta-D-galactosidase deficient *E. coli* BW25113 originating from *E. coli* K-12.

Acute oral toxicity study in rats (Gurung et al. 2018) Regulatory status: GLP; conducted in accordance with US FDA guidelines

The acute oral toxicity of 6′-SL was evaluated in Sprague Dawley rats. Rats (5/sex/group; age 6 – 7 weeks) were administered single oral gavage doses of 0, 5000, 10,000, 15,000 or 20,000 mg/kg bw 6′-SL and monitored for 14 days. The vehicle control was water. At the end of the study animals were killed and organs were weighed and examined macroscopically.

No animals died during the 14-day observation period and no clinical signs of toxicity were observed. There were no differences in body weight, food consumption and water intake, organ weights or macroscopic observations between treated animals and controls. It was concluded that the LD_{50} of 6′-SL was > 20,000 mg/kg bw.

13-week oral gavage toxicity study in rats (Gurung et al. 2018) Regulatory status: GLP; conducted in accordance with US FDA guidelines

Sprague-Dawley rats (11/sex/group; age 6-7 weeks) were administered 6′-SL at doses of 0, 1000, 2500 or 5000 mg/kg bw/day by oral gavage for 13 weeks. Water was used as the vehicle control. Animals were observed twice daily for clinical signs. Body weight and food consumption were recorded weekly. All animals underwent ophthalmoscopic examination pre-study, and the eyes of control and high-dose animals were examined towards the end of the study. Blood samples were collected at the end of the study for haematology and clinical chemistry analysis. Urine samples were collected during the last week of the study for urinalysis. Animals were killed at the end of the study and subjected to external and internal gross pathological examination. Organ weights were recorded and any gross lesions identified in tissues from animals in the control and high-dose groups were examined microscopically.

All animals survived to the end of the study and no clinical signs of toxicity were observed. There were no treatment-related adverse effects on body weight, food consumption, ophthalmology, haematology, clinical chemistry or urinalysis parameters. Organ weights were not affected by treatment and no treatment-related macroscopic changes were observed.

It was concluded that the NOAEL in this study was 5000 mg/kg bw/day, the highest dose tested.

21-day dietary study in neonatal piglets (Monaco et al. 2020) Regulatory status: Non-GLP, non-guideline

Two-day old male and female piglets (12/group; strain unspecified) were provided sow-milk replacer formula containing 0, 300, 600 or 1200 mg/L 6′-SL for 21 days. Feed intake and body weight were recorded daily. Blood samples were collected on study days 8 and 22 for haematology, clinical chemistry and coagulation time analyses. Urine samples for urinalysis were collected on study day 22. At the end of the study brain, spleen, stomach, kidneys, heart, lungs, intestine and liver weights were recorded. Selected tissues from the control and high dose animals were subjected to microscopic histological analysis.

One animal in the control group was removed from the study following observations of watery diarrhoea over three days. No other clinical signs were reported. There were no differences in formula consumption, body weight or body weight gain between treated piglets and controls. No treatment-related changes in haematology, clinical chemistry, coagulation and urinalysis parameters were observed. There were no treatment-related changes in absolute or relative (to body weight) organ weights, intestinal length, pH of the colonic contents or histological analyses.

It was concluded that formula containing 6′-SL supported normal growth and development of neonatal piglets at concentrations up to 1200 mg/L, the highest concentration tested.

Genotoxicity studies

Genotoxicity studies have also been conducted with 6′-SL produced by GeneChem Inc. These studies were GLP compliant and conducted according to US Food and Drug Administration Redbook guidelines. The positive controls in these studies produced the expected responses. As summarised in Table 5, 6′-SL showed no evidence of mutagenicity, clastogenicity or aneugenicity in these studies.

Table 5 Summary of genotoxicity studies with other 6'-SL preparations

Test	**Test system**	**Test article**	**Concentration or dose range**	**Result**	**Reference**
Bacterial reverse mutation assay (plate incorporation method)	*S. enterica* ser. Typhimurium strains TA97, TA98, TA100, TA102 & TA1535	6′-SL (>98% purity) Vehicle: saline Positive controls: 4-Nitro-o-phenylenediamine, daunomycin, sodium azide & methyl methanesulfonate (-S9); 2-aminofluorene, 1,8-dihydroxyanthraquino	100 – 5000 µg/plate	Negative ± S9 No cytotoxicity	(Gurung et al. 2018)

		ne & 2-aminoanthracene (+ S9)			
In vitro mammalian chromosomal aberration test	Chinese hamster lung cells	6′-SL (>98% purity) Vehicle: saline Positive controls: mitomycin C (- S9); cyclophosphamide (+ S9)	225 – 900 µg/plate	Negative ± S9	(Gurung et al. 2018)
In vivo mammalian erythrocyte micronucleus test	Kunming mice (age 4-5 weeks) Oral gavage 24h and 48h sampling times	6′-SL (>98% purity) Vehicle: water Positive control: cyclophosphamide	500, 1000 & 2000 mg/kg bw/day for 2 days; samples collected 24 & 48h after dosing	Negative No clinical signs of toxicity	(Gurung et al. 2018)

3.2.5 Toxicological studies with 3′-SL

The same test item was used in all toxicity and genotoxicity studies of 3′-SL produced by the Applicant (Batch No. CPN5115 1000516 FD; 90.3 % w/w 3′-SL). Doses/concentrations used in these studies were adjusted to account for the presence of other carbohydrates in the test item, and are expressed in terms of pure 3′-SL.

In addition, studies with 3′-SL produced by GeneChem Inc. (Republic of Korea) are available in the scientific literature. These studies have also been reviewed.

In vivo toxicity studies with 3′-SL produced by the Applicant

14-day dose-range finding study in neonatal rats (Stannard 2019b) Regulatory status: non-GLP; non-guideline

In a dose-range finding study, groups of neonatal Crl:CD(SD) rats (8/sex/group) were administered 0, 4000 or 5000 mg/kg bw/day 3′-SL by oral gavage from Day 7 of age for 14 days. Water was used as the vehicle control. The total dose of the test item was adjusted to account for the presence of other carbohydrates (i.e. total doses of 0, 4264 or 5330 mg/kg bw/day test item were administered). The study was conducted in a facility operating in accordance with GLP principles, although GLP compliance was not claimed for this study. All animals were weighed and observed daily for changes in clinical condition. At the end of the study animals were subjected to a gross necropsy.

There were no treatment-related deaths. One male administered 4000 mg/kg bw/day 3′-SL was found dead on Day 14 of dosing. This animal had shown no changes in clinical condition but gained slightly less weight than the other males in this group between Days 13 and 14 of dosing. No abnormalities were observed on macroscopic examination, and there was no evidence of dosing trauma. In the absence of any other deaths during the study, the death was considered incidental and unrelated to treatment. No adverse clinical signs and no

treatment-related differences in body weight were observed. No treatment-related macroscopic abnormalities were observed at necropsy.

It was concluded that 3′-SL was well tolerated and that 5000 mg/kg bw/day 3′-SL would be an acceptable high dose for a 90-day toxicity study in neonatal rats.

90-day oral gavage toxicity study in neonatal rats (Phipps et al. 2019a; Stannard 2019a); Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

Groups of neonatal Crl:CD(SD) rats (10/sex/group) were administered 0, 1000, 3000 or 5000 mg/kg bw/day 3′-SL by oral gavage from postnatal day (PND) 7 for at least 90 days. The vehicle control was water. An additional reference control group (10/sex/group) was administered 5000 mg/kg bw/day oligofructose. Additional groups of 5 males and 5 females were administered 0 or 5000 mg/kg bw/day 3′-SL, or 5000 mg/kg bw/day oligofructose, for at least 90 days and then kept without dosing for 4 weeks to assess the reversibility of any observed effects.

Clinical observations were performed daily, with detailed physical examinations performed daily from PND 7 – 20 and weekly thereafter. Body weights were recorded daily until weaning on PND 21 and twice weekly thereafter. Food intake was recorded twice weekly from weaning. Water consumption was recorded daily during Week 13 of treatment for main study animals and during Week 4 of the treatment-free period for recovery phase animals. Ophthalmological examinations were performed in the control, high-dose and reference control groups during Week 13. Pre-weaning reflex development (eye opening, air righting, startle response and pupil closure response), ulna length and sexual maturation (balano-preputial separation and vaginal opening) were recorded for all animals. All animals were subjected to a functional observational battery in Weeks 11 and 12, comprising observations in-hand and in a standard arena, in addition to assessments of grip strength and learning and memory (using the Morris water maze). Blood samples were collected for haematology, coagulation and clinical chemistry analysis during Week 13. Blood samples for clinical chemistry were collected from recovery phase animals in Week 4 of the treatment-free period. Urine samples were collected for urinalysis during Week 13 and from recovery animals at the end of the treatment-free period. All surviving animals were subjected to gross necropsy at the end of the treatment or recovery periods. Organ weights were recorded for all surviving animals at the end of the treatment period. Tissues from the vehicle control and high-dose main phase groups, as well as any tissues with abnormalities from other groups, were examined microscopically.

There were no test item-related deaths. One female in the low dose group was killed on welfare grounds on PND 80 (Day 74 of dosing), due to clinical signs of rapid respiration, thin build and whole body pallor. Histopathology found a number of changes including marked haemorrhagic necrosis of the adrenal cortex, which was considered to be the major factor contributing to death. This isolated death was considered to be incidental and unrelated to treatment.

There were no test item-related clinical signs and body weight, body weight gain and food consumption were similar in treated groups compared with controls. No treatment-related differences in ophthalmic findings, pre-weaning development, ulna growth and sexual maturation were observed. There were no test item-related differences in the functional observational battery, with clear evidence of learning and memory observed. No treatment-related adverse effects on haematology, coagulation, clinical chemistry or urinalysis parameters were observed. Organ weights were unaffected by treatment with the test item. No treatment-related macroscopic or microscopic abnormalities were observed.

The NOAEL in this study was 5000 mg/kg bw/day 3′-SL, the highest dose tested.

Genotoxicity studies with 3'-SL produced by the Applicant

Bacterial reverse mutation assay (Phipps et al. 2019a; Šoltésová 2019) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

Test systems for this assay were *Salmonella enterica* ser. Typhimurium strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli* strain WP2 *uvr*A. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method, and both assays were performed in the presence and absence of metabolic activation (S9 mix). Concentrations of up to 5000 µg/plate 3′-SL were tested. Water was used as the solvent and negative control. Positive controls in the absence of metabolic activation were sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide. In the presence of metabolic activation positive controls were 2-aminoanthracene and benzo[a]pyrene.

No increases in the number of revertant colonies compared with negative controls were observed following treatment with 3′-SL in either test, in the presence or absence of metabolic activation. The positive controls produced the expected increases in revertant colonies, confirming the validity of the test system.

It was concluded that 3′-SL was not mutagenic at concentrations up to 5000 µg/plate.

In vitro micronucleus assay in human lymphocytes (Gilby 2019; Phipps et al. 2019a) Regulatory status: GLP; conducted in accordance with OECD TG 487 (2016)

3′-SL was assessed for its potential to induce micronuclei in cultured human peripheral lymphocytes. Cells were exposed to 3′-SL for 3 hours in the absence or presence of metabolic activation (S9 mix), and for 20 hours in the absence of S9. At the end of the 3-hour exposures the medium was replaced and cells were treated with the cytokinesis inhibitor cytochalasin B for an additional 17 hours until harvest. For cell cultures under long-term exposure, cytochalasin B was added at the beginning of treatment. The vehicle control was water. Positive controls were mitomycin C and colchicine in the absence of S9 and cyclophosphamide in the presence of S9. Based on the results of a preliminary cytotoxicity test, 3′-SL concentrations up to 2000 µg/mL were evaluated for the induction of micronuclei.

No evidence of cytotoxicity was observed following exposure to 3′-SL. The test item did not induce any increases in the numbers of micronucleated binucleate cells (MNBC) compared with vehicle controls in any of the tests. The positive controls produced substantial increases in the number of MNBC compared with vehicle controls, confirming the sensitivity of the assay and the metabolic activity of the S9 preparations.

It was concluded that 3′-SL was not clastogenic or aneugenic under the conditions of this study.

Toxicity studies with 3'-SL produced by other manufacturers

Several toxicity studies were conducted with 3′-SL produced by GeneChem Inc., Republic of Korea. The test item (> 98% purity; 0.25% lactose; 0.46% 3′-sialylgalactose; 0.49% sialic acid) was produced by enzymatic synthesis using a strain of beta-D-galactosidase deficient *E. coli* BW25113.

Acute oral toxicity study in rats (Kim et al. 2018) Regulatory status: GLP; conducted in

accordance with US FDA Redbook guidelines (2000)

Sprague Dawley (Crl:CD[SD]) rats (age 6 weeks; 5/sex/group) were administered a single dose of 0, 5000, 10,000 or 20,000 mg/kg bw 3′-SL by oral gavage (vehicle not reported). Animals were observed for 14 days for changes in clinical signs, body weight and food and water consumption. At the end of the study animals were killed and major organs examined macroscopically and microscopically.

All animals survived to the end of the study and no abnormal clinical signs were observed. There were no treatment-related changes in body weight, food and water intake, organ weights and no treatment-related macroscopic or microscopic findings. The LD_{50} of 3′-SL was > 20,000 mg/kg bw, the highest dose tested.

Dose escalation oral toxicity study in dogs (Kim et al. 2018) Regulatory status: GLP; conducted in accordance with US FDA Redbook guidelines

Beagle dogs aged 4-5 months (1-2/sex/group) were administered single escalating doses of 500, 1000 and 2000 mg/kg bw 3′-SL at 4 day intervals. Doses were administered by oral gavage. A vehicle control group were administered water at the same doses. Animals were observed for general condition, motor activity, autonomic nerve activity and excretion at 0.5, 1, 2, 4 and 6 hours after substance administration on each dosing day. From the next day dogs were observed twice daily for 2 weeks for any morbidity or mortality. Body weights were recorded before each dose was administered, the day after dosing and on days 1, 3, 7 and 14 after final dosing.

No deaths occurred during the study. With the exception of transient diarrhoea observed for 4 hours after dosing in the 2 males and 1 female given 2000 mg/kg bw, no treatment-related effects on clinical signs, body weights or food consumption were observed. Ophthalmic examinations found no abnormalities.

It was concluded that the maximum tolerated dose of 3′-SL in male and female Beagle dogs was > 2000 mg/kg bw.

28-day toxicity study in rats (Kim et al. 2018) Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

Sprague Dawley (Crl:CD[SD]) rats (age 6 weeks; 10/sex/group) were administered 0, 500, 1000 or 2000 mg/kg bw/day 3′-SL by oral gavage for 28 days. Water was used as the vehicle control. Rats were assessed for clinical signs, body weight, food consumption, clinical chemistry, urinalysis and organ weights. Ophthalmic and gross post mortem examinations were performed and selected tissues underwent histopathological investigation.

The authors reported that no treatment-related abnormalities were observed in any of the parameters assessed.

90-day oral toxicity study in rats (Kim et al. 2018) Regulatory status: GLP; conducted in accordance with OECD TG408

3′-SL was administered to Sprague Dawley (Crl:CD[SD]) rats (age 6 weeks; 10/sex/group) at doses of 0, 500, 1000 or 2000 mg/kg bw/day for 90 days. The vehicle control was water. All animals were assessed for clinical signs, body weights, food consumption, ophthalmic abnormalities, haematology, clinical chemistry and organ weights. Gross examinations were performed post mortem and selected tissues underwent histopathological examinations.

All animals survived to the end of the study and no treatment-related clinical signs were observed. There were no treatment-related adverse effects on body weight, food consumption, ophthalmology, haematology, clinical chemistry, urinalysis, organ weights, gross necropsy or histopathology examinations.

The NOAEL in this study was 2000 mg/kg bw/day 3′-SL, the highest dose tested.

21-day dietary study in neonatal piglets (Monaco et al. 2019) Regulatory status: Non-GLP, non-guideline

Male and female piglets (12 per group; sex ratio and strain not reported) were fed sow-milk replacer formula containing 0, 140, 200 or 500 mg/L 3′-SL from two days of age for 21 days. Body weight and formula intake was recorded daily. Blood samples were collected on study day 8 for clinical chemistry and coagulation time analyses, and on study day 22 for analysis of haematology, clinical chemistry and coagulation time. Urine samples were collected at the end of the study. At study termination organ weights and length of the small and large intestine were recorded, and microscopic histological analysis was performed on selected tissues from the control and high dose groups.

Sow-milk replacer containing 3′-SL was well tolerated over the study period. Three piglets in the low dose group were removed on study days 6-8 due to watery diarrhoea, this was considered incidental as it was not observed at higher concentrations. No treatment-related differences in body weight, body weight gain or formula consumption were observed. There were no treatment-related changes in haematology, clinical chemistry, coagulation time or urinalysis parameters. Absolute and relative (to body weight) organ weights and intestinal length were similar in all groups. No treatment-related changes in histological findings were observed.

It was concluded that swine milk replacer formula supplemented with 3′-SL supported normal growth and development at concentrations up to 500 mg/L, the highest concentration tested.

Genotoxicity studies

Genotoxicity studies have also been conducted with 3′-SL produced by GeneChem Inc. These studies were GLP compliant and conducted according to OECD test guidelines. The positive controls in these studies produced the expected responses. As summarised in Table 6, 3′-SL showed no evidence of mutagenicity, clastogenicity or aneugenicity in these studies.

Table 6 Summary of genotoxicity studies with other 3'-SL preparations

Test	Test system	Test article	Concentration or dose range	Result	Reference
Bacterial reverse mutation assay	*S. enterica* ser. Typhimurium strains TA98, TA100, TA1535 & TA1537; *E. coli* strain WP2*uvr*A	3′-SL (98.8% purity) Vehicle: not reported Positive controls: sodium azide, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide,	313 – 5000 µg/plate	Negative ± S9 No cytotoxicity	(Kim et al. 2018)

		2-nitrofluorene & 9-aminoacridine (-S9); 2-aminoanthracene (+ S9)			
In vitro mammalian chromosomal aberration test	Chinese hamster lung cells	3′-SL (98.8% purity) Vehicle: not reported Positive controls: mitomycin C (-S9); benzo[a]pyrene (+ S9)	1250 – 5000 µg/plate	Negative ± S9	(Kim et al. 2018)
In vivo mammalian erythrocyte micronucleus test	ICR mice (age 8 weeks) Oral gavage 24h, 48h & 72h sampling times	3′-SL (98.8% purity) Vehicle: saline Positive control: mitomycin C	500, 1000 & 2000 mg/kg bw/day for 3 days; samples collected 24, 48 and 72h after dosing	Negative	(Kim et al. 2018)

3.2.6 Human tolerance studies

A range of clinical studies of 2′-FL in infants have been reviewed by FSANZ as part of Applications A1155, A1190, A1233 and A1251. For the present evaluation, the Applicant submitted results from an unpublished clinical study evaluating the Applicant's 2′-FL/DFL, LNT, 3′-SL and 6′-SL. In addition, two clinical studies of formula containing 2′-FL, 3-fucosyllactose (3-FL), LNT, 3′-SL and 6′-SL are available in the scientific literature.

The methodology used in these studies is detailed in Attachment 1. Safety outcomes are reviewed below.

Clinical study of infant formula, follow-up formula and growing-up milk supplemented with 2′-FL/DFL, LNT, 6′-SL and 3′-SL (Cohen 2022)

Healthy term infants aged 7 – 21 days and exclusively consuming cow's milk formula were recruited and randomised to a Control Group, Test Group 1 or Test Group 2. A group of exclusively breastfed infants of the same age was also enrolled as a Reference Group. The test formulas were identical to the control formula except part of the lactose content was replaced with a blend of the 5 HiMOs 2′-FL/DFL, LNT, 3′-SL and 6′-SL. Infant formula was given from enrolment to 6 months of age, follow-up formula from 6 to 12 months and growing-up milk from 12 to 15 months. In Test Groups 1 and 2 the infant formula contained 1.5 g/L or 2.5 g/L of the HiMO blend, respectively. In both test groups the follow-up formula contained 0.5 g/L total HiMOs and growing-up milk contained 0.4 g/L total HiMOs. Complementary foods were allowed from 4 months of age.

Measures of gastrointestinal tolerance, including stool frequency, difficulty passing stool, flatulence, fussing and Infant Gastrointestinal Symptom Questionnaire (IGSQ) composite

scores were not statistically different between any of the formula-feeding groups up to 12 months of age. Infants from Test Group 1 had significantly lower stool consistency scores (indicating softer stools) compared to infants from the Control Group at 2 months ($p<0.05$), but were comparable between formula-fed groups at all other time points. The odds of adverse events (total, "related", and "related/probably related" to the study product) were similar in all formula-fed groups at 6 and 12 months of age. The odds of a serious adverse event were significantly more likely in Test Group 1 compared to the Control Group at both timepoints ($p<0.05$; most commonly classified as "infections and infestations"), but were not different from the breastfed reference group. There was no difference in the odds of serious adverse events between Test Group 2 and the Control Group. Furthermore, all serious adverse events in the Test Groups were classified as "unlikely" or "unrelated" to study formulas containing HiMOs.

It was concluded that tolerance outcomes and safety were similar between all formula-fed groups up to 12 months of age.

Clinical study of infant formula supplemented with 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL (Parschat et al. 2021)

Healthy term infants ≤ 14 days of age were randomised to consume either control infant formula or formula supplemented with the 5 HiMO mixture for 4 months followed by a voluntary 8-week follow-up period. The test formula contained 5.75 g/L total HiMO (2.99 g/L 2′-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3′-SL and 0.28 g/L 6′-SL), partially replacing the carbohydrate maltodextrin in the control formula. A reference group of breastfed infants was also included.

Breastfed infants passed more stools per day during the first 28 days of intervention compared with both formula-fed groups ($p = 0.0000$), but there were no differences between infants fed the test and control formula. Stool frequency from 2 months to the end of the intervention was similar between the test group and breastfed reference group, while infants in the control formula group passed fewer stools per day than infants in the test or breastfed groups ($p = 0.0428$ and $p = 0.0136$, respectively). Soft stools were observed more frequently in the test group than the control group at the four visits during the first two months ($p = 0.023$, $p = 0.003$, $p = 0.004$ and $p = 0.0136$, respectively), while breastfed infants had a higher number of soft stools than both formula-fed groups at most timepoints ($p < 0.05$; $p < 0.01$ or $p < 0.001$ for various comparisons). There was no difference in flatulence, vomiting or fussiness without crying between the formula-fed groups. Regurgitation was higher in the test group compared to the control group ($p < 0.05$), but similar to breastfed infants. Crying was less frequent in the test group compared to breastfed infants at most time points ($p < 0.05$), but no significant differences were observed between formula-fed groups. Both groups of formula-fed infants woke less frequently at night than breastfed infants ($p < 0.05$; $p < 0.01$ or $p < 0.001$ for various comparisons).

The total incidence of adverse events was similar in all groups. A higher incidence of gastrointestinal disorders was observed in the test and control formula-fed groups compared to the reference group ($p = 0.0008$ and $p = 0.0180$, respectively), but there was no difference between the two formula-fed groups ($p = 0.3975$). Haematochezia was more frequently reported in the test group compared with the breastfed group, but the overall incidence was low and the study authors considered it may have been caused by factors unrelated to the intervention. The total incidence of serious adverse events was similar in the test group and reference group, with both being lower than in the control formula group. Two serious adverse events in each of the test and control formula-fed groups were deemed to be related to the investigational product. In the test group, one infant was hospitalised due to choking and gastrointestinal reflux but recovered and continued with the study. The second infant in

the test group experienced severe diarrhoea and was treated with hydrolysed milk and removed from the study. Both serious adverse events in the control group resulted in diagnosis of bovine milk protein allergy.

It was concluded that infant formula supplemented with the 5 HMiO mixture at 5.75 g/L was safe and well tolerated by healthy term infants.

Clinical study of infant formula supplemented with 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL (Lasekan et al. 2022)

Healthy term infants aged ≤ 14 days were randomised to receive either a control formula or test formula containing 5.75 g/L HiMOs (3.0 g/L 2′-FL, 0.8 g/L 3-FL, 1.5 g/L LNT, 0.2 g/L 3′-SL and 0.3 g/L 6′-SL) from enrolment to 4 months of age. A nonrandomised breastfed group was also included.

The percentage of feedings with spit-up/vomit was similar in all three groups. Infants given test formula produced more frequent stools ($p < 0.001$), softer stool consistencies, more yellow-predominant stool colour and less green-, brown- or black-predominant stool colour compared to infants given control formula (all $p < 0.05$). The human milk-fed group had more frequent stools, softer stools, yellow stools and less green or brown stools than either formula-fed group, but the test formula group's stool characteristics were closer to those of the breastfed group than the control formula infants.

The incidence and severity of adverse events was similar in all three groups. Seven participants reported serious adverse events, one from the test formula group, three from the control formula group and three from the breastfed group. All of the serious adverse events reported in either formula-fed group were medically confirmed as 'not related' to the study formulas. The proportion of infants seen by healthcare professionals for illness was significantly lower in the test group compared to the control group ($p \leq 0.044$), but similar to that of infants fed human milk.

It was concluded that infant formula supplemented with the blend of 5 HiMOs (2′-FL, 3-FL, LNT, 3′-SL and 6′-SL) at a concentration of 5.75 g/L supported gastrointestinal tolerance and safe use in healthy term infants.

3.2.7 Post-marketing surveillance

The Applicant provided post-marketing surveillance data collected between 2017 to 2021 from 36 countries in which stage 1 infant formula containing 2′-FL and LNnT has been commercialised, as well as data collected throughout 2021 from 21 countries in which IFPs containing 2′-FL/DFL, LNT, 6′-SL and 3′-SL have been commercialised. No safety concerns were identified from these data.

3.2.8 Safety assessments by other agencies

The European Food Safety Authority (EFSA) has issued scientific opinions on the safety of 2′-FL/DFL, LNT, 6′-SL and 3′-SL produced by the Applicant, as well as LNT, 6′-SL and 3′-SL produced by Chr. Hansen, as novel foods. Uses evaluated included addition to infant formula and follow-on formula (EFSA 2019a; EFSA 2019b; EFSA 2020b; EFSA 2020a; EFSA 2022b; EFSA 2022c; EFSA 2022a). EFSA has also published an opinion on the safety of an extension of use for the Applicant's 2′-FL/DFL and LNT in food supplements for infants (EFSA 2022d).

EFSA concluded that all of these substances are safe under the proposed conditions of use.

2′-FL/DFL and LNT are authorised for use as novel foods in the UK under retained EU law following the exit of the UK from the EU. As 6′-SL and 3′-SL were approved in the EU after the UK had exited, Glycom made Applications requesting their permission as novel foods in the UK. The Food Standards Agency/Food Standards Scotland (FSA/FSS) reviewed the EFSA opinions on these substances and agreed with EFSA's conclusions regarding their safety (FSA/FSS 2022). The FSA/FSS also reviewed EFSA's opinion on an extension of use and change in the specifications of 2′-FL/DFL and agreed with EFSA's conclusions (FSA/FSS 2022).

The US Food and Drug Administration (FDA) has responded that it has 'no questions' to Glycom's self-assessments that 2′-FL/DFL, LNT, 3′-SL and 6′-SL produced by microbial fermentation are Generally Recognized as Safe (GRAS) (FDA 2020c; FDA 2020d; FDA 2020b; FDA 2020a).

Glycom's 2′-FL/DFL, LNT, 3′-SL and 6′-SL have also been approved as novel foods in several food categories including infant formula in Singapore and Israel, and LNT, 3′-SL and 6′-SL have been approved in Brazil.

3.2.9 Summary of the toxicology assessment

Glycom's 2′-FL/DFL, LNT, 6′-SL and 3′-SL are structurally and chemically identical to the forms of these substances present in human milk. As such, no differences in pharmacokinetics between the naturally occurring and manufactured forms of these HMOs are expected.

FSANZ's previous evaluation of 2′-FL and LNnT considered the available data on absorption, distribution, metabolism and excretion of HMOs (FSANZ 2019). Overall, the available data indicate that intestinal absorption is limited, and a significant proportion of HMOs reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces.

2′-FL/DFL, LNT, 6′-SL and 3′-SL produced by Glycom were not mutagenic, clastogenic or aneugenic *in vitro*. 3′-SL and 6′-SL produced by microbial fermentation by another company were also not genotoxic in *in vitro* and *in vivo* studies.

In 90-day oral toxicity studies in neonatal rats, no adverse effects were observed with Glycom's 2′-FL/DFL, LNT, 6′-SL or 3′-SL at doses up to 5000 mg/kg bw/day (2′-FL/DFL, 6′-SL and 3′-SL) or 4000 mg/kg bw/day (LNT), the highest doses tested.

Toxicity studies with 6′-SL and 3′-SL produced by another company are also available. For 6′-SL, no adverse effects were observed in an acute oral toxicity study in rats at doses up to 20,000 mg/kg bw, in a 90-day oral toxicity in rats at doses up to 5000 mg/kg bw/day, or in a 21-day study in neonatal piglets given formula at concentrations up to 1200 mg/L. For 3′-SL, no adverse effects were observed in an acute oral toxicity study in rats at doses up to 20,000 mg/kg bw, a 90-day oral toxicity in rats at doses up to 2000 mg/kg bw/day, a dose-escalation study in dogs at doses up to 2000 mg/kg bw or in a 21-day study in neonatal piglets given formula at concentrations up to 500 mg/L.

In a human clinical study, consumption of infant formula containing 1.5 or 2.5 g/L of a mixture of 2′-FL/DFL, LNT, 6′-SL and 3′-SL, followed by follow-up formula and growing-up milk containing 0.5 g/L or 0.4 g/L of the HMO blend, respectively, was safe and well tolerated.

Two human clinical studies with infants fed formula containing 5.75 g/L of an alternative

mixture of 5 HMOs, including 2′-FL, LNT, 6′-SL and 3′-SL, also found the formula was safe and well tolerated.

The Applicant also provided post-marketing surveillance data that found no safety concerns following consumption of infant formula containing the mixture of 2′-FL/DFL, LNT, 6′-SL and 3′-SL, or infant formula containing 2′-FL in combination with LNnT.

3.3 Microbiology Assessment

The objective of this assessment is to review the microbiological safety of the addition of 2′-FL/DFL, LNT 3′-SL and 6′-SL to infant formula products.

A literature review covering the period 2017-2022 (i.e. the period after FSANZ's finalisation of Application A1155) confirmed that there are no specific microbiological adverse impacts from the addition of 2′-FL, LNT, 3′-SL and 6′-SL at the proposed concentrations to infant formula. FSANZ's assessments of Applications A1155, A1190, A1233 and A1251 (FSANZ 2019; FSANZ 2020; FSANZ 2021a; FSANZ 2021b; FSANZ 2022a; FSANZ 2022b) identified no microbiological safety concerns from the addition of 2′-FL to IFP previous to this, and subsequent studies have not raised any additional safety concerns. In addition, EFSA has issued scientific opinions on the safety of 2′-FL/DFL, LNT, 6′-SL and 3′-SL produced by the Applicant, as well as LNT, 6′-SL and 3′-SL produced by Chr. Hansen including a microbiological risk assessment and no issues were identified (summarised in Section 3.2.8 (EFSA 2019a; EFSA 2019b; EFSA 2020b; EFSA 2020a; EFSA 2022b; EFSA 2022d; EFSA 2022c; EFSA 2022a).

3.4 Dietary Intake Assessment

The objective of this dietary intake assessment is to estimate the dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from the proposed addition to infant formula products as defined in Standard 2.9.1 (infant formula, follow-on formula and infant formula products for special dietary use). Estimated dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from mature human milk will also be determined and used as a reference against which estimated intakes from the proposed addition to infant formula products will be compared.

3.4.1 Approach to estimating dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL

Calculating estimates of dietary intake requires data on concentrations of the proposed substances in foods (including any naturally-occurring sources and current permissions) as well as food consumption data. Dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL were estimated using proposed maximum use levels in infant formula products and estimates of concentrations in mature human milk from scientific literature, combined with consumption data from model diets for infants aged 3 and 9 months.

A summary of the general FSANZ approach to conducting the dietary intake assessment for this Application is outlined below. A detailed discussion of the FSANZ methodology and approach to conducting dietary intake assessments is set out in Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes (FSANZ 2009).

3.4.1.1 Previous FSANZ dietary intake assessment of 2'-FL

FSANZ has previously conducted a dietary intake assessment of 2′-FL (FSANZ 2019; FSANZ 2020; FSANZ 2021a; FSANZ 2022a; FSANZ 2022b), assessing a maximum amount of 96 mg/100 kJ in infant formula, follow-on formula and Formulated Supplementary Foods

for Young Children (FSFYC, commonly referred to as 'toddler milks'). A new dietary intake assessment of 2′-FL has been conducted for this Application as substance composition specifications and proposed maximum amounts are not directly comparable across Applications, and more recent literature is available on HMO concentrations in human milk.

3.4.1.2 Consumption data used

The hazard identification and characterisation did not identify any population sub-groups for which there were specific safety considerations in relation to intake of 2′-FL, DFL, LNT, 6′-SL and 3′-SL. The population groups that were used for the dietary intake assessment are:

- Infants aged 3 months – representing exclusively formula-fed / breastfed infants
- Infants aged 9 months – representing infants who consume food as well as follow-on formula or human milk.

Food consumption data used in dietary intake assessments is preferably obtained from a national nutrition survey, however the scope of Australian and New Zealand national nutrition surveys to date does not include infants. Model diets were therefore used to represent consumption of infant formula products and human milk for 3- and 9- month old infants. Details on the model diet methodology are outlined later in this section.

As the Application only requested addition of the HiMOs to infant formula products, intakes from FSFYC were not estimated in this assessment.

3.4.1.3 Concentrations of 2'-FL, DFL, LNT, 6'-SL and 3'-SL in infant formula products

The proposed maximum permitted amounts of the HiMOs in infant formula products provided in the Application are listed in Table 7.

Table 7 Proposed maximum use levels of 2'-FL, DFL, LNT, 6'-SL and 3'-SL in infant formula products, from the Application

Substance	Maximum permitted amount (mg/100 kJ)
2′-FL/DFL mixture	96
LNT	32
6′-SL	16
3′-SL	8

For LNT, 6′-SL and 3′-SL, the maximum use levels provided by the Applicant were used for the FSANZ dietary intake assessment. For 2′-FL and DFL, a minimum, mean and maximum value were derived based on information provided in the Application, as outlined in Table 8. Mean values were based on Table D.3.1-2 of the Application, where DFL was reported to be 12% of the mixture weight on a HiMO basis. Assuming the mixture is 100% 2′-FL and DFL, the mean 2′-FL composition was therefore 88%. The minimum composition specifications of 2′-FL and DFL provided by the Applicant (Table B.5.1-1 of the Application) of ≥ 75.0 and ≥ 5.0 w/w % water-free weight, respectively, were used to determine representative lower and upper concentrations considered in the dietary intake assessment.

Table 8 Use levels (mg/100 kJ) for 2'-FL and DFL used in the FSANZ dietary intake assessment

	2′-FL		DFL	
Composition in mixture	% in mixture	Concentration used for FSANZ dietary intake assessment[1] (mg/100 kJ)	% in mixture	Concentration used for FSANZ dietary intake assessment[1] (mg/100 kJ)
Low	75.0 %	72.0	5.0 %	4.8
Mean	88.0%	91.2	12.0%	24.0
High	95.0 %	84.5	25.0 %	11.5

[1] Calculated by applying % in mixture to proposed maximum use level of 96 mg/100 kJ for 2′-FL/DFL mixture.

3.4.1.4 Concentrations of 2'-FL, DFL, LNT, 6'-SL and 3'-SL in human milk

In their dietary intake assessment, the Applicant used concentrations of 2′-FL in Secretor human milk from A1155 (FSANZ 2019) derived by that Applicant from a number of published studies. For DFL, LNT, 6′-SL and 3′-SL, the Applicant used concentrations from Secretor human milk from a systematic review by Thurl et al. (Thurl et al. 2017). Mean of means and 95% confidence level of means were reported for two groups in this review: term infants from a lactation period of 0-100 days, and pre-term infants from a lactation period of 0-60 days. For each HMO the Applicant selected the group with the highest mean and used this value for the infant model calculations.

Table 9 Mean concentrations (g/L) of 2'-FL, DFL, LNT, 6'-SL and 3'-SL in human milk used by the Applicant, from A1155 (FSANZ 2019) and Thurl et al. (2017)

	Mean concentration in human milk (g/L)	
HMO	3 month old model diet	9 month old model diet
2′-FL	3.0	3.0 and 2.4
DFL	0.42	
LNT	1.04	
6′-SL	0.66	
3′-SL	0.29	

FSANZ reviewed the recent literature on HMO concentrations in human milk and identified a more recent systematic review (Soyyılmaz et al. 2021) with methodology deemed better suited to the dietary intake assessment for this Application. FSANZ selected concentrations from this study to use for the dietary intake assessment based on the following review study characteristics that align with the objectives of the dietary intake assessment:

- Reports mean concentrations for all of the HMOs in this Application
- Reports a measure of dispersion (e.g. range, 95% confidence internal, interquartile range)
- Adjusted for study sample size
- Reflects the proportion of Secretors and non-Secretors in the global population
- Reports concentrations by lactation periods relevant to 3- and 9- month old infant

models.

Information on these characteristics in reviews by Thurl et al. (Thurl et al. 2017), Soyyilmaz et al. (Soyyılmaz et al. 2021) and another recent review considered by FSANZ (Conze et al. 2022) is summarised in Table 10.

Table 10 Summary of review study characteristics

	Thurl et al. 2017	Soyyilmaz et al. 2021	Conze et al. 2022
A1265 HMOs assessed	All	All	2′-FL, LNT, 6′-SL, 3′-SL
Average and distribution statistics	Mean, 95% CL (of mean values)	Mean, median, SD, range, 95% CI (of mean values)	Mean, SD, median, IQR, P90
Weighted for study sample size	No	Yes	Yes
Secretor status	Reported by secretors only and pooled	Pooled and adjusted to reflect global proportion (80% Secretor)	Secretors only for 2′-FL, pooled for other HMOs
Lactation stages	0-100 days, 0-60 days	0-5 days, 6-14 days, 15-90 days, 90+ days	Not reported

Mean and high concentrations of 2′-FL, DFL, LNT, 6′-SL and 3′-SL in mature human milk from the review by Soyyilmaz et al. are provided in Table 11.

Table 11 Concentrations (g/L) of 2'-FL, DFL, LNT, 6'-SL and 3'-SL in mature human milk from Soyyilmaz et al. 2021, used in FSANZ dietary intake assessment

Lactation period	15 to 90 Days		90+ Days	
Use in infant model	3 month old		9 month old	
Concentration	Mean[1] (g/L)	High[2] (g/L)	Mean[1] (g/L)	High[2] (g/L)
2′-FL	2.279	4.28	1.65	4.27
DFL	0.293	0.54	0.27	0.58
LNT	0.793	1.60	0.64	1.37
6′-SL	0.403	0.74	0.30	1.00
3′-SL	0.192	0.7	0.13	0.30

[1] Weighted mean of individual study means.
[2] Highest individual study mean.

For all HMOs assessed, mean concentrations used by FSANZ are lower than those used by the Applicant for dietary intake assessments. High concentrations used by FSANZ are all greater than the mean concentrations used by the Applicant.

3.4.1.5 Concentrations of milk oligosaccharides in cows' and goats' milk

FSANZ's assessment of Application A1155 identified cows' and goats' milk as a naturally occurring sources of milk oligosaccharides, and their contribution to infant intakes was considered because many infant formula products are made with cows' or goats' milk bases

(FSANZ 2019). A comprehensive dietary intake assessment was not undertaken for infants for the assessment of A1155 due to the very low concentrations in cows' and goats' milk relative to human milk and proposed maximum use levels in the Application. As reported in A1155 the total concentration of milk oligosaccharides in cows' milk is around 30 – 60 mg/L. Compared with cows' milk, goats' milk has a higher oligosaccharide concentration of 60 – 650 mg/L (van Leeuwen et al. 2020), still significantly lower than the concentration in mature human milk of 8,600 – 16,800 mg/L (Soyyılmaz et al. 2021).

A review of the literature conducted for this Application confirms that similar to 2′-FL, the concentration of DFL, LNT, 3′-SL and 6′-SL is significantly lower in cows' milk compared with human milk due to the low concentration of oligosaccharides overall in cows' milk. The relative abundance of fucosylated oligosaccharides (such as 2′-FL and DFL) is especially low in cows' milk compared with human milk, and the relative abundance of sialylated oligosaccharides (such as 3′-SL and 6′-SL) is higher. The literature suggests the oligosaccharide in this Application with the highest relative abundance in cows' milk compared with human milk is 3′-SL, with around 25% and 1% relative abundance, respectively (Aldredge et al. 2013). Applying an energy value of 2728 kJ/L for *Milk, cow, fluid, regular fat (~3.5%)* (FSANZ 2016) to the upper value of total oligosaccharide concentration in cows' milk reported in FSANZ's assessment of A1155 (60 mg/L) provides a concentration of 2.2 mg/100 kJ. Applying a relative abundance of 25% to this value, the concentration of 3′-SL in cows' milk would be up to 0.53 mg/100 kJ. This concentration is around 15-fold lower than the maximum proposed use level in this Application.

Similar to cows' milk, goats' milk is proportionally higher in sialylated oligosaccharides than human milk. The literature indicates the oligosaccharide in this Application with the highest relative abundance in goats' milk compared with human milk is 6′-SL. A review by Sousa et al. (Sousa et al. 2019) estimated 6′-SL concentrations in goats' milk around 50 - 70 mg/L, a relative abundance of around 17% using the midpoint of van Leeuwen et al's (2020) total oligosaccharide estimate. Human milk, in comparison, has an estimated relative abundance of 6′-SL of around 1% (Aldredge et al. 2013). Applying an energy value of 2010 kJ/L for *Milk, goat, fluid, regular fat* (FSANZ 2016) to the upper concentration of 6′-SL (650 mg/L) in goats' milk (Sousa et al. 2019) provides a concentration of 3.5 mg/100 kJ, approximately 4.5 times lower than the maximum proposed use level in this Application.

While infant formula products from a cows' or goats' milk base would contain naturally occurring milk oligosaccharides, intake from these sources is well below the proposed maximum permitted amounts in this Application, and any permitted maximum amount for the substances in this Application would apply to intake from all sources. Other foods consumed by infants, such as cheese and yoghurt, may also contain cows' or goats' milk, however these consumption amounts would not differ by feeding type (infant formula product or human milk fed). Further, a comprehensive dietary intake assessment of milk oligosaccharides from cows' or goats' milk was therefore not undertaken for this Application.

3.4.1.6 Sodium in 6′-SL and 3′-SL Application substances

As the proposed substances 6′-SL and 3′-SL are sodium salts, FSANZ considered the contribution of sodium from these substances to total sodium intake from infant formula products. The composition specifications provided in the Application (Tables B.5.1-3 and B.5.1-4) note the proposed 6′-SL and 3′-SL substances are 4.5 - 6.0 w/w % sodium, equivalent to 0.72 - 0.96 mg sodium per 100 kJ for 6′-SL and 0.27 - 0.48 mg sodium per 100 kJ for 3′-SL when added at proposed maximum use levels. Schedule S29-9 of the Code requires the total sodium content of infant formula products from all sources to be between 5 and 15 mg per 100 kJ. If 6′-SL and 3′-SL were both added at the proposed maximum use levels, this would contribute 1.44 mg sodium per 100 kJ formula, equivalent to around 10% of

the maximum or 29% of the minimum sodium content in the Code. A comprehensive dietary intake assessment was therefore not required for sodium for this Application, as there is a maximum permitted amount of sodium in infant formula products specified in the Code and infants would not be expected to have higher sodium intakes from formula containing 3′-SL and/or 6′-SL at the proposed maximum use levels compared with products not containing the substances.

3.4.2 Dietary intake assessment methodology

As there are no food consumption data available from the 2011-12 Australian National Nutrition and Physical Activity Survey or the 2002 New Zealand National Children's Nutrition Survey for children aged less than 2 years, model diets were constructed to estimate dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL for the target groups of infants aged 3 and 9 months. The same model diets were used for Australia and New Zealand.

As the 3 and 9 month old infant model diets are based on mean consumption amounts only, a distribution of consumption could not be calculated. Therefore, 90th percentile (P90) dietary intakes were estimated using the calculation shown in Equation 1, where high (P90) consumption is estimated by multiplying the mean consumption by 2.

Equation 1: 90th percentile dietary intake calculation for the 3 and 9 month old infant model diets

$$90^{th} \text{ Percentile intake} = \text{Concentration} \times (\text{Mean consumption} \times 2)^{\wedge}$$

^ (WHO 1985)

For infant/follow-on formula, two estimated dietary intake scenarios were calculated using the infant model diets:

- Mean consumption * Proposed maximum use level
- High (P90) consumption * Proposed maximum use level.

For human milk, four estimated dietary intake scenarios were calculated using the infant model diets:

- Mean consumption * Mean concentration in human milk (represents a 'best estimate' of mean intake)
- High consumption * Mean concentration in human milk
- Mean consumption * High concentration in human milk
- High consumption * High concentration in human milk (represents a 'best estimate' of high intake).

The energy content of human milk is required for the calculation of the amount of human milk consumed in model diets for 3 and 9 month old infants. AUSNUT 2011-13 is the latest nutrient data set published for Australian foods. In this dataset, the energy content of *Milk, human/breast, mature, fluid* is 286 kJ/100g (FSANZ 2016). This energy content value is used to estimate the dietary intake of HMOs from human milk in this assessment. For assessment of milk oligosaccharides in cows' and goats' milk, the energy content values of 281 kJ/100 g for *Milk, cow, fluid, regular fat (~3.5%)* and 207 kJ/100 g for *Milk, goat, fluid, regular fat* were used, respectively (FSANZ 2016).

The recommended energy intake for a three-month-old boy (343 kJ/kg bw/day) (United Nations University and World Health Organization 2004) and the 50th percentile weight

(6.4 kg) (World Health Organization 2006) for the same age and sex were used as the basis for the model diet. Boys' weights were used because boys tend to be heavier than girls at the same age and therefore have higher overall energy and food requirements. The entire energy requirement in the 3 month old infant diet is derived from infant formula or human milk, depending on the assessment. The body weight of 6.4 kg was used to estimate dietary intakes for 3 month old infants on a body weight basis.

By the age of 9 months, infants are consuming a mixed diet of solids and follow-on formula / human milk. The model diet was constructed based on recommended energy intakes, mean body weight and the proportion of milk and solid foods in the diet for a 9 month old infant. The recommended energy intake for a 9 month old boy (330 kJ/kg bw/day) (United Nations University and World Health Organization 2004) and the 50th percentile weight (8.9 kg) (World Health Organization 2006) for the same age and sex was used as the basis for the model diet. It was assumed that 50% of energy intake was derived from follow-on formula / human milk and 50% from solids and other fluids (Hitchcock et al. 1986; Pan American Health Organization and World Health Organization 2003; Butte et al. 2004). The body weight of 8.9 kg was used to estimate dietary intakes for 9 month old infants on a body weight basis.

A set of model diets was not established for infants consuming infant formula products for special dietary uses as the energy and/or fluid requirements can vary depending on the medical conditions of the infant. Additionally, the energy content of the various infant formula products for special dietary uses can be variable. The assessment of A1155 included an examination of products, including formulas for premature infants, formulas for use by infants with inborn errors of metabolism, and formulas for use by infants with severe food allergies, which found the range of energy contents was 269 – 415 kJ/100 g (FSANZ 2019). If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a similar energy content to that used in the model diets, then their intake of 2′-FL, DFL, LNT, 6′-SL and 3′-SL is anticipated to be similar to that outlined in the assessment for this Application. If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a higher energy content to that used in the model diets, then their intake of 2′-FL, DFL, LNT, 6′-SL and 3′-SL is anticipated to be similar to or lower than that outlined in this assessment. Further to these considerations, infants consuming infant formula products for special dietary uses are generally under medical and dietetic supervision given their specific needs. Short term dietary exposures to food additives in excess of those estimated may be of a lesser priority than medical and dietetic considerations in their overall case management.

3.4.2.1 Assumptions and limitations of the dietary intake assessment

The aim of the dietary intake assessment was to make the most realistic estimation of dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL as possible. However, where significant uncertainties in the data existed, conservative assumptions were generally used to ensure that the estimated dietary intake was not an underestimate of intake.

Assumptions made in the dietary intake assessment included:

- The proposed substances are composed solely of the HiMOs of interest (i.e. the maximum use levels are equal to the amount of each HiMO present in infant/follow-on formula if the proposed substances are added at this level)
- Unless otherwise specified, all infant/follow-on formula and human milk contains 2′-FL, DFL, LNT, 6′-SL and 3′-SL at the concentrations specified in Tables 7, 8 and 11

- 1 litre of infant or follow-on formula equals 1,050 grams
- 1 litre of human milk equals 1,040 grams
- 1 litre of cows' or goats' milk equals 1,030 grams
- there is 100% market penetration of the use of 2′-FL, DFL, LNT, 6′-SL and 3′-SL into the infant/follow-on formula markets
- infants aged 3 months exclusively consume infant formula or human milk
- Infants aged 9 months consume follow-on formula or human milk in amounts that meet 50% of their energy requirements, with the other 50% of energy requirements obtained from consuming solids and other fluids
- the model diets represent current consumption amounts of infant formula, follow-on formula and human milk for Australian and New Zealand infants aged 3 and 9 months
- there is no contribution to 2′-FL, DFL, LNT, 6′-SL and 3′-SL intakes through foods and beverages other than from infant formula, follow-on formula and human milk
- there is no contribution to 2′-FL, DFL, LNT, 6′-SL and 3′-SL intakes through the use of complementary or other medicines.

In addition to the specific assumptions made in this assessment, a discussion of additional limitations associated with consumption data and dietary intake assessments is included in Section 6 of the Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes (FSANZ 2009).

3.4.3 Estimated dietary intakes

3.4.3.1 FSANZ estimated dietary intakes from infant/follow-on formula and human milk

Tables 12 and 13 summarise estimated dietary intakes for 3 month olds of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from infant formula and mature human milk, respectively.

For 3 month olds, based on the proposed maximum use level the mean estimated dietary intake of 2′-FL from infant formula (0.29 (0.25 - 0.31) g/kg bw/day) is comparable to that from human milk (0.26 g/kg bw/day). Similarly, based on the proposed maximum use level the mean estimated dietary intake of DFL from infant formula (0.040 (0.016 - 0.082) g/kg bw/day) is comparable to that from human milk (0.033 g/kg bw/day). For LNT, 6′-SL and 3′-SL at the proposed maximum use levels, mean estimated dietary intakes from infant formula were higher than mean estimated dietary intakes from human milk, but were below estimates from human milk based on high consumption or high concentration.

For all HiMOs assessed except for DFL, estimated dietary intakes from infant formula at high (P90) consumption and proposed maximum use levels were lower than estimated intakes from human milk based on high concentration and high (P90) consumption. The estimated dietary intake of DFL from infant formula at high consumption was 0.079 (0.033 - 0.165) g/kg bw/day; the upper end of this range, which assumes 25% of the 2′-FL-DFL mixture is DFL, is above the high concentration, high consumption estimate for human milk of 0.12 g/kg bw/day.

Table 12 Estimated dietary intakes (g/kg bw/day) for 3 month old infants[#] of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from infant formula at proposed maximum use levels from the Application

	Estimated dietary intake (g/kg bw/day)[1]	
HiMO	Mean	P90[2]
2′-FL	0.29 (0.25 - 0.31)	0.058 (0.49 -0.63)
DFL	0.040 (0.016 - 0.082)	0.079 (0.033 - 0.16)
LNT	0.11	0.22
6′-SL	0.055	0.11
3′-SL	0.027	0.055

[#] Assuming P50 body weight of 6.4 kg (World Health Organization 2006), recommended energy intake of 343 kJ/kg bw/day (United Nations University and World Health Organization 2004) and 100% of dietary energy is obtained from infant formula.
[1] Estimated intakes for 2′-FL and DFL reflect mean and (minimum - maximum) concentration values based on composition specifications provided in the Application document (Table D.3.1-2 and B.5.1-1, respectively).
[2] Based on mean consumption amount * 2.

Table 13 Estimated dietary intakes^ (g/kg bw/day) for 3 month old infants[#] of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from mature human milk

	Estimated dietary intake (g/kg bw/day)			
HMO concentration[1] Human milk consumption amount	Mean		High	
	Mean	P90[2]	Mean	P90[2]
2′-FL	0.26	0.52	0.49	0.98
DFL	0.034	0.067	0.062	0.12
LNT	0.085	0.17	0.18	0.37
6′-SL	0.046	0.093	0.085	0.17
3′-SL	0.022	0.044	0.08	0.16

^ Assuming energy content of human milk is 286 kJ/100 g and 1,000 mL human milk is equivalent to 1,040 g (FSANZ 2016).
[#] Assuming P50 body weight of 6.4 kg (World Health Organization 2006), recommended energy intake of 343 kJ/kg bw/day (United Nations University and World Health Organization 2004) and 100% of dietary energy is obtained from human milk.
[1] Mean and high concentrations from Table 11, 15 to 90 days lactation period.
[2] Mean consumption amount * 2.

Tables 14 and 15 provide estimated dietary intakes for 9 month olds of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from follow-on formula and mature human milk, respectively.

For 9 month olds, based on the proposed maximum use level the mean estimated dietary intake of 2′-FL from follow-on formula (0.14 (0.12 - 0.15) g/kg bw/day) was higher than the mean estimated intake from human milk (0.092 g/kg bw/day), but lower than estimated intake based on high concentration (0.24 g/kg bw/day) or high consumption (0.18 g/kg bw/day). Mean estimated intake of DFL from follow-on formula (0.019 (0.0079 - 0.040) g/kg bw/day) was comparable with mean estimated intake from human milk (0.015 g/kg bw/day). Similar to 2′-FL, mean estimated dietary intakes of LNT, 6′-SL and 3′-SL from follow-on formula were higher than estimated intakes from human milk based on mean concentration and mean consumption, but lower than estimated intakes based on high concentration or high consumption.

For 2′-FL, LNT, 6′-SL and 3′-SL, estimated dietary intake from follow-on formula at high (P90) consumption and proposed maximum use levels was below the estimated intake from human milk based on high concentration and high consumption. For DFL, the estimated dietary intake of DFL from follow-on formula at high consumption was 0.038 (0.016 - 0.079) g/kg bw/day; the upper end of this range estimate, which assumes 25% of the 2′-FL-DFL mixture is DFL, is above the high concentration, high consumption estimate for human milk of 0.065 g/kg bw/day. Estimates based on the mean or minimum percent composition of DFL in the mixture are below the high concentration, high consumption estimate for human milk.

Table 14 Estimated dietary intakes (g/kg bw/day) for 9 month old infants# of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from follow-on formula at proposed maximum use levels from the Application

	Estimated dietary intake (g/kg bw/day)[1]	
HiMO	Mean	P90[2]
2′-FL	0.14 (0.12 - 0.15)	0.28 (0.24 - 0.30)
DFL	0.019 (0.0079 - 0.040)	0.038 (0.016 - 0.079)
LNT	0.053	0.11
6′-SL	0.026	0.053
3′-SL	0.013	0.026

Assuming P50 body weight of 8.9 kg (World Health Organization 2006), recommended energy intake of 330 kJ/kg bw/day (United Nations University and World Health Organization 2004) and 100% of dietary energy is obtained from follow-on formula.
1 Estimates intakes for 2′-FL and DFL reflect mean and (minimum - maximum) concentration values based on composition specifications provided in the Application document (Table D.3.1-2 and B.5.1-1, respectively).
2 Mean consumption amount * 2.

Table 15 Estimated dietary intakes (g/kg bw/day) for 9 month old infants# of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from mature human milk

	Estimated dietary intake (g/kg bw/day)			
HMO concentration[1]	Mean		High	
Human milk consumption amount	Mean	P90[2]	Mean	P90[2]
2′-FL	0.092	0.18	0.24	0.48
DFL	0.015	0.03	0.032	0.065
LNT	0.036	0.071	0.076	0.15
6′-SL	0.017	0.033	0.056	0.11
3′-SL	0.0072	0.014	0.017	0.033

^ Assuming energy content of human milk is 286 kJ/100 g and 1,000 mL human milk is equivalent to 1,040 g (FSANZ 2016).
# Assuming P50 body weight of 8.9 kg (World Health Organization 2006), recommended energy intake of 330 kJ/kg bw/day (United Nations University and World Health Organization 2004) and 100% of dietary energy is obtained from human milk.
1 Mean and high concentrations from Table 11, 90+ days lactation period.
2 Mean consumption amount * 2.

Some estimated intakes of DFL from IFP were higher than those from human milk. These were based on the representative maximum composition of 25% for DFL, which is the most conservative figure that assumes the mixture is 100% 2'-FL and DFL and contains only the minimum composition of 2'-FL listed in the mixture composition specifications (75%). This conservative assumption results in a DFL composition markedly higher than the mean composition from analysed samples provided by the Applicant (12%). In addition, for the

human milk calculations the high concentration values came from study means, therefore there would be higher intakes from human milk in reality for some individuals compared to the estimates presented. Estimated intakes of DFL based on the mean analysed concentrations of DFL in the 2'-FL/DHL mixture or minimum percent composition of DFL in the mixture are similar to or below the high concentration, high consumption estimate for human milk. Mean intakes, which are reflective of longer term intakes, of DFL from IFP are similar to the estimated mean intake from human milk at mean concentrations.

3.4.3.2 Comparison of FSANZ and Applicant estimated dietary intakes from infant/follow-on formula

As shown in Tables 16 and 17 below, estimated dietary intakes of HiMOs from infant/follow-on formula were similar between FSANZ and Applicant calculations. For 2′-FL, Applicant estimates were consistently slightly above range estimates calculated by FSANZ. This corresponds with the different proposed maximum use levels applied; FSANZ values reflect an estimated composition of the 2′-FL/DFL mixture as 75-95% 2′-FL, whereas Applicant reported intake estimates are from A1155 where a proposed maximum use level for 2′-FL of 96 mg/100 kJ was assessed, equivalent in this assessment to a composition of the 2′-FL-DFL mixture as 100% 2′-FL. The Applicant's estimates for DFL were within the range estimated by FSANZ. The Applicant's estimates for LNT, 6′-SL and 3′-SL were essentially identical to FSANZ (only differences in rounding).

Table 16 Estimated dietary intakes (g/kg bw/day) for 3-month old infants of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from infant formula at proposed use levels^, FSANZ and Applicant estimates for A1265

	Estimated dietary intake (g/kg bw/day)			
	Mean (g/kw bw/day)		High (g/kw bw/day)	
Calculated by	FSANZ	Applicant	FSANZ	Applicant
2′-FL	0.29 (0.25 - 0.31)	0.33	0.058 (0.49 - 0.63)	0.66
DFL	0.040 (0.016 - 0.082)	0.04	0.079 (0.033 - 0.16)	0.08
LNT	0.11	0.11	0.22	0.22
6′-SL	0.055	0.05	0.11	0.11
3′-SL	0.027	0.03	0.055	0.05

^ For 2′-FL, FSANZ and the Applicant use concentrations of 84.48 (72 – 91.2) mg/100 kJ, and 96 mg/100 kJ respectively. For DFL, FSANZ and the Applicant use concentrations of 11.52 (4.8 - 24.0) mg/100 kJ, and 11.52 mg/100 kJ, respectively. For LNT, 6′-SL and 3′-SL, the same concentrations are used by FSANZ and the Applicant.

Table 17 Estimated dietary intakes (g/kg bw/day) for 9-month old infants of 2'-FL, DFL, LNT, 6'-SL and 3'-SL from follow-on formula at proposed use levels^, FSANZ and Applicant estimates for A1265

	Estimated dietary intake (g/kg bw/day)			
	Mean (g/kw bw/day)		High (g/kw bw/day)	
Calculated by	FSANZ	Applicant	FSANZ	Applicant
2′-FL	0.14 (0.12 - 0.15)	0.16	0.028 (0.24 - 0.30)	0.32
DFL	0.019 (0.0079 - 0.040)	0.02	0.038 (0.016 - 0.079)	0.04
LNT	0.053	0.05	0.11	0.11
6′-SL	0.026	0.03	0.053	0.05
3′-SL	0.013	0.01	0.026	0.03

^ For 2′-FL, FSANZ and the Applicant use concentrations of 84.48 (72 – 91.2) mg/100 kJ, and 96 mg/100 kJ respectively. For DFL, FSANZ and the Applicant use concentrations of 11.52 (4.8 - 24.0) mg/100 kJ, and 11.52 mg/100 kJ, respectively. For LNT, 6′-SL and 3′-SL, the same concentrations are used by FSANZ and the Applicant.

4 Nutrition Assessment

4.1 Approach for the nutrition assessment

The objective of the nutrition assessment is to determine the effect on infant growth (if any) from the addition of 2′-FL/DFL, LNT, 6′-SL and 3′-SL to infant formula products. A comparison of human milk concentrations to the amounts requested by the Applicant is also shown.

The nutrition assessment examined the three human clinical studies that were provided by the Applicant. A summary of study parameters for the intervention studies are provided in Appendix 1.1. FSANZ notes that only one of the submitted studies (Cohen et al., 2022) included the complete blend of the HiMOs requested in the Application (i.e. 2′-FL/DFL, LNT, 6′-SL and 3′-SL). No additional human clinical studies were identified in the published literature.

The nutrition assessment evaluated the effect of the requested HiMOs on growth using weight gain (g/day) as the primary outcome. This measure has been the most informative in previous FSANZ assessments examining effects on infant growth and was the primary endpoint in all three clinical studies.

All three studies pre-specified a non-inferiority margin of ±3 g/day when comparing HiMO-containing formulas against control formulas and a breastfed reference group (AAP 1988). That is, a group fed the HiMO-containing formulas that showed a difference in weight gain compared to a control formula or breastfed was not deemed to show inferior or excessive growth if within this margin. This assumption is consistent with FSANZ's previous assessments of nutritive substances in IFP. Other growth measures were considered where warranted (such as when a significant effect on weight gain was identified).

4.2 Previous FSANZ assessments of 2′-FL and LNnT

FSANZ has previously assessed the effect of the addition of two HiMOs – 2 ′-FL and LNnT (a constitutional isomer of LNT), and a mixture of these HiMOs.

Addition of 2′-FL to IFP on infant growth has been examined in 4 applications (FSANZ 2019; FSANZ 2020; FSANZ 2021a; FSANZ 2022a; FSANZ 2022b). The body of evidence for these assessments included infant cohort and clinical studies where infants fed formula with or without added 2′-FL were evaluated against breastfed reference groups for significant differences in weight, length or head circumference during the study period. In all prior assessments it was concluded that 2′-FL does not affect growth at levels normally found in human milk.

LNnT was assessed by FSANZ in A1155 (FSANZ 2019; FSANZ 2020) with the conclusion that there is no effect on growth when LNnT is added at levels typically observed in human milk and in combination with 2′-FL.

4.3 Effect of HiMO blend on infant growth

4.3.1 Parschet et al. (2021)

Parschat et al (2021) investigated the effect of a blend of 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL on infant growth with a comparison of mean daily body weight gain (g/day) over 4 months of the intervention as the primary endpoint. Calculating from baseline to the final visit at 4 months, there was no clinically relevant difference in mean body weight gain between infants fed the HiMO-containing formula versus the non-HiMO formula. That is, differences in mean body weight gain were within the non-inferiority margin of ±3 g/day. The authors cited reports showing similar weight gain (approx. 28.7 g/day) for infants consuming formulas containing 2′-FL + GOS (Marriage et al. 2015), 2′-FL + LNnT (Puccio et al. 2017), and 2′-FL + 3′-GL and FOS/GOS (Vandenplas et al. 2020).There were also no significant differences in mean weight at all time points, with mean weights at 4 months of 6578 ± 697.6 g for the HiMO-group versus 6557.3 ± 672.8 g for the non-HiMO group.

Comparing to the breastfed reference group, statistically significant differences in weight gain (g/day) for the formula fed groups compared to the breastfed control were observed at one time point (Day 112±3). It was reported that this is consistent with many studies showing increased weight gain for infants bottle-fed with formula. However, the differences in weight gain between the formula and breastfed groups disappeared when subjects that deviated from the study protocol were removed. Higher weight-to-age z-scores were also observed with both formula fed groups compared to breastfed which is also consistent with bottle-fed infants gaining weight faster than breastfed infants. Therefore the differences in weight could not be attributed to the presence or absence of the HiMO blend.

There were no statistically significant differences between the two formula fed groups in secondary measures of growth (body length and head circumference).

4.3.2 Lasekan et al. (2022)

Lasekan et al. (2022) investigated the effect of a blend of 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL on infant growth by comparing weight gain between infants fed the HiMO formula, non-HiMO formula and a breastfed control group as the primary outcome. Weight gain was measured at multiple time points from Day 14 (D14) to D119. There was no statistically significant difference in weight gain between any of the groups of infants except for the male cohort at

D84-D119. At this time point the HiMO group showed statistically significantly higher mean weight gain compared to the control group. However the difference in weight gain was less than 3 g/day which is not regarded as clinically significant. There were also no statistically significant differences between all three groups on secondary growth outcome measures (head circumference and length gain per day).

4.3.3 Cohen et al (2022)

Cohen et al. (2022) investigated the effect of the HiMO blend requested in this Application on infant growth. The study compared infant weight gain from enrolment to 4 months of age for two HiMO-containing formulas (1.5 and 2.5 g/L total HiMO content – see Attachment 1) compared to control formula (no HiMO) and the breastfed reference group. Measuring from baseline to 4 months of age, there were no statistically significant differences in daily body weight gain for the two HiMO-containing formulas compared to the control formula. That is, statistical differences were within the non-inferiority margin of ±3 g/day.

Other anthropometric measures for body weight, length, and head circumference were assessed through 12 months of age and compared to World Health Organisation (WHO) median values. All three formula fed groups were consistent with WHO standard growth curves (within 0.5 standard deviations).

4.4 Effect of individual HiMOs on infant growth

No human intervention studies for 6′-SL, 3′-SL and DFL as single HiMOs were provided by the Applicant. A search of the literature identified only studies where test formulas contained various mixtures of the relevant HiMOs (Parschat et al. 2021; Bosheva et al. 2022; Lasekan et al. 2022) and we were unable to identify additional published clinical studies testing the effects of 6′-SL, 3′-SL and DFL as individual HiMOs on infant growth.

As noted in section 4.2, FSANZ's previous assessments showed that 2′-FL, LNnT (a structural isomer of LNT), and a mixture of 2′-FL and LNnT, do not affect growth when present in IFP at levels normally found in human milk. Likewise a mixture of 2′-FL with FOS/GOS was also determined to have no effect on infant growth. Therefore, based on the best available evidence, addition of individual HiMOs (3′-SL, 6′-SL and DFL), or different mixture of these to that presented in section 4.3, would be unlikely to adversely affect infant growth as long as the HiMOs are added at levels that are normally found in human milk. This conclusion is consistent with recent EFSA opinions (EFSA 2020a, 2020b, 2022a, 2022b) which noted the lack of clinical studies for the individual HiMOs (3′-SL and 6′-SL) but concluded the substances were safe under the proposed conditions of use (which included addition to infant formula and follow-on formula).

4.5 Comparison to human milk concentrations

A review of human milk concentrations of 2′-FL/DFL, LNT, 6′-SL and 3′-SL is reported in section 3.4.2.4 of this report. Table 18 compares these concentrations to the amount requested by the Applicant for addition to IFP. Conversion of human milk concentrations to g/100 kJ was calculated using 286 kJ/100 g as the energy density of human milk (FSANZ 2016). For all HiMOs, the amount requested to be added to IFP is within the range found in human milk.

Table 18 Comparison of requested HiMO amounts to human milk concentrations[1]

HiMO	Requested	Human milk concentration at 15 - 90 days	Human milk concentration at 90+ days

		Mean		P90[3]		Mean		P90[3]	
	mg/100 kJ	g/L[2]	mg/100kJ	g/L[2]	mg/100kJ	g/L[2]	mg/100kJ	g/L[2]	mg/100kJ
2′-FL	96 (sum of 2′-FL and DFL)	2.279	76.7	4.28	144.1	1.65	55.6	4.27	143.8
DFL		0.293	9.9	0.54	18.2	0.27	9.1	0.58	19.5
LNT	32	0.793	26.7	1.6	53.9	0.64	21.5	1.37	46.1
6′-SL	16	0.403	13.6	0.74	24.9	0.3	10.1	1	33.7
3′-SL	8	0.192	6.5	0.7	23.6	0.13	4.4	0.3	10.1

[1] Adapted from Table 11 in section 3.4.2.4 of this report. Energy density from AUSNUT(2016): 286 kJ/100 g = 297 kJ/100 mL based on the specific gravity of human milk of 1.04 g/mL
[2] Soyyilmaz et al., 2021
[3] Mean consumption amount * 2.

4.6 Key findings of the nutrition assessment

The amounts of 2′-FL/DFL, LNT, 6′-SL and 3′-SL requested to be added to IFP is within the range of human milk concentrations as reported in a recent systematic review (Soyyilmaz et al. 2021).

From the three human clinical trials provided with the Application, only one used a combination of HiMOs containing the five HiMOs requested in the Application to be added to infant formula products. However the results of this study as well as the accumulating body of evidence indicates that infants achieve normal growth when they are fed IFP containing HiMOs at the levels that are normally present in human milk. Therefore, the nutrition assessment concludes that the HiMO blend added to IFP does not pose a risk to the normal growth of infants

5 Beneficial Health Effects Assessment

The objective of this assessment is to review reported health benefits of the addition of 2′-FL/DFL, LNT, 3′-SL and 6′-SL to IFP on the development of the gut microbiota, in terms of composition (bifidogenic), anti-pathogenic, and immunomodulation effects for a formula-fed infant.

5.1 Introduction

Microbiological benefit to an infant of an additive to a base infant formula, has been determined to be where the gut microbiota development is more closely aligned to that of a breast-fed infant compared to one fed with the original base formula.

The link between HMO consumption and gut microbiota has been reported through *in vitro* and *in vivo* mechanistic studies, animal studies and human intervention studies (as recently reviewed by Zhang et al. 2021). The majority of HMOs are able to be metabolized by gastrointestinal bacteria which can lead to changes in composition and/or activity of the microbiota as a whole through the release of metabolites including short chain fatty acids (SCFA), which are essential for gut health (Walsh et al. 2020). HMO metabolism is thought to be the result of adaptive co-evolution of symbiotic commensals with the human host (Duranti et al. 2019) and cross-feeding of substrates produced by *Bifidobacteria* that utilise sialyllactoses, which are considered to contribute to the growth of other important species

that colonise the infant gut (Cheng et al. 2020; Chia et al. 2020; Chia et al. 2021).

Early stages of development of the gut microbiota has been linked to host well-being and health throughout life (Bode 2015). Since HMO composition in breast milk varies between women, it can be hypothesized that breast milk from different women affects the infant gut microbiome differently. This may relate to short-term infant health outcomes, but also have long-term consequences for health status and disease risk later on in life. HMOs are at their highest concentration in colostrum. A recent study by Kumbhare et al. (2022) demonstrated that milk source (mother versus donor) was strongly associated with microbiome composition, less gut inflammation for pre-term infants and potential for a decrease in necrotizing enterocolitis occurrence (Kumbhare et al. 2022). This variability adds a level of complexity to determining microbial benefit of specific HMOs. However, in a review Bering (2018) noted that although the health benefits from breast feeding can be explained by the abundance of the HMOs present acting as prebiotics and immunomodulators, the effect may be mitigated by the maturity of the infant gut (Bering 2018). Animal models suggest that HMO supplementation of formula does not aid maturation of the gut in pre-term births but would be more beneficial once the gut has reached a level of maturity where breast milk is tolerated.

This assessment covers health effects of individual HMO components as well as the overall effect of the Applicant's HiMO blend. Key recent studies used in the assessment are summarised below.

Natividad et al (2022) examined the impact of a 6 HiMO mix (2′-FL, LNnT, DFL, LNT, 3' and 6' SL; Glycom) on infant microbiota and intestinal barrier integrity using an *in vitro* model for short exposure trial assessment; M-SHIME® was used for the long-term trial (Natividad et al. 2022). Five faecal donors were selected based on the following criteria – age (2-4 months), exclusively breastfed, no antibiotics or other medications, no pre-or probiotic intake. All samples were used in a short term exposure trial and from that one, was selected for a longer term trial. 2′-FL, and 2′-FL/LNnT and 6′-SL had a strong bifidogenic effect, with an associated increase in acetate. This result is in line with other HMO combinations that included 2′-FL and 2′-FL/LNnT.

Ding et al (2022) investigated the role of sialylated oligosaccharides (3′-SL and 6′-SL) on the initial colonisation of the infant gut within the first week of life and compared that to the microbiome found in human milk (Ding et al. 2022). *Staphylococcus, Enterococcus* and *Bacteroides* were present in both breast milk and the gut microbiota. *Bacteroides* similarly metabolised 3′-SL in both breast milk and faeces. However, this interaction was moderated by the faecal microbiota potentially resulting in modification of the faecal metabolome. Over time, the proportion of *Bacteroides* in the faecal microbiota decreased. It appears that 3′-SL and 6′-SL from neonatal breastmilk is more interactive with the faecal microbiota than for older (5 month old) infants. This may in part be due to changes in HMO concentrations and the lower levels of *Bacteroides* spp. which can metabolise sialylated oligosaccharides unlike *Bifidobacterium* which preferentially utilises 2'FL.

Bosheva et al (2022) reported microbiological data from a randomised, controlled, double-blind, multi-centre 15-month clinical trial for participants' first 6 months (see Attachment 1) (Bosheva et al. 2022). The trial was carried out at 32 sites located in Poland, Hungary and Bulgaria between 2018 and 2021 and consisted of three randomised formula-fed groups and a non-randomised human milk fed reference group. Participants were healthy infants between 7-10 days of age, and their gut microbiota composition and activity were evaluated at the start of the study, and at 3 and 6 months (approximately n=155 test and control group infants; n=69 breast-fed infants). The study evaluated gut maturation effects of 5 HMOs (2′-FL, DFL, LNT, 3′-SL and 6′-SL) at a total concentration of either 1.5g/L or 2.5g/L added to

starter formula milk using a proportional profile that was based on human milk (see Table 18). Microbial DNA was extracted from frozen faecal samples taken at the start, after 3 and 6 months and analysed by shot gun- based metagenomic sequencing and relative abundances determined. Initially there were no significant differences in the gut microbiome between the groups, with significant differences in diversity appearing at 3 and 6 months for both supplemented formulas, the level of significance decreased with time from $P < 0.001$ to $P = 0.04$), indicating that the test groups transitioned towards a closer alignment to the (vaginally-delivered) human milk group.

Estorninos et al (2021) performed a clinical study to look at the effects of a bovine milk-derived oligosaccharides (MOS)-supplemented infant formula on gut microflora and intestinal immunity (Estorninos et al. 2022). Participating infants (n=226, of which 112 were in the control group and the remainder in the test group) were healthy full term infants aged between 21-26 days that had been formula-fed. 70 infants that had been exclusively breast-fed were included as a reference point. The test group were given the same formula as the control group with the addition of 7.2g MOS/L until the age of 6 months. Faecal samples were collected at the start of the trial for the baseline, 2.5 and 4 months and assessed microbiota using 16S-RNA, metabolites and biomarkers of gut health and immune response. Detection and quantification was undertaken for selected genes of pathogenic bacteria species including *Clostridioides difficile* 16S and toxB, *Clostridium perfringens*, *Klebsiella pneumonia*, enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC) heat-labile toxin and ETEC heat-stable toxin, *Salmonella* species, *Campylobacter jejuni* and *Campylobacter coli*. Addition of MOS had a significant effect on the composition of the microbiota with an increase in level of *Bifidiobacterium* as compared to the control group. The authors note that there were proportional difference in the species of *Bifidobacterium* present which may be due to a competitive growth advantage for those able to metabolise the specific HMOs.

5.2 Bifidogenic effects

Bifidobacterium are the predominant genera of healthy breast-fed infants. Colonisation of the gastrointestinal tract starts at birth with bacteria derived from the mother during birth, through breastfeeding and the environment. The gut microbiota composition is highly individual, but predominantly comprises *Bifidobacterium* and *Lactobacilli* in the first few weeks of life. This provides competitive based protection from pathogenic bacteria which are prevented from causing illness. It has been noted that altered or delayed colonization is observed in clinical cases of inflammatory conditions of the gut or other related immune disorders (Tojo et al. 2014).

The gut microbiota of exclusively formula-fed infants is characterised by a microflora profile that more closely resembles the digestive tract of adults. In contrast, the microbiota of infants supplemented with HMOs are enriched with *Bifidobacterium* and generally resemble that of breastfed infants.

HMOs support the growth of *Bifidobacterium*, with specific strains (*B. breve, B. bifidum, B. longum, and B. infantis*) housing genes for HMO-degrading enzymes (*e.g.*, glycosyl hydrolases, fucosidases, and sialidases), transporters, and carbohydrate-binding proteins (Zhang et al. 2021). HMOs are also metabolised by other gastrointestinal bacteria (including *Bacteroides* and *Lactobacilli*), and metabolites from HMO digestion also interact to shape the overall composition and activity of intestinal microbiota.

Beneficial *Bifidobacteria spp*. and *Bacteroides spp*. have been demonstrated *in vitro* to grow selectively in the presence of fucosylated HMOs (including 2′-FL, DFL, and 3’-FL) (Yu et al.

2013a; Yu et al. 2013b; Zabel et al. 2020; Salli et al. 2021) and reduce culture pH through the production of lactate and SCFAs (Yu et al. 2013a; Yu et al. 2013b).The significance of the bifidogenic effects of 2'FL and LNnT has been discussed in previous Applications to FSANZ (FSANZ 2019; FSANZ 2021a; FSANZ 2022a; FSANZ 2022b).

The ability of *Bifidobacteria* to utilise sialyllactoses (including 6′-SL and 3′-SL) has been demonstrated in a range of culture experiments for common species of *Bifidobacterium* present in the microbiota of breast-fed infants (Nakano 2001; Vester Boler et al. 2013; Yu et al. 2013a; Bunesova et al. 2016; Moon et al. 2016; Thongaram et al. 2017; Cheng et al. 2020; Chia et al. 2021). However, not all commensal bacterial species and type strains are able to utilise 6′-SL (Yu et al. 2013a; Bunesova et al. 2016; Cheng et al. 2020). 6′-SL and 3′-SL are hydrolysed by sialidases (also referred to as neuraminidases) which cleave the terminal sialic acid (reviewed in (Juge et al. 2016; Coker et al. 2021)). Sialyllactoses do not provide a mechanism for the growth of potentially pathogenic bacteria, including *Clostridium perfringens*, *Clostridioides difficile*, *Enterobacter aerogenes*, *Cronobacter sakazakii*, and *Escherichia coli* O1:K1:H7 (Yu et al. 2013a; Hoeflinger et al. 2015; Moon et al. 2016).

Vester Boler et al (2013) examined the fermentation potential of HMOs by faecal microbiota from breast-fed and formula fed infants (Vester Boler et al. 2013). Faecal samples were mixed with media containing 2'- FL, 6′-SL and LNnT and incubated for 12 hours. Samples were taken at 0, 3, 6 and 12 hours and analysed for the presence of short chain fatty acids, lactate and microbial species (through use of quantitative analysis). This study showed that LNnT and 2′-FL in particular were fermented by microbiota from both breast-fed and formula fed infants; 6′-SL was fermented but at a lower rate. Microbiota from formula fed infants fermented the substrates more rapidly than breast-fed which was likely due to the increased diversity of bacterial species and the higher proportion of *Bacteriodes* present in formula fed faeces. Acetate was the main short chain fatty acid detected.

Natividad et al (2022), using an *in vitro* cell culture assay with infant faecal samples, concluded from their study that the increased complexity of HMOs added to infant formula may benefit the infant gut microbiota by supporting a wider range of different *Bifidobacteria* spp, protecting the gut barrier against pro-inflammatory imbalances (Natividad et al. 2022).

Estorninos et al (2022) showed that addition of MOS had a significant effect on the composition of the microbiota with an increase in level of *Bifidobacterium* as compared to the control group, and a microbiota profile closer to that of breast-fed infants Estorninos et al. 2022). The authors noted that there were proportional differences in the species of *Bifidobacterium* present in the MOS-supplemented formula group which may be due to a competitive growth advantage where species which can metabolise the MOS components dominate. This also aligns with the observed higher concentration of lactate and acetate present in this group. For the control group, a higher concentration of propionate and butyrate was noted which may reflect the increased diversity of the microbiota, given that these are produced by *Bacteriodes* and Firmicutes. Both acetate and lactate lower the gut pH which may suppress pathogen growth, but acetate has been shown to directly promote host innate responses against *C. difficile* (Fachi et al. 2020). This supports the positive impact that *Bifidobacterium* spp. have on pathogen control in the infant colon.

Bosheva et al (2020) noted that at 6 months of age there was a significant increase in the relative abundance of *Bifidobacterium* for both 5 HiMO blend supplemented formula-fed groups ($P < 0.001$) and was comparable to that in breast-fed infants (Bosheva et al. 2022). In particular, *Bifidobacterium longum* subsp. *Infantis* (*B. infantis*) demonstrated a significant increase, with a relative abundance at 3 and 6 months in TG1 ($P < 0.0001$ and $P = 0.010$, respectively) than in the control group, and was more similar to the breast-fed group. This study also demonstrated a significant increase in the proportion of the faecal organic acids

acetate and lactate (the dominant end products of bifidobacteria catabolism), most similar to the human milk group, with the introduction of the 5 HiMO blend into the formula fed group. Butyrate and isobutyrate levels were lower than that observed for the unsupplemented formula group, which would be expected given the increased diversity in the microbiome. Dogra et al (2021) also demonstrated *in vitro* that *B. longum* subsp. *infantis* type strain (ATCC 15697) grew on 2′-FL, 6′-SL, LNT, and LNnT, with LNT generating the highest bacterial growth (Dogra et al. 2021). The highest amounts of acetate production were observed from *B. longum* subsp. *infantis* conditioned with LNT and LNnT as substrates. Strain-level differences of commercialised strains have however been found to differ in their ability to utilise HiMOs. Some strains of *B.infantis* have a fitness advantage through possession of a complete set of HMO utilisation genes (H5-positive strains), whilst H5-negative strains lack an ABC-type transporter which binds to core HMO structures (Duar et al. 2020).

5.3 Anti-pathogenic effects

Attachment of bacteria and viruses to the epithelial cells lining the gut is the first step for colonisation or invasion which is dependent on the common target carbohydrate structures on the host cell surface. Oligosaccharides in milk have been shown to be analogues of the cell surface receptors and thus can act as ligand decoys by binding to the bacterial cell surface facilitating an anti-adhesive function to pathogen surface receptor glycans and virulence agents. To examine this further, Lane et al (2011) developed a chip-based sensor with 2'FL and demonstrated binding *C. jejuni* to the chip-bound 2′-FL, that was inhibited by introduction of unbound 2'- FL (Lane et al. 2011). However, little to no binding was observed for *Staphylococcus aureus, Salmonella* Typhyimurium*, Cronobacter sakazakii, Pseudomonas aeruginosa,* and *Listeria monocytogenes.*

2′-FL has been shown in both animal and *ex-vivo* studies to inhibit the binding of *Campylobacter jejuni*, limiting colonisation and illness (Lane et al. 2011; Weichert et al. 2013; Yu et al. 2016; Ruiz et al. 2017). An early study by Morrow et al (2004) following breast-fed infants for the first two years postpartum showed that the incidence of *C. jejuni* illness was inversely correlated with levels of naturally occurring 2′-FL in human milk (Morrow 2004). *In vitro* assays demonstrate that synthetic 2′-FL compete with enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar Fyris, and *Pseudomonas aeruginosa,* which bind to α-1,2,fucosylated moieties of the H-2 antigens on the intestinal epithelial cells (Weichert et al. 2013). 3′-FL was shown to inhibit enteropathogenic *E. coli* and *P. aeruginosa* attachment in *ex vitro* studies of human intestinal and respiratory cell lines (Weichert et al. 2013). The authors acknowledge that there are some limitations to these studies due to the simple oligosaccharides that were used, however the study does support the concept of the use of bioengineered HMOs as anti-infective agents. Approximately 1% of ingested HMOs are absorbed into the circulation system and are excreted intact in the urine (reviewed in (Bode 2015)). The pathogen-binding inhibitory effect of 2′-FL against *C. jejuni* was assessed previously by FSANZ in Application A1155 (FSANZ 2019).

Antibiofilm plate-based assays demonstrated that DFL reduced the growth of *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Craft et al. 2018). DFL was reported to have significant antimicrobial activity against Group B Streptococcus by increasing cellular permeability, with the highest average growth reduction over 24 hours (51%) and the second highest average viability reduction (17%) of the HMOs evaluated (Chambers and Townsend 2020).

LNT and LNnT have been demonstrated to bind the "VP8*" glycan binding domain of the VP4 spike protein of several rotavirus strains (Yu et al. 2014; Sun et al. 2018), however

these HMOs may not be sufficient to act as decoy receptors against neonatal strain G10P[11] rotaviruses (Ramani et al. 2018). Similar decoy mechanisms have been proposed for viruses including norovirus by sialylated HMOs and rotavirus by 2′-FL (reviewed in (Walsh et al. 2020)).

6′-SL and 3′-SL have also been shown to bind viral strains in binding assays (Kubota et al. 2016; Wegener et al. 2017; Kubota et al. 2019). Both sialylactoses reduced infectivity of rotavirus strains in African green monkey kidney epithelial cells (Vero cells) (Laucirica et al. 2017), while only 3′-SL inhibited mumps virus entry (Kubota et al. 2019). 6′-SL and 3′-SL have been reported (through *in vitro* studies) to also have an anti-adhesive effect against bacterial pathogen strains associated with diarrhoea (enteropathogenic *E. coli* serotypes O127:H6 and O119, *Salmonella enterica* subspecies *enterica* serotype Fyris, and *Vibrio cholerae* ATCC 14034) (Angeloni et al. 2005; Coppa et al. 2006). More recently, Facinelli et al (2019) demonstrated that 6′-SL significantly inhibited the adhesion *of E. coli* serotype O119 to Caco-2 cells at a concentration occurring in human milk (0.6 g/L) compared to control without 6′-SL (Facinelli et al. 2019).

Piotrowski et al (2022) investigated the impact of 3′-SL and 6′-SL on attachment of *C. difficile* to 3 human colon cells lines *in vitro*. HiMOs were added at 1% (w/v) to the adhesion assay (Piotrowski et al. 2022). Adhesion of *C. difficile* was significantly inhibited to all three cell lines by the addition of both 3′-SL and 6′-SL, however there were differences in the degree of inhibition depending on the cell line used. Furthermore, there were differences in biofilm development by *C. difficile,* depending on the presence of 3′-SL and 6′-SL, through modulation of transcription of a component of biofilm formation, Cwp84 - a recognised virulence factor for of *C. difficile.*

Lin et al (2014) provided evidence to suggest that HMOs were also active in the urinary tract with decreased invasion by uropathogenic *Escherichia coli* (Lin et al. 2014). Further evidence suggested that in both the gastro-intestinal tract and the urinary tract, anti-adhesive properties of HMOs prevented bacterial attachment to the surface of the epithelial cells. HMOs were also demonstrated to supress intracellular apoptotic pathway signalling in epithelial cells.

A clinical trial of IF supplemented with a blend of 5 HiMOs demonstrated significantly reduced detection of toxigenic *Clostridioides difficle* in faecal samples taken at 3 and 6 months of age ($P \leq 0.069$ and $P \leq 0.001$ respectively) for both concentrations of HMOs as compared to supplemented infant formula (Bosheva et al. 2022). Furthermore, at 6 months the supplemented formula fed groups had lowered detection of *C. difficile* comparable to human milk fed infants. The prevalence of other pathogens was low in the faecal samples and so were not further investigated.

5.4 Immunomodulation effects

The gastrointestinal immune system of neonates is immature and breast milk, in addition to nutrition, is purported to regulate immune-related homeostasis and provide a protective mechanism for inducing tolerance to antigens (Duijts et al. 2010). Bioactive compounds in human milk are important for the development of appropriate immune responses (Cheng et al. 2021a; Chia et al. 2021) and antigenic memory (Bering 2018).

Goehring et al (2016) investigated the effect of 2′-FL, on immune function in healthy full term infants (Goehring et al. 2016). A sub-study nested within a randomized, double-blind, controlled growth and tolerance study was undertaken with infants less than 5 days old and either formula-fed (n = 317) or breastfed (n = 107) to 4 months old. Blood samples were taken at 6 weeks and analysed for a range of immune biomarkers. Infants on formula

supplemented with 2′-FL at either 0.2 g or 1 g/L were compared to breast-fed and a control group fed non-supplemented formula. Both breast-fed and infants fed formula containing 2′-FL had lower concentrations of plasma inflammatory cytokines as compared to non-supplemented. Further, the control group infants had lower levels of T-cells compared to breast-fed infants. Infants fed 2′-FL containing formulas exhibited innate cytokine profiles that were intermediate between breast-fed and control formula infants and more like breastfed infants. There was no difference between the two concentrations of 2′-FL compared to breast-fed infants. The authors concluded that supplementation with 2′-FL modified innate and adaptive immune profiles to be more aligned to the breast-fed group.

He et al (2014) showed that HMOs, especially 2′-FL, supressed expression of CD14 expression in by intestinal epithelial cells, attenuating LPS-induced inflammation (He et al. 2014). This observed inhibition supports the hypothesis that HMOs have a role in regulating the innate immune system to protect the infant through breast milk.

Ayechu-Muruzabal et al (2018) developed an *in vitro* model to examine the modulation of host responses to viral exposure (Ayechu-Muruzabal et al. 2018). They were able to show that 2'- FL in the presence of poly I:C (used to mimic viral exposure) has a role in increasing galectin secretion from intestinal epithelial cells and immune cells, which is part of the immune system for viral defence. They were also able to show changes in some cytokine expression in the presence of 2' FL with poly I:C stimulation, particularly IL-13, a central mediator for physiological changes induced by allergic inflammation.

The effect of individual HMOs on the attenuation of intestinal inflammation was studied by Cheng et al (2021b) in foetal (and adult) epithelial cells (Cheng et al. 2021b). Modulatory effects of the HMOs (2′-FL, 3'-FL, LNnT, LNT and 6'SL) were strongly structure-dependent and inflammatory responses were mostly seen in immature foetal epithelial cells that express more tumour necrosis factor receptor 1.

Bosheva et al (2022) used faecal secretory immunoglobulin A (sIgA) as a marker of intestinal immune response (Bosheva et al. 2022). sIgA levels for 5HiMO supplemented formula groups at 3 months were significantly higher than the unsupplemented formula group ($p=0.004$ and $p=0.016$ for the lower and higher supplemented feeding groups respectively). This difference continued to 6 months for the higher level of HMO supplemented media. In all cases, sIgA levels were significantly less than that produced by the human milk group ($p<0.001$) at both 3 and 6 months. This may be due to the difference in the range and concentration of HMOs that would be present in human milk.

Gut barrier function was also investigated through levels of faecal alpha-1-antitrypsin (ATT), a marker of intestinal permeability (Bosheva et al. 2022). Although concentrations of AAT were significantly lower in supplemented formulas than in the control group at 3 months, levels of AAT in breastfed infants were not significantly different from those in the formula-fed groups and at 6 months i.e. ATT was the same between all feeding groups.

Estorninos et al. (2022) observed a 2-fold increase in sIgA concentration in the MOS-supplemented formula group at 4 months compared to the supplemented formula in their study (Estorninos et al. 2022). This was positively correlated with *Bifidobacterium* abundance in the whole study population. This increase in sIgA is most likely associated with the increased proportion of *Bifidobacterium* in the microbiota, as they have been shown in other studies to interact directly with the immune cells and to modulate the innate and adaptive immune responses (Ruiz et al. 2017).

5.5 Summary of health effects

The addition of HiMOs to IFP is documented in the literature from the early 2000s, with *in vitro*, *ex vitro* and *in vivo* animal studies and clinical trials building a credible evidence base on the health effects of HiMOs used to supplement IFP.

There is evidence of a direct relationship between bacteria within the mothers' own milk, and the infant faecal profile, leading to the conclusion that these are unique to the individual and are, in part at least, supported by the human milk oligosaccharide profile of the milk. The effect of HiMOs on bacterial growth are bacterial strain as well as HiMO structure-dependent and different growth patterns were observed for different bacterial strains when exposed to the same HiMO. As an extension of this, HiMOs have different effects on microbiota composition, supporting a strong bifidogenic response as they can metabolise some HiMOs that reach the colon. Taking a weight of evidence approach, each HiMO investigated in this assessment demonstrated a bifidogenic effect, to a greater or lesser extent, both individually and as a 5HiMO blend.

There is some evidence to support a direct effect of HiMOs on pathogenic bacteria and some viruses (e.g. norovirus and rotavirus) by fucosylated and sialylated HiMOs via competitive inhibition by binding to the gut epithelial cell receptors, or mimicry of those receptors leading to direct binding of free HMOs to the bacteria preventing attachment. A clinical trial with a blend of 5 HiMOs demonstrated a notable reduction in toxigenic *C. difficile* which would reasonably be associated with a decreased risk of diarrheal illness in formula-fed infants.

These data suggest that the variety of human milk oligosaccharides in milk are able to collectively provide an anti-infective protection effect against a range of pathogenic microorganisms which a new born infant is likely to be exposed to. This would support the hypothesis that a mixture of HMOs added to infant formula is likely to provide a wider range of anti-infective activity.

There is evidence to support a role for some HiMOs, particularly 2′-FL, in inflammatory suppression and facilitating appropriate immune responses and antigenic memory. sIgA concentration has been shown to be positively correlated with the proportion of *Bifidobacterium* spp in the gut microbiota, which in turn is supported by the presence of human milk oligosaccharides in breast milk. Immunomodulatory functions have been postulated as a result of interactions between increased levels of bifidobacterial, human immune cells and their surface-associated molecules and metabolites.

From a microbiological aspect, the development of a "healthy microbiota" is supported by the inclusion of a wider range of HiMOs to IFP enabling the microbiota profile to closer resemble that of breast-fed infants.

6 Conclusions

FSANZ has undertaken an assessment of the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of 2′-FL/DFL, LNT, 6′-SL and 3′-SL to IFP.

Information reviewed in the food technology assessment demonstrates 2′-FL/DFL, LNT, 6′-SL and 3′-SL are chemically and structurally identical to the naturally occurring forms of these substances in human milk. The substances were shown to be stable in IFP with an adequate shelf-life. Multi-batch analyses showed the oligosaccharides can be consistently produced to meet their proposed specifications.

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and poses no risks to humans. Analyses of the gene donors also confirmed there are no safety concerns. The production strains are genetically and phenotypically stable.

Mean estimated dietary intakes of 2′-FL, DFL, LNT, 6′-SL and 3′-SL from IFP were comparable to mean estimated dietary intakes from mature human milk. High (90th percentile) estimated dietary intakes from IFP did not exceed estimated dietary intakes from mature human milk at high consumption and high concentration levels, except for DFL when assuming a representative maximum composition of 25% in the proposed 2′-FL/DFL mixture. The maximum composition of 25% for DFL is the most conservative concentration, and is markedly higher compared to the mean composition from analysis provided by the Applicant (12%). Based on the mean analysed concentration of DFL, the estimated mean intakes, which are more reflective of longer term intakes, from IFP are similar to that from human milk.

Based on the available toxicological and clinical data, and considering the dietary intake assessments, it was concluded that there are no public health and safety concerns associated with the addition of 2′-FL/DFL, LNT, 6′-SL and 3′-SL to IFP at the proposed use levels. No microbiological safety concerns were identified.

Post-marketing surveillance data have also found no safety concerns following consumption of infant formula containing the mixture of 2′-FL/DFL, LNT, 6′-SL and 3′-SL.

The weight of evidence supports health effects of HiMOs added to IFP through an increase in the abundance of *Bifidobacterium* spp. in the infant gut microbiota, anti-pathogenic effects, inflammatory suppression and facilitating appropriate immune responses and antigenic memory. The inclusion of a wider range of HiMOs to IFP enables the microbiota profile to more closely resemble that of breast-fed infants.

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Attachment 1

Study parameters for human intervention studies

Three human intervention studies that included the specified HiMOs (2′-FL/DFL, LNT, 6′-SL and 3′-SL) were provided by the Applicant to demonstrate safety (toxicological, microbiological and nutritional) and efficacy (supporting gut microbiome development).

FSANZ notes that two of the studies used a HiMO blend that included 3'-FL and no DFL (Parschat et al, 2021 and Lasekan et al, 2022).

The composition of the HiMO blend tested in each trial is shown in Table A-1. Study parameters (number of subjects etc.) are summarised in Table A-2.

Table A-1: Comparison of HiMO content in test formulas and the requested permission

HiMO	Parschat 2021 (E=2800 kJ/L)		Lasekan 2022 (E=2828 kJ/L)		Bosheva 2022[1] (E=2800 kJ/L)		Requested permission	Permitted in S29
Units	*g/L*	*mg/100 kJ*	*g/L*	*mg/100 kJ*	*g/L*	*mg/100 kJ*	*mg/100 kJ*	*mg/100 kJ*
2′-FL	2.99	107	3.0	106	0.87 1.45	31 52	Comb. not exceeding 96	96
DFL	—	—	—	—	0.10 0.14	3.6 5.0		—
3-FL	0.75	27	0.8	28	—	—	—	—
LNT	1.5	54	1.5	53	0.29 0.48	10 17	32	—
3′-SL	0.23	8.2	0.2	7.1	0.11 0.18	3.9 6.4	8	—
6′-SL	0.28	10	0.3	11	0.14 0.24	5.0 8.6	16	—
Total	5.75	206	5.8	205	1.5 2.5	54 89	152	96

[1] Bosheva et al (2022) is the published report of this clinical trial. It is the same clinical trial as Cohen et al (2022, unpublished) (identifier NCT03722550)

Table A-2 Summary of human intervention trials

Study design	IF composition	Study population and allocation	Outcomes	Other
Lasekan (2021) United States (multisite)				
Randomised, double-blind, controlled parallel trial. Randomisation method: computer-generated using a dynamic minimization algorithm with a random component. Randomisation was stratified by sex.	Base composition: standard commercial formula that met US FDA requirements HiMO source: N.A. See Table A-1 for HiMO composition	Healthy term infants ≤ 14 days fed control or test formulas from enrolment to 119 days of age. 366 enrolled 222 completed HiMO formula: 130 non-HMO formula: 129 Human milk reference group (non-randomised): 104 (N=3 not randomised because deemed non-eligible)	***Growth:*** Primary outcome: weight gain per day from D14 to D119. Secondary variables were weight, interval weight gain per day, length, interval length gain per day, head circumference (HC) and interval HC gain per day. Mean weight for age, mean length for age and mean HC for age were plotted on the WHO growth charts. ***Safety/Tolerance:*** Records collected by parents: spit up and vomit with feeding, stool characteristics (frequency, consistency, color), infant feeding and stool pattern questionnaires, infant behaviour questionnaires, formula satisfaction questionnaires. Physician confirmed: non-serious and serious adverse effects. Due to COVID-19, the anthropometric measurement protocol was adjusted.	The study was registered at clinicaltrials.gov (# NCT04105686). "A higher proportion of participants in the formula-fed groups exited the study early compared to the participants who were fed HM [human milk] (all p = 0.009)." Primary analysis: protocol-evaluable analysis (n=222). Secondary analysis: ITT (n=366 enrolled. N=356 ITT. N=3 not randomised because deemed non-eligible. N=7 randomised but never consumed study formula. Therefore n=10 excluded from ITT.) Conflict of interest: study was funded by Abbott Nutrition and all authors are current employees of the study sponsor.
Parschat (2021) Multicentre (Germany, Italy and Spain)				
Randomised, double-blind, controlled, multi-centre, parallel nutritional noninferiority study performed across Germany, Italy and Spain Randomisation method: a randomisation list was generated by an independent statistician in a 1:1 ratio in blocks of four and stratified by sex and site.	Base composition: The infant formula was manufactured by Töpfer (Dietmannsried) to meet EU regulations HiMO source: The 5HMO-Mix was produced by Chr. Hansen HMO GmbH, Rheinbreitbach, Germany.	Healthy term infants ≤ 14 days fed control or test formulas from enrolment to 112 days. Voluntary 8-week follow-up period. Enrolled: 341 HiMO formula: 113 non-HMO formula: 112 Human milk reference group (non-randomised): 116	***Growth:*** Primary outcome: weight gain per day over 4 months period (to 112 days). Secondary outcomes included other anthropometric data such as (changes in) weight, length, and head circumference, their increments, and their respective WHO growth standard z-scores (weight-for-age, length-for-age, head circumference-for-age, and weight-for-length). ***Safety/Tolerance:*** Records collected by parents: stool frequency and consistency, digestive tolerance (regurgitation,	The study was registered at clinicaltrials.gov (# NCT03513744). Initial drop-outs (reason: "did not receive allocated intervention"): C, n=0; I (non-HMO) n=3; and, I (HMO), n=4. Drop-outs during study: C, n=28; I (non-HMO) n=21; and, I (HMO), n=27. Reasons provided (see Fig 2 in publication). Differences in number of drop-outs between groups was not statistically analysed. Conflict of interest: study was funded by Chr. Hansen HMO GmbH, Rheinbreitbach, Germany

Study design	IF composition	Study population and allocation	Outcomes	Other
	See Table A-1 for HiMO composition	Completed: 222 HiMO formula: 72 non-HMO formula: 77 Human milk reference group (non-randomised): 73	vomiting, flatulence), behaviour (fussiness, crying, awakening at night. Adverse events (AEs): any medical occurrence during the intervention period; concomitant medication, medical treatment or healthcare utilization were recorded. Due to COVID-19, the anthropometric measurement protocol was adjusted.	and all authors are employees of Chr. Hansen or the former affiliation Jennewein Biotechnologie.
Cohen (2022, unpublished)				
Double-blind, randomised, controlled multi-centre trial across Bulgaria, Hungary and Poland. Infants exclusively consuming cow's milk formula were recruited and randomised to a Control Group, Test Group 1 or Test Group 2. A group of exclusively breastfed infants of the same age was also enrolled as a Reference Group	Base composition: bovine-milk-based, whey predominant starter infant formula, and follow-up formula HiMO source: N.A. See Table A-1 for HiMO composition	Healthy term infants aged 7 – 21 days . Enrolled: 789 Randomised to 3 formula groups: 686 (HiMO formula 1, HiMO formula 2 and non-HMO formula) Human milk reference group (non-randomised): 96 Completed at 4 months: 577 Completed at 12 months: 573	***Growth:*** Primary outcome: weight gain per day from V1 to V6 (4 mo period). Secondary outcomes included other anthropometric data such as (changes in) weight, length, and head circumference, their increments, and their respective WHO growth standard z-scores (weight-for-age, length-for-age, head circumference-for-age, and weight-for-length). ***Safety/Tolerance:*** Stool frequency, difficulty passing stool, flatulence, fussing, and Infant Gastrointestinal Symptom Questionnaire (IGSQ) composite scores ***Efficacy:*** Faecal microbiota, pH, organic acids, and markers of intestinal immune response, permeability, and inflammation	The study was registered at clinicaltrials.gov (# NCT 03722550) Study duration to 12 months. Complementary foods were allowed after the age 4 months. Additional study details were provided in the Application as CCI.