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Te Mana Kounga Kai - Ahitereiria me Aotearoa

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Supporting document 1

Risk, technical and benefit assessment – Application A1339

A1339 - 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL from *Escherichia coli* BL21 for use as nutritive substances in infant formula products

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Executive summary

FSANZ received an application from Chr. Hansen A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit the addition of 5 human-identical milk oligosaccharides (HiMO) for use as nutritive substances, alone or in combination, in infant formula products. FSANZ has assessed the public health and safety risks associated with:

- Permitting the addition of the applicant's HiMO for use as nutritive substances, alone or in combination, in infant formula products. The applicant's HiMO are produced by microbial fermentation using genetically modified (GM) strains of *Escherichia coli* BL21.
- Increasing the maximum amount of the HiMO from all permitted GM sources in the Code.

The HiMO and their proposed maximum amounts are:

- 2′-fucosyllactose (2′-FL) (3.0 g/L in infant formula; 3.64 g/L in follow-on formula and special medical purpose product for infants)
- 3-fucosyllactose (3-FL) (0.9 g/L in infant formula; 1.2 g/L in follow-on formula and special medical purpose product for infants)
- lacto-N-tetraose (LNT) (1.82 g/L in infant formula products)
- 3′-sialyllactose (3′-SL) sodium salt (0.28 g/L in infant formula products)
- 6′-sialyllactose (6′-SL) sodium salt (0.7 g/L in infant formula products)

FSANZ has previously assessed and approved the applicant's 2′-FL for use as a nutritive substance. FSANZ has determined that the applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt are chemically, structurally and functionally identical to the naturally occurring forms of these substances in human milk.

The food technology assessment demonstrated that the applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt were shown to be stable in infant formula products with an adequate shelf-life. Multi-batch analyses showed the oligosaccharides can be consistently produced to meet their proposed specifications.

FSANZ's microbiological risk assessment did not identify any public health and safety concerns associated with the use of *E. coli* BL21 as a host organism. The GM *E. coli* BL21 strain used to produce 2′-FL conforms to the permitted source organism listed in Schedule 26 of the Code and was therefore not further assessed. The GM *E. coli* BL21 production strains used to manufacture 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt were characterised to confirm the presence of the introduced genes and to demonstrate that each production strain was genetically and phenotypically stable.

Building on previous FSANZ assessments, recent literature and a weight of evidence approach, the associated health benefits from the use of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt as nutritive substances in infant formula products at the proposed amounts are recognised as: (1) an anti-pathogenic effect; (2) immunomodulation and (3) development of the gut microbiome through supporting growth of *Bifidobacteria* spp. There is evidence from clinical and *in vitro* studies that the HiMO concentrations discussed in this application can provide these benefits.

The public health and safety associated with the requested maximum amounts of the following HiMO from all sources permitted in the Code to be used as nutritive substances in infant formula products were considered:

2′-FL
No public health and safety concerns have been identified associated with the addition of 2′-FL in infant formula products at the proposed amounts. Toxicological studies confirm that 2′-FL is safe and well tolerated in infant formula products. The addition of 2′-FL at concentrations up to 3 g/L in infant formula and 3.64 g/L in follow-on formula, which is within the range found in human milk, is unlikely to affect normal infant growth.

3-FL
No public health and safety concerns have been identified associated with the addition of 3-FL to infant formula products at the proposed amounts. Toxicological studies confirm that 3-FL is safe and well tolerated in infant formula products. The addition of 3-FL at a concentration of 0.9 g/L in infant formula and 1.2 g/L in follow-on formula, which is within the range found in human milk, is unlikely to affect normal infant growth.

LNT
Toxicological studies confirm that LNT is safe and well tolerated in infant formula products. The addition of LNT at concentrations up to 1.6 g/L in infant formula and up to 1.37 g/L in follow-on formula is unlikely to affect normal infant growth. However, the requested concentration of 1.82 g/L in infant formula products is greater than that found in human milk, and therefore its effect on infant growth at these concentrations could not be determined.

3′-SL sodium salt
No public health and safety concerns have been identified associated with the addition of 3′-SL sodium salt to infant formula products at the proposed amount. Toxicological studies confirm that 3′-SL sodium salt is safe and well tolerated in infant formula products. The addition of 3′-SL sodium salt at a concentration of 0.28 g/L in infant formula products, which is within the range found in human milk, is unlikely to affect normal infant growth.

6′-SL sodium salt
No public health and safety concerns have been identified associated with the addition of 6′-SL sodium salt to infant formula products at the proposed amount. Toxicological studies confirm that 6′-SL sodium salt is safe and well tolerated in infant formula products. The addition of 6′-SL sodium salt at a concentration of 0.7 g/L in infant formula products, which is within the range found in human milk, is unlikely to affect normal infant growth.

Combination of 5 HiMO
The safety of the 5 HiMO in combination was also considered. No adverse effects of the applicant's "5HMO-Mix" were observed in a 13-week dietary toxicity study in rats or a 21-day neonatal piglet study, and no treatment-related adverse events were observed in infants consuming a formula containing the 5HMO-Mix for four months. The requested maximum amounts of the 5 HiMO are already approved in the EU, with no reports of adverse effects in that population.

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Glossary

2′-FL	2′-fucosyllactose
3-FL	3-fucosyllactose
3′-SL	3′-sialyllactose
6′-SL	6′-sialyllactose
DFL	difucosyllactose
HiMO	Human-identical milk oligosaccharide(s)
HMO	Human milk oligosaccharide(s)
LNT	Lacto-N-tetraose
P50	50th percentile
P90	90th percentile
SMPPi	Special medical purpose product for infants

1 Introduction

FSANZ received an application from Chr. Hansen A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit the addition of 5 human-identical milk oligosaccharides (HiMO) for use as nutritive substances, alone or in combination, in infant formula products. The HiMO are produced by microbial fermentation using genetically modified strains of *Escherichia coli* BL21. The HiMO and the levels requested are shown in Table 1.

Table 1: HiMO and their requested maximum amounts

HiMO	Infant formula (g/L)	Follow-on formula and SMPPi (g/L)
2′-fucosyllactose (2′-FL)	3.0	3.64
3-fucosyllactose (3-FL)	0.9	1.2
lacto-N-tetraose (LNT)	1.82	1.82
3′-siallylactose (3′-SL) sodium salt	0.28	0.28
6′-siallylactose (6′-SL) sodium salt	0.7	0.7

The stated purpose for adding the HiMO to infant formula products is to align their composition more closely to that of human milk.

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

2 Food technology assessment

The objective of the food technology assessment was to determine whether the applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt are identical to that present in human milk. The assessment also considered the manufacturing process, stability, specifications and analytical methods of analysis used to quantify and characterise these substances.

FSANZ has assessed recent applications requesting permissions for human-identical milk oligosaccharides (HiMO) for use in infant formula products The information in this section has built on the assessment under those applications, i.e. A1155, A1190, A1233, A1251, A1265, A1277, A1283, A1308, and A1324 (FSANZ 2020, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024, FSANZ 2025a, FSANZ 2025b).

As FSANZ has assessed and approved the applicant's 2′-FL for use as a nutritive substance and included a relevant specification in the Code (under Application A1190), further food technology assessment has not been carried out on that substance.

2.1 Chemical and physical properties

The substances 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt are part of the human milk oligosaccharide (HMO) fraction found in human milk. A brief description of each substance is included below. The structural formula of each substance is provided in

previous supporting documents for the relevant applications referenced above and can be found in Table 5 of the application dossier for this application.

3-FL is a trisaccharide consisting of D-glucose, D-galactose and L-fucose, derived from lactose by addition of a fucose sugar to the glucose unit by an alpha (1-3) linkage). 3-FL is an isomer of 2′-FL with the fucose sugar linked to the galactose unit of lactose.

LNT is a constitutional isomer of another permitted oligosaccharide lacto-N-neotetraose (LNnT) (permitted under Application A1155). LNT was permitted under Application A1265. LNT 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. Both 6′-SL and 3′-SL are in the form of sodium salts.

Each of the individual HiMOs are readily soluble in water, 500g/L at ambient temperature.

Table 6 of the application dossier provides a summary of the chemical and physical properties of each HiMO.

2.1.1 Equivalence to human milk

The application presented analytical data, which was deemed Confidential Commercial Information (CCI) under Section 114 of the *Food Standards Australia New Zealand Act 1991 (*the Act)*,* to substantiate that 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt produced via microbial fermentation are chemically and structurally equivalent to their counterparts found naturally in human milk. FSANZ's assessment of these data confirmed that each of the substances are chemically and structurally identical to those occurring in human milk.

2.1.2 Stability of 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt

The applicant provided details of stability studies carried out on each individual HiMO. Some of this information is deemed CCI under the Act and therefore cannot be disclosed in this report. Based on the assessment of this information, FSANZ concludes the following:

- Analytical data for representative batches of each the applicant's HiMO support stability for 24 months.
- Analytical data for each of the applicant's HiMO when added to infant formula milk powder indicate stability for up to 24 months at ambient temperature.

2.2 Manufacturing processes

The applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt are produced by microbial fermentation using GM strains of *E. coli* BL21 which are described in section 3.1. The production process consists of 3 main stages: upstream processing (USP), downstream processing (DSP) and drying. The USP consists of the fermentation steps while the DSP encompasses the purification, isolation and concentration steps.

Details of the manufacturing flow and processes and food safety management systems are CCI. While these details have been evaluated by FSANZ, they cannot be disclosed in this report.

2.3 Specifications

Section 1.1.1—15 of the Code states that any substance used as a nutritive substance must meet the applicable identity and purity specification in Schedule 3.

While Schedule 3 contains specifications for 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt, these specifications were developed following assessment of previous applications, where the production strain was not *E. coli* BL21.

The applicant has proposed specifications for 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt, consistent with the relevant EU regulations. The analytical results for 5 independent representative batches of each of these substances provided in the application indicate that they can meet the comparable specification.

Table 2 below shows the proposed specification parameters for inclusion in Schedule 3 of the Code. The proposed specification parameters are based on information provided in the application and the specifications in the EU Regulations listed below:

- 3-FL: Commission Implementing Regulation (EU) 2023/52 - Implementing regulation - 2023/52 - EN - EUR-Lex
- LNT: Commission Implementing Regulation (EU) 2023/7 - Implementing regulation - 2023/7 - EN - EUR-Lex
- 3′-SL sodium salt: Commission Implementing Regulation (EU) 2023/113 - Implementing regulation - 2023/113 - EN - EUR-Lex
- 6′-SL sodium salt: Commission Implementing Regulation (EU) 2023/948 - Implementing regulation - 2023/948 - EN - EUR-Lex

The HiMO preparations consist of the stated HiMO (i.e. 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt for which a minimum purity value is included in the specification), together with smaller amounts of other carbohydrates and ash (which are subject to maximum values in the specification). The parameter ‘sum of other carbohydrates’ ensures that the HiMO preparation consists primarily of the HiMO, together with the other (quantified) carbohydrates listed in the table.

Specification parameters have been included in Table 2 for lead, cadmium, arsenic and mercury. The values included in Table 2 are consistent with the applicant’s 2′-FL (listed in S3—45).

Microbiological limits are in place in Schedule 27 for *Cronobacter* (not detected in 10 g – for Infant formula) and *Salmonella* (not detected in 25 g - for Infant formula and follow-on formula). Therefore, these parameters are not listed in the proposed specifications in Table 2. Limits for Enterobacteriaceae, yeasts, moulds, and residual endotoxins were provided by the applicant, and are consistent with specifications for similar HiMO substances permitted in the Code.

Table 2: Proposed specifications for 3-FL, LNT, 3'-SL sodium salt and 6'-SL sodium salt from E. coli BL21

Parameter	3-FL	LNT	3′-SL sodium salt	6′-SL sodium salt
Chemical name	β-D-Galactopyranosyl-(1→4)- [α-L-fucopyranosyl-(1→3)]- D-glucopyranose	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose	N-Acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt	N-Acetyl-α-D-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt
Chemical formula	$C_{18}H_{32}O_{15}$	$C_{26}H_{45}NO_{21}$	$C_{23}H_{38}NO_{19}Na$	$C_{23}H_{38}NO_{19}Na$
Molecular mass	488.44 g/mol	707.63 g/mol	655.53 g/mol	655.53 g/mol
CAS No.	41312-47-4	14116-68-8	128596-80-5	157574-76-0
Appearance Colour	White to off-white powder	White to off-white powder	White to off-white powder or agglomerate	White to off-white powder or agglomerate
3-FL (3-fucosyllactose)	Not less than 90% (water free)	-	-	-
Water	Not more than 9.0%	Not more than 9.0%	Not more than 9.0%	Not more than 9.0%
L-fucose	Not more than 3.0% (water free)	-	-	-
D-galactose	Not more than 3.0% (water free)	-	-	-
D-glucose	Not more than 3.0% (water free)	-	-	-
Sum of D-glucose and D-galactose	See separate entries	Not more than 5.0% (water free)	See separate entries	See separate entries
D-lactose	Not more than 5.0% (water free)	Not more than 5.0% (water free)	Not more than 5.0% (water free)	Not more than 5.0% (water free)

Parameter	3-FL	LNT	3′-SL sodium salt	6′-SL sodium salt
Sum of other carbohydrates[1]	Not more than 5.0% (water free)	Not more than 15.0% (water free)	Not more than 5.0% (water free)	Not more than 5.0% (water free)
LNT (lacto-N-tetraose)	-	Not less than 75% (water free)	-	-
Lacto-N-triose II	-	Not more than 5.0% (water free)	-	-
Para-lacto-N-hexaose	-	Not more than 5.0% (water free)	-	-
3′-SL sodium salt (3′-sialyllactose sodium salt)	-	-	Not less than 88.0% (water free)	-
3′-Sialyl-lactulose	-	-	Not more than 5.0% (water free)	-
6′-SL sodium salt (6′-sialyllactose sodium salt)	-	-	-	Not less than 90% (water free)
6′-Sialyl-lactulose	-	-	-	Not more than 3.0% (water free)
Sialic acid–(acetyl-D-glucosamine)	-	-	Not more than 1.5 % (water free)	Not more than 2.0% (water free)
N-acetyl-D-glucosamine	-	-	Not more than 1.0% (water	Not more than 3.0% (water

[1] Sum of other carbohydrates = 100 (% (w/w) of dry matter) minus quantified carbohydrates (% (w/w) of dry matter) minus Ash (% (w/w) of dry matter). Quantified carbohydrates are those listed in the table for the applicable HiMO, and includes the relevant HiMO (3-FL, LNT, 3′-SL sodium salt or 6′-SL sodium salt).

Parameter	3-FL	LNT	3′-SL sodium salt	6′-SL sodium salt
			free)	free)
Sodium	-	-	Not more than 4.2%	Not more than 4.2%
Ash, sulphated	Not more than 1.0 %	Not more than 1.0 %	Not more than 8.5 %	Not more than 8.5 %
Residual proteins	Not more than 0.01%	Not more than 0.01%	Not more than 0.01%	Not more than 0.01%
Arsenic	Not more than 0.2 mg/kg	Not more than 0.2 mg/kg	Not more than 0.2 mg/kg	Not more than 0.2 mg/kg
Cadmium	Not more than 0.1 mg/kg	Not more than 0.1 mg/kg	Not more than 0.1 mg/kg	Not more than 0.1 mg/kg
Mercury	Not more than 0.5 mg/kg	Not more than 0.5 mg/kg	Not more than 0.5mg/kg	Not more than 0.5 mg/kg
Lead	Not more than 0.02 mg/kg	Not more than 0.02 mg/kg	Not more than 0.02 mg/kg	Not more than 0.02 mg/kg
Microbiological				
Aerobic mesophilic total plate count	Not more than 1000 cfu/g	Not more than 1000 cfu/g	Not more than 1000 cfu/g	Not more than 1000 cfu/g
Enterobacteriaceae	Not more than 10 CFU/g	Not more than 10 CFU/g	Not more than 10 CFU/g	Not more than 10 CFU/g
Yeasts and moulds	Not more than 100 cfu/g	Not more than 100 cfu/g	Not more than 100 cfu/g	Not more than 100 cfu/g
Aflatoxin M1	Not more than 0.025 ug/kg	Not more than 0.025 ug/kg	Not more than 0.025 ug/kg	Not more than 0.025 ug/kg
Residual endotoxins (Endotoxin units)	Not more than 10 EU/mg	Not more than 10 EU/mg	Not more than 10 EU/mg	Not more than 10 EU/mg

2.4 Analytical methods of detection

The analytical methods of analyses to detect and quantify 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt in milk products are based on methods deemed CCI under the Act (Section B.5 of the application states that the methods are HPAEC-PAD for carbohydrate analyses). The application also provided analytical methods used to quantify the parameters listed in their specifications for each of the oligosaccharides which were considered by FSANZ and also deemed CCI.

2.5 Food technology conclusion

The applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt produced by a microbial fermentation method of production are chemically and structurally identical to the naturally occurring substances present in human milk.

The applicant has proposed specifications for 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt consistent with the relevant EU regulations. This information was used to develop specifications for inclusion in Schedule 3 of the Code. The analytical results for each of these substances provided in the application indicate they can meet these specifications.

Stability studies of the applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt concluded that these nutritive substances in powder form are stable for 24 months. The applicant's 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt when added to powdered infant formula products are stable for 24 months under ambient conditions.

3 Safety assessment

FSANZ has assessed all information relevant to the safety of the material that is the subject of this application. Only information not deemed CCI under the Act is provided below.

3.1 Genetically modified (GM) production strain assessment

3.1.1 Host organism

The applicant uses distinct production strains to produce each of the 5 HiMO, all derived from *Escherichia coli* BL21 (DE3). BL21 (DE3) is a direct derivative of *E. coli* BL21 with minimal, well-characterised divergence. Due to this and the minimal divergence between the 5 production strains, FSANZ's microbiological assessment will focus on the safety of the parent strain, *E. coli* BL21.

Escherichia coli is a facultative anaerobe, gram-negative, rod-shaped bacteria found in the gut of mammals (Guerra et al. 2019). Some *E. coli* strains can be pathogenic to humans causing a wide range of diseases, which can be fatal (Guerra et al. 2019, Mir and Kudva 2019). However, there are strains of *E. coli*, termed safe strains, that are used in research and industry specifically because of their inability to cause disease in humans (Bauer et al. 2008). BL21 is one of these safe strains. It lacks well-recognised pathogenic mechanisms (Chart et al. 2000). As such it is classified as non-pathogenic and is categorised as a Biosafety Level 1 and Risk Group 1 organism (Bauer et al. 2008, Pinske et al. 2011).

BL21 is widely used in biotechnology, molecular biology, and industrial applications (Pinske et al. 2011, Kim et al. 2017). Originally developed for its utility as an expression host, BL21 has a long history of safe use in research and production of recombinant proteins and other bioproducts. Because of its extensive industrial use, BL21 has previously been assessed by regulatory agencies, including the U.S. Food and Drug Administration (FDA), the European

Food Safety Authority (EFSA) and FSANZ (in applications A1190 and A1318), for use in industrial processes under Good Manufacturing Practices (GMP). The use of BL21 as the production organism of food ingredients has been concluded to be 'generally recognised as safe' (GRAS) numerous times over the past decade (e.g. GRNs 485, 571, 876, 921, 922, 923, 925, 1015, and 1016).

The *E. coli* BL21 production strains assessed here have been genetically modified to contain well-characterised synthetic genes for the purpose of HiMO production. See section 3.1.2 for more detail. The taxonomic identity of the production strains were confirmed by data deemed as CCI under the Act. . Furthermore, batch analyses demonstrate that viable cells as well as residual DNA from each of the production strains are absent from the final product.

Overall, no public health and safety concerns were identified for the use of *E. coli* BL21 as a production organism.

3.1.2 Characterisation of the GM production organisms

The modifications specific to each production strain were:

- 3-SL: insertion of the gene for alpha-1,3-fucosyltransferase from *Bacteroides fragilis*
- LNT: insertion of the genes for beta-1,3-N-acetylglucosaminyltransferase from *Neisseria meningitidis* and for beta-1,3-galactosyltransferase from *Salmonella enterica*
- 3′-SL sodium salt: insertion of the gene for alpha-2,3-sialyltransferase from *Haemophilus parahaemolyticus*
- 6′-SL sodium salt: insertion of the gene for alpha-2,6-sialyltransferase from *Streptococcus suis.*

3.1.2.1 Development of the GM production strains

The GM *E. coli* BL21 strain used to produce 2′-FL conforms to the permitted source organism listed in Schedule 26 of the Code (*Escherichia coli* BL21 containing the gene for alpha-1,2-fucosyltransferase from *Escherichia coli* O126). Therefore, based on FSANZ's previous assessment as part of Application A1190, an assessment of this production strain was not required.

To create the production strains for 3-FL, LNT, 3′-SL sodium salt, and 6′-SL sodium salt, a series of common genetic modifications were first introduced into the parental *E. coli* BL21 strain. This base strain was then further modified for each HiMO by inserting the genes required its biosynthesis. All heterologous genes were inserted into the genome using standard molecular biology techniques.

All inserted genes were chemically synthesised prior to insertion. This avoids the possibility of any extraneous DNA from the gene donor organisms being inadvertently transferred to the production strain.

Data provided by the applicant and analysed by FSANZ confirmed the identity of each of the heterologously expressed genes.

3.1.2.2 Characterisation of introduced DNA

Polymerase chain reaction (PCR) was used to verify the presence of the inserted DNA in the production strains. These analyses confirmed the presence of the intended genomic insertions in each of the production strains.

3.1.2.3 Genetic stability and inheritance of the introduced DNA

The PCR analyses that were used to demonstrate the presence of the inserted DNA in the production strains also showed that these insertions were stable over 5 successive generations. Targeted proteomic analyses of selected proteins involved in HiMO synthesis in each production strain confirmed consistent expression of all proteins over 5 generations.

Mass spectrometric data was also provided showing that HiMO production by each production strain was stable over 5 generations, providing further evidence of the stability and inheritance of the inserted DNA over this period.

3.1.3 Conclusion

FSANZ's microbiological risk assessment did not identify any public health and safety concerns associated with the use of *E. coli* BL21 as a production organism for 3-FL, LNT, 3′-SL sodium salt, and 6′-SL sodium salt. Characterisation of the GM production strains confirmed all introduced genes were inserted as expected and were genetically stable and functional.

Based on the data provided, no safety concerns were identified in the assessment of the 3-FL, LNT, 3′-SL sodium salt, or 6′-SL sodium salt production strains.

3.2 Toxicology assessment

3.2.1 Kinetics and metabolism

The metabolism of the individual HiMO has been previously reviewed by FSANZ. Briefly, the HiMO are chemically identical to the forms present naturally in human milk (HMO) and therefore are expected to share the same kinetics. Data indicate that intestinal absorption in the small intestine is limited, and a significant proportion of the HiMO reaches the large intestine where they are either fermented by the microbiota or excreted unchanged in the faeces.

3.2.2 Status regarding safety

3.2.2.1 2′-FL

Current permission

2′-FL is currently permitted in the Code at 2.4 g/L in infant formula products, based on previous assessments, of which the first was under A1155 (2019). The applicant's 2′-FL has already been approved (A1190; 2021) and is listed in Schedules 3 and 26 of the Code. The existing approval for 2′-FL at up to 2.4 g/L, reached in A1155, is based on the average level in human milk and on overseas approvals at up to that level. In the current application, the applicant has requested 2′-FL at higher levels; a level of 3.0 g/L for infant formula and 3.64 g/L for follow-on formula.

Safety

The safety of 2′-FL has previously been reviewed by FSANZ under applications A1155, A1190, A1233, A1251, A1265, A1277, A1283, and A1308 (FSANZ 2019, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024a, FSANZ 2025a).

FSANZ found that 2′-FL is not mutagenic or genotoxic *in vitro* and has no adverse effects in neonatal animals. In clinical studies, consumption of infant formula containing 2′-FL is safe and well tolerated.

Safety assessments by other agencies

In the EU, 2′-FL is permitted for use in infant formula at up to 3.0 g/L, and in toddler 'follow on' formula at up to 3.64 g/L. These are the same levels that the applicant is requesting in the current application.

3.2.2.2 3-FL

Current permission

FSANZ assessed 3-FL at concentrations up to 0.8 g/L in application A1324 (FSANZ 2025b) The applicant has requested approval for 3-FL at a level of 0.90 g/L for infant formula and 1.20 g/L for follow-on formula.

Safety

FSANZ found that 3-FL does not pose concerns regarding genotoxicity. No adverse effects were observed in neonatal rats or neonatal piglets. In clinical studies with human infants, formula containing up to 0.8 g/L of 3-FL in combination with other HiMO was safe, well tolerated and did not affect growth. Post-marketing surveillance data from other countries have also found no safety concerns from consumption of infant formula containing 3-FL in combination with up to 5 other HiMO.

Safety assessments by other agencies

In the EU, 3-FL is permitted for use in infant formula and in toddler 'follow on' formula at up to 1.75 g/L. These are higher levels than the applicant is requesting in the current application.

3.2.2.3 LNT

Current permission

LNT was assessed by FSANZ under application A1265 and is currently permitted in the Code at 0.8 g/L in infant formula products (FSANZ 2023a). The applicant has requested permission for a higher level of 1.82 g/L for LNT in infant formula products.

Safety

No public health and safety concerns associated with LNT to infant formula were identified in A1265.

Safety assessments by other agencies

In the EU, LNT is permitted for use in infant formula and in toddler 'follow on' formula at up to 1.82 g/L. This is the same level that the applicant is requesting in the current application.

3.2.2.4 3'-SL sodium salt

Current permission

3′-SL sodium salt was assessed by FSANZ under A1265 and is permitted in the Code at 0.2 g/L in infant formula products (FSANZ 2023a). The applicant has requested 3′-SL sodium salt at a level of 0.28 g/L in infant formula products.

Safety

No public health and safety concerns associated with the addition of 3′-SL sodium salt to infant formula were identified in A1265.

Safety assessments by other agencies

In the EU, 3′-SL sodium salt is permitted for use in infant formula and in toddler 'follow on' formula at up to 0.28 g/L. This is the same level that the applicant is requesting in the current application.

3.2.2.5 6′-SL sodium salt

Current permission

6′-SL sodium salt was assessed by FSANZ under A1265 (FSANZ 2023a) and is permitted in the Code at 0.4 g/L infant formula products. The applicant has requested 6′-SL sodium salt at a level of 0.7 g/L in infant formula products.

Safety

No public health and safety concerns associated with the addition of 6′-SL sodium salt to infant formula were identified in A1265.

Safety assessments by other agencies

In the EU, 6′-SL sodium salt is permitted for use in infant formula and in toddler 'follow on' formula at up to 0.70 g/L. This is the same level that the applicant is requesting in the current application.

3.2.3 Combination of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt from *Escherichia coli* BL21

Toxicology data for the applicant's combination of HiMO, characterised as 5HMO-Mix, are presented in Appendix 1. No adverse effects were observed in a 13-week dietary toxicity study in rats, or in a 21-day study in neonatal piglets.

The calculated intakes of 5HMO-Mix in the rat study were 5.67 g/kg bw/day for males and 6.97 g/kg bw/day for females. Group mean intakes of individual HiMO were higher in female rats than in males, at 3.28 g/kg bw/day for 2′-FL, 1.12 g/kg bw/day for 3-FL, 1.65 g/kg bw/day for LNT, 0.29 g/kg bw/day for 3′-SL sodium salt, and 0.28 g/kg bw/day for 6′-SL sodium salt. The mean levels of 2′-FL, LNT, and 6′-SL sodium salt consumed by the rats were lower than the estimated dietary intakes from the levels requested in the current application, whereas the mean doses of 3-FL consumed by the rats were slightly higher than estimated dietary intakes from the levels requested in the application. The mean doses of 3′-SL sodium salt were comparable to the estimated dietary intakes from the requested level.

In the piglet study, the calculated intakes of 5HMO-Mix were 3.6 and 3.7 g/kg bw/day in males and females, respectively, corresponding to intakes of approximately 1.8 g 2′-FL/kg bw/day, 0.4 g 3-FL/kg bw/day, 0.7 g LNT/kg bw/day, 0.1 g 3′-SL sodium salt/kg bw/day, and 0.2 g 6′-SL sodium salt/kg bw/day. These levels of intake are all lower than the estimated dietary intakes at the maximum levels requested in the application.

No evidence of genotoxicity was found in a bacterial reverse mutation assay or in an *in vitro* mammalian chromosomal aberration assay in human peripheral blood lymphocytes.

The applicant's 5HMO-Mix was safe and well-tolerated in a multi-centre, randomised, double-blinded, controlled, parallel group clinical study conducted in healthy term infants ≤14 days of age over a period of four months.

3.2.4 Toxicology assessment conclusions

All 5 HiMO in the applicant's 5HMO-Mix have previously been assessed and approved by FSANZ, although at lower levels than those requested in the current application. HMO and HiMO reach the large intestine where they are either fermented by the microbiota or excreted unchanged in the faeces. Results of genotoxicity assays of the individual HiMO, and the 5HMO-Mix, are consistently negative.

No adverse effects of the 5HMO-Mix were observed in a 13-week rat dietary toxicity study or a 21-day neonatal piglet study, although calculated levels of intake were generally less than those expected from the maximum use levels requested by the applicant. No treatment-related adverse events were observed in infants consuming a formula containing the 5HMO-Mix, at the requested concentration, for four months. In addition, the maximum levels of the five HiMO are already approved in the EU, with no reports of adverse effects in that large population.

3.3 Dietary intake assessment

3.3.1 Objective

The objective of this dietary intake assessment is to estimate the dietary intakes of 2′-FL, 3-FL, LNT, 3′-SL sodium salt, and 6′-SL sodium salt from the proposed addition to infant formula and follow-on formula. Infant formula is defined in Standard 2.9.1 of the Code as being suitable for infants aged 0–6 months and follow-on formula for infants from the age of 6 months. Estimated dietary intakes of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL from mature human milk will also be determined and used as a reference to which estimated intakes from the proposed addition to infant formula products will be compared.

3.3.2 Approach to estimating dietary intakes of HiMO

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. The dietary intakes of the HiMO in this assessment were estimated using the proposed maximum use levels provided in the application and estimates of concentrations in mature human milk from scientific literature, combined with consumption data from model diets for infants aged 3 months 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 2024b).

3.3.2.1 Previous FSANZ dietary intake assessments of HiMO

FSANZ has previously assessed the dietary intake of 2′-FL as part of several applications, most recently under A1265 and A1308 (FSANZ 2023a, FSANZ 2025a). The applicant has requested permission to add 2′-FL at 3.0 g/L in infant formula and 3.64 g/L in follow-on formula and SMPPi. These concentrations are higher than the current permitted level, which is 2.4 g/L across infant formula products.

FSANZ has previously assessed the dietary intake of 3-FL as part of A1324 (FSANZ 2025b). As a result of this application, 3-FL is permitted to be added to infant formula products at a maximum of 2 g/L. This is higher than the levels requested by the applicant in A1339 (0.9 g/L in infant formula and 1.2 g/L in follow-on formula and SMPPi).

The dietary intakes of LNT, 3′-SL sodium salt, and 6′-SL sodium salt were assessed by FSANZ as part of A1265 (FSANZ 2023a). The applicant has requested permission to add these HiMO to infant formula products at 1.82, 0.28 and 0.7 g/L respectively. These levels are higher than those currently permitted in the Code (0.8, 0.2, and 0.4 g/L respectively).

3.3.2.2 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 the combined intake of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt. The population groups used for the dietary intake assessment are:

- infants aged 3 months – representing exclusively formula-fed or 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.

3.3.2.3 Proposed maximum amount of HiMO in infant formula products

The maximum proposed levels provided in the application in g/L are listed in Table 3. The equivalent values in g/kg have been calculated by FSANZ for use in its dietary modelling. The values were converted using the specific gravity of infant formula of 1.05 from AUSNUT 2023 (FSANZ 2025c).

Table 3: Proposed maximum proposed amount of HiMO as provided in the application (g/L) and calculated by FSANZ (g/kg)[1]

Substance	Proposed maximum amount			
	Infant formula		Follow-on formula and SMPPi	
	g/L	g/kg	g/L	g/kg
2′-FL	3.0	2.90	3.64	3.50
3-FL	0.9	0.86	1.2	1.10
LNT	1.82	1.70	1.82	1.70
3′-SL sodium salt	0.28	0.27	0.28	0.27
6′-SL sodium salt	0.7	0.67	0.7	0.67

[1]Converted using specific gravity of 1.05 from AUSNUT 2023 (FSANZ 2025c).

3.3.2.4 Concentrations of HMO in human milk

The applicant used the HMO concentrations in the mature milk of mothers (lactation day 15 to 90) reported by Soyyılmaz et al. (2021) to calculate the estimated dietary intake of these HMO for infants exclusively breastfed. FSANZ reviewed original data published since the review by Soyyılmaz et al. (2021) and the most recent reviews of each relevant HMO included in the scoping literature review against FSANZ's inclusion criteria (lactation period (>10 days), term birth, units (g/L)) and determined that the mean and maximum mean concentrations from the review by Soyyılmaz et al. (2021) remain current and therefore have been used for this dietary intake assessment.

Mean and high concentrations of 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL in mature and late (90+ days) human milk from the review by Soyyılmaz et al. (2021) are provided in Table 4.

Table 4: Concentrations of HMO in mature and late human milk from Soyyılmaz et al. (2021)^ and maximum proposed levels of HiMO provided by the applicant* used in FSANZ's dietary intake assessment

Use in infant model	**3-month-old**			**9-month-old**		
	Mature human milk (15 to 90 days)^		**Infant formula***	**Late human milk (90+ days)^**		**Follow-on formula and SMPPi***
Concentration	**Mean[1] (g/L)**	**High[2] (g/L)**	**Maximum proposed amount (g/L)**	**Mean[1] (g/L)**	**High[2] (g/L)**	**Maximum proposed amount (g/L)**
2′-FL	2.28	4.28	3.0	1.65	4.27	3.64
3-FL	0.72	1.9	0.9	0.92	2.57	1.2
LNT	0.74	1.60	1.82	0.64	1.37	1.82
3′-SL	0.19	0.7	0.28	0.13	0.30	0.28
6′-SL	0.40	0.74	0.7	0.30	1.00	0.7

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

As shown in Table 4, the requested concentrations of 2′-FL, 3-FL, 3′-SL sodium salt, and 6′-SL sodium salt in infant formula, and follow-on formula and SMPPi, are higher than the mean concentrations found by Soyyılmaz et al. (2021) in mature and late human milk respectively, but are lower than the corresponding highest mean concentrations reported in the review.

The requested concentration of LNT in infant formula products is higher than the mean and highest mean concentrations found in both mature and late human milk by Soyyılmaz et al. (2021).

3.3.2.5 Concentrations of oligosaccharides in domestic mammalian milks

Many infant formula products use either cows' milk or goats' milk as a base. As identified in previous FSANZ assessments (FSANZ 2019, FSANZ 2023, FSANZ 2025b), the milk oligosaccharide content of cows' milk and goats' milk is much lower than that in human milk. As reported in A1155 the total concentration of milk oligosaccharides in cows' milk is around 30–60 mg/L. Goats' milk has a higher oligosaccharide concentration than cows' milk of 60–650 mg/L (van Leeuwen et al. 2020). This is still significantly lower than the concentration in mature human milk of 8,600–16,800 mg/L (Soyyılmaz et al. 2021). Levels of oligosaccharides in cows' and goats' milk are also substantially lower than the proposed maximum levels of HiMO to be added to infant formula products in this application.

As the total concentration of oligosaccharides is lower in cows' and goats' milk than in human milk, the same is true for each specific substance. The relative abundance of fucosylated oligosaccharides (such as 2′-FL) 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). FSANZ's assessment of A1155 found an upper value of 0.06 g/L total oligosaccharide concentration in cow's milk. Applying a relative abundance of 25% to this value, the concentration of 3′-SL in cows' milk would be up to 0.015 g/L. This concentration is around 20-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. (2019) estimated 6′-SL concentrations in goats' milk to be 0.05–0.07 g/L, which is one-tenth of 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 the amount 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.

3.3.3 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, 3-FL, LNT, 3′-SL and 6′-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^

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

^ (WHO 1985)

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

1. mean consumption × proposed maximum use level
2. high (P90) consumption × proposed maximum use level.

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

1. mean consumption × mean concentration in human milk (represents a 'best estimate' of mean intake)
2. high consumption × mean concentration in human milk
3. mean consumption × high concentration in human milk
4. 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 2023 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 2025c). This energy content value is used to estimate the dietary intake of HMO from human milk in this assessment.

The recommended energy intake for a 3-month-old boy (343 kJ/kg bw/day) (United Nations University and World Health Organization 2004) and the 50th percentile body 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 summary of the model diet data used to estimate dietary intakes of the relevant oligosaccharides is presented in Table 5.

Table 5: Data used to calculate estimated dietary intakes of oligosaccharides

	Units	3 months	9 months
Recommended energy intake	kJ/kg bw/day	343	330
P50 body weight	kg	6.4	8.9
Recommended energy intake	kJ/day	2,195	2,937
100% of energy requirements	kJ/day	2,195	n/a
50% of energy requirements	kJ/day	n/a	1,469
Amount of human milk[1]	g/day	765[2]	515[3]
Amount of infant formula[4]	g/day	805	n/a
Amount of follow-on formula[4]	g/day	n/a	540

[1]Calculated using energy content of human milk (286 kJ/100 g) and weight of human milk (1,040 g/L) from AUSNUT 2023.
[2]Amount of human milk required to meet 100% of energy requirements.
[3]Amount of human milk required to meet 50% of energy requirements.
[4]Calculated using energy content of infant formula (272 kJ/100 g) and weight of infant formula (1,050 g/L) from AUSNUT 2023.
P50: 50th percentile

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, 3-FL, LNT, 3′-SL and 6′-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, 3-FL, LNT, 3′-SL and 6′-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.

3.3.3.1 Assumptions and limitations of the dietary intake assessments

The aim of the dietary intake assessment was to make the most realistic estimation of dietary intakes of the HiMO 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 HiMO 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, 3-FL, LNT, 3′-SL and 6′-SL at the concentrations specified in Table 3 and Table 4 respectively
- 1 L of infant or follow-on formula equals 1,050 g
- 1 L of human milk equals 1,040 g
- there is 100% market penetration of the use of 2′-FL, 3-FL, LNT, 3′-SL and 6′-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, 3-FL, LNT, 3′-SL and 6′-SL intakes through foods and beverages other than from infant formula, follow-on formula and human milk
- there is no contribution to 2′-FL, 3-FL, LNT, 3′-SL and 6′-SL intakes through the use of complementary or other medicines.

In addition to the specific assumptions made in relation to this dietary intake assessment, there are some limitations associated with the nutrition surveys from which the food consumption data used for the assessment are based. A discussion of these limitations is included in Section 6 of the Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes (FSANZ 2024b).

3.3.4 Estimated dietary intakes

The common information used by FSANZ to calculate all the estimated dietary intakes in this section is presented in Table 5.

3.3.4.1 Estimated dietary intakes of 2'-FL from infant formula products and human milk

The applicant has requested that 2′-FL be permitted to be added at 3.0 g/L in infant formula and 3.64 g/L in follow-on formula. Both concentrations have been used to estimate dietary intake for infants aged 3 months and 9 months (see Table 6 and Figure 1).

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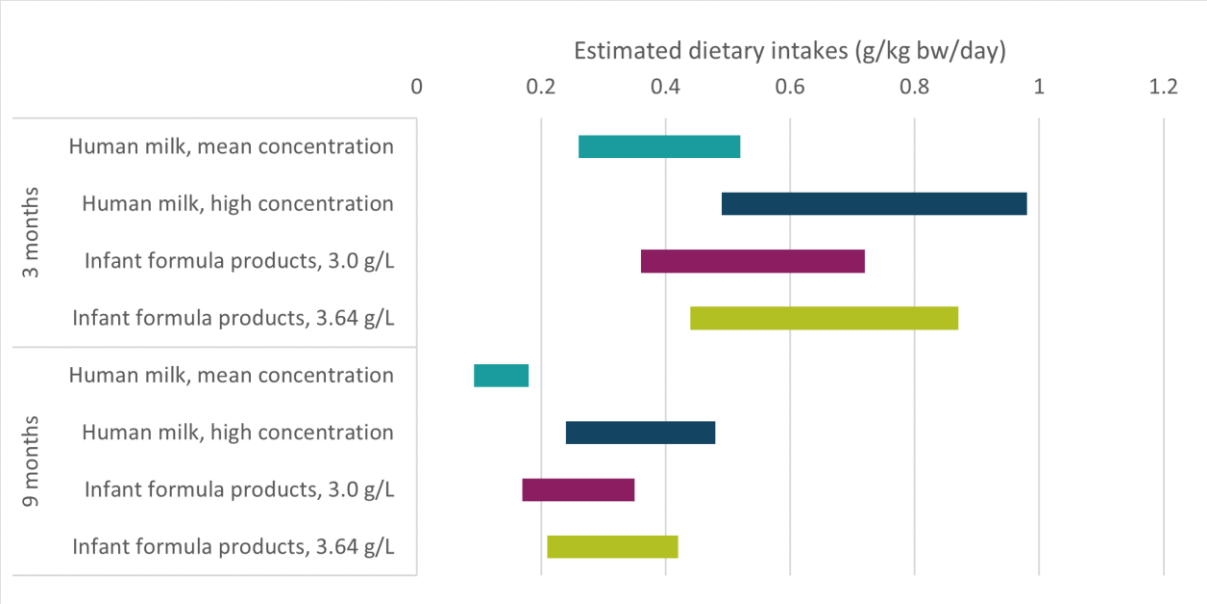
For 3-month-old infants, estimated mean and 90th percentile dietary intakes of 2′-FL from mature human milk were 0.26 g/kg bw/day and 0.52 g/kg bw/day for mean concentrations, and 0.49 g/kg bw/day and 0.98 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes from infant formula were 0.36 g/kg bw/day and 0.72 g/kg bw/day if the formula contained 3.0 g/L, and 0.44 g/kg bw/day and 0.87 g/kg bw/day if the formula contained 3.64 g/L.

For 9-month-old infants, estimated mean and 90th percentile dietary intakes of 2′-FL from late human milk were 0.092 g/kg bw/day and 0.18 g/kg bw/day for mean concentrations, and 0.24 g/kg bw/day and 0.48 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes from follow-on formula were 0.17 g/kg bw/day and 0.35 g/kg bw/day if the formula contained 3.0 g/L, and 0.21 g/kg bw/day and 0.42 g/kg bw/day if the formula contained 3.64 g/L.

Table 6: Estimated dietary intakes of 2'-FL from infant formula products and human milk for infants aged 3 months and 9 months

		Estimated intakes (g/kg bw/day)	
		3 months	9 months
Human milk, mean concentration	**Mean dietary intake**	0.26	0.092
	P90 dietary intake	0.52	0.18
Human milk, high concentration	**Mean dietary intake**	0.49	0.24
	P90 dietary intake	0.98	0.48
Infant formula products, 3.0 g/L	**Mean dietary intake**	0.36	0.17
	P90 dietary intake	0.72	0.35
Infant formula products, 3.64 g/L	**Mean dietary intake**	0.44	0.21
	P90 dietary intake	0.87	0.42

P90: 90th percentile



Note: Bars represent the range of estimated intakes for infants with mean to 90th percentile consumption of human milk or infant formula.

Figure 1: Estimated dietary intakes of 2'-FL from infant formula products and human milk for infants aged 3 months and 9 months

3.3.4.2 Estimated dietary intakes of 3-FL from infant formula products and human milk

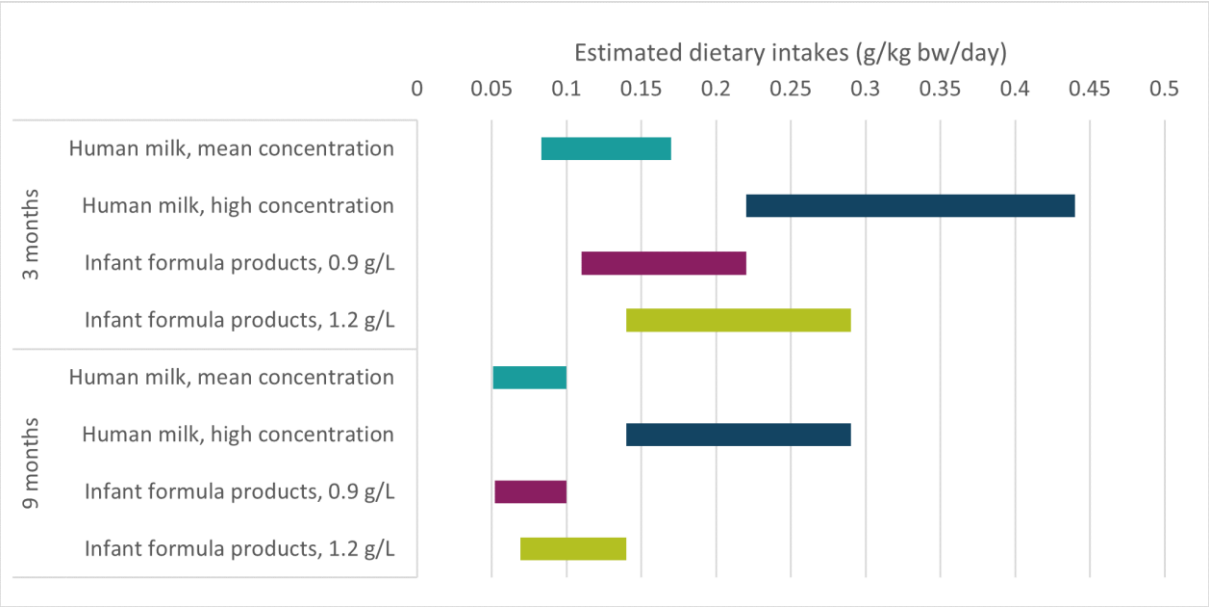
The applicant has requested that 3-FL be permitted to be added at 0.9 g/L to infant formula and 1.2 g/L to follow-on formula. Both concentrations have been used to estimate dietary intake for infants aged 3 months and 9 months (see Table 7 and Figure 2).

For 3-month-old infants, estimated mean and 90th percentile dietary intakes of 3-FL from mature human milk were 0.083 g/kg bw/day and 0.17 g/kg bw/day for mean concentrations, and 0.22 g/kg bw/day and 0.44 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes from infant formula were 0.11 g/kg bw/day and 0.22 g/kg bw/day if the formula contained 0.9 g/L, and 0.14 g/kg bw/day and 0.29 g/kg bw/day if the formula contained 1.2 g/L.

For 9-month-old infants, estimated mean and 90th percentile dietary intakes of 3-FL from late human milk were 0.051 g/kg bw/day and 0.10 g/kg bw/day for mean concentrations, and 0.14 g/kg bw/day and 0.29 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes from follow-on formula were 0.052 g/kg bw/day and 0.10 g/kg bw/day if the formula contained 0.9 g/L, and 0.069 g/kg bw/day and 0.14 g/kg bw/day if the formula contained 1.2 g/L.

Table 7: Estimated dietary intakes of 3-FL from infant formula products and human milk for infants aged 3 months and 9 months

		Estimated intakes (g/kg bw/day)	
		3 months	**9 months**
Human milk, mean concentration	**Mean dietary intake**	0.083	0.051
	P90 dietary intake	0.17	0.10
Human milk, high concentration	**Mean dietary intake**	0.22	0.14
	P90 dietary intake	0.44	0.29
Infant formula products, 0.9 g/L	**Mean dietary intake**	0.11	0.052
	P90 dietary intake	0.22	0.10
Infant formula products, 1.2 g/L	**Mean dietary intake**	0.14	0.069
	P90 dietary intake	0.29	0.14



Note: Bars represent the range of estimated intakes for infants with mean to 90th percentile consumption of human milk or infant formula.

Figure 2: Estimated dietary intakes of 3-FL from infant formula products and human milk for infants aged 3 months and 9 months

3.3.4.3 Estimated dietary intakes of LNT from infant formula products and human milk

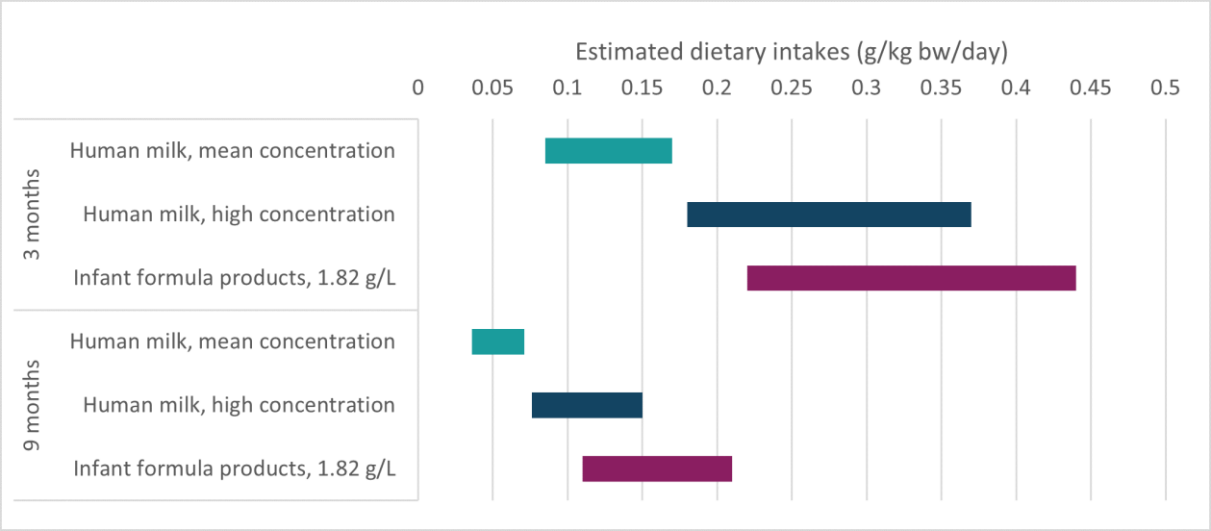
The estimated dietary intakes of LNT from infant formula products at the requested concentrations are higher than the corresponding estimated intakes from human milk (see Table 8 and Figure 3).

For 3-month-old infants, estimated mean and 90th percentile dietary intakes of LNT from mature human milk were 0.085 g/kg bw/day and 0.17 g/kg bw/day for mean concentrations, and 0.18 g/kg bw/day and 0.37 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of LNT from infant formula were 0.22 g/kg bw/day and 0.44 g/kg bw/day.

For 9-month-old infants, estimated mean and 90th percentile dietary intakes of LNT from late human milk were 0.036 g/kg bw/day and 0.071 g/kg bw/day for mean concentrations and 0.076 g/kg bw/day and 0.15 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of LNT from follow-on formula were 0.11 g/kg bw/day and 0.21 g/kg bw/day.

Table 8: Estimated dietary intakes of LNT from infant formula products and human milk for infants aged 3 months and 9 months

		Estimated intakes (g/kg bw/day)	
		3 months	9 months
Human milk, mean concentration	**Mean dietary intake**	0.085	0.036
	P90 dietary intake	0.17	0.071
Human milk, high concentration	**Mean dietary intake**	0.18	0.076
	P90 dietary intake	0.37	0.15
Infant formula products, 1.82 g/L	**Mean dietary intake**	0.22	0.11
	P90 dietary intake	0.44	0.21



Note: Bars represent the range of estimated intakes for infants with mean to 90th percentile consumption of human milk or infant formula.

Figure 3: Estimated dietary intakes of LNT from infant formula products and human milk for infants aged 3 months and 9 months

3.3.4.4 Estimated dietary intakes of 3'-SL sodium salt from infant formula products and human milk

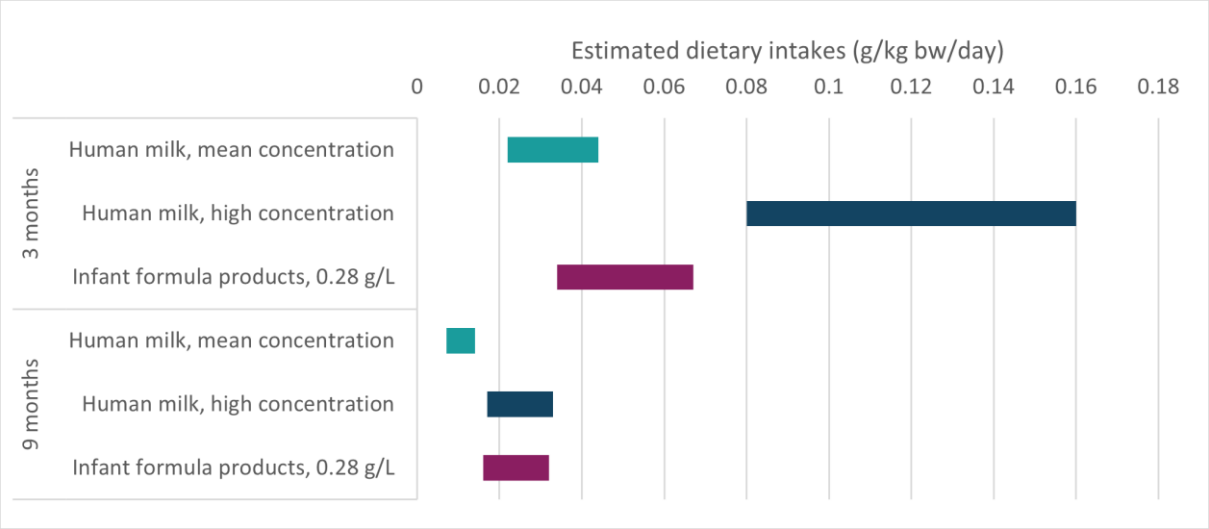
The estimated dietary intakes of 3′-SL sodium salt from infant formula products at the requested concentrations are higher than the corresponding estimated intakes from human milk (see Table 9 and Figure 4).

For 3-month-old infants, estimated mean and 90th percentile dietary intakes of 3′-SL sodium salt from mature human milk were 0.022 g/kg bw/day and 0.044 g/kg bw/day for mean concentrations and 0.08 g/kg bw/day and 0.16 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of 3′-SL sodium salt from infant formula were 0.034 g/kg bw/day and 0.067 g/kg bw/day.

For 9-month-old infants, estimated mean and 90th percentile dietary intakes of 3′-SL sodium salt from late human milk were 0.0072 g/kg bw/day and 0.014 g/kg bw/day for mean concentrations and 0.017 g/kg bw/day and 0.033 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of 3′-SL sodium salt from follow-on formula were 0.016 g/kg bw/day and 0.032 g/kg bw/day.

Table 9: Estimated dietary intakes of 3'-SL sodium salt from infant formula products and human milk for infants aged 3 months and 9 months

		Estimated intakes (g/kg bw/day)	
		3 months	**9 months**
Human milk, mean concentration	**Mean dietary intake**	0.022	0.0072
	P90 dietary intake	0.044	0.014
Human milk, high concentration	**Mean dietary intake**	0.08	0.017
	P90 dietary intake	0.16	0.033
Infant formula products, 0.28 g/L	**Mean dietary intake**	0.034	0.016
	P90 dietary intake	0.067	0.032



Note: Bars represent the range of estimated intakes for infants with mean to 90th percentile consumption of human milk or infant formula.

Figure 4: Estimated dietary intakes of 3'-SL sodium salt from infant formula products and human milk for infants aged 3 months and 9 months

3.3.4.5 Estimated dietary intakes of 6'-SL sodium salt from infant formula products and human milk

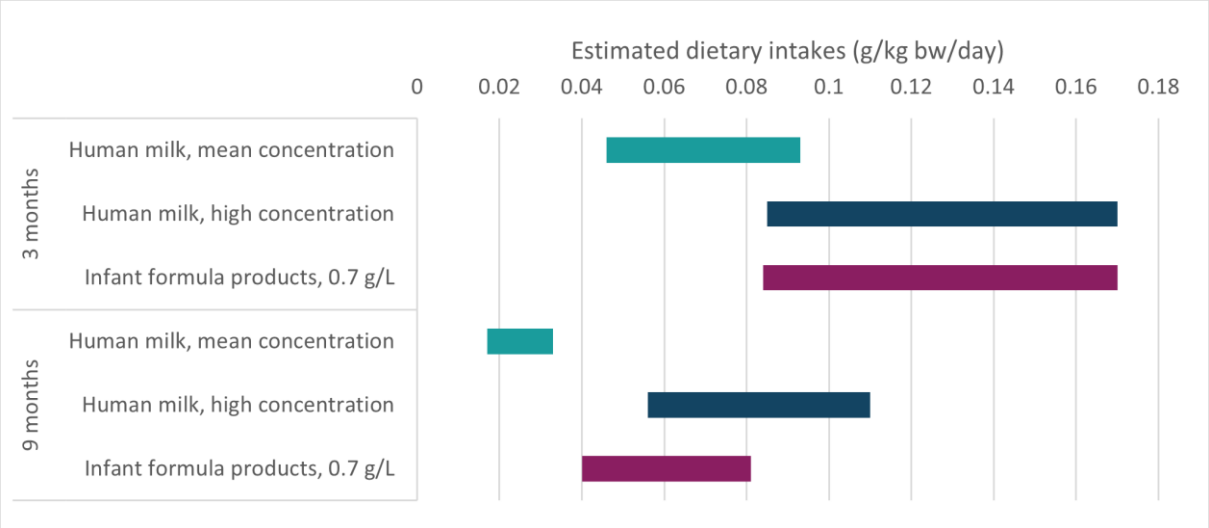
The estimated dietary intakes of 6′-SL sodium salt from infant formula products at the requested concentrations are higher than the corresponding estimated intakes from human milk (see Table 10 and Figure 5).

For 3-month-old infants, estimated mean and 90th percentile dietary intakes of 6′-SL sodium salt from mature human milk were 0.046 g/kg bw/day and 0.093 g/kg bw/day for mean concentrations and 0.085 g/kg bw/day and 0.17 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of 6′-SL sodium salt from infant formula were 0.084 g/kg bw/day and 0.17 g/kg bw/day.

For 9-month-old infants, estimated mean and 90th percentile dietary intakes of 6′-SL sodium salt from late human milk were 0.017 g/kg bw/day and 0.033 g/kg bw/day for mean concentrations and 0.056 g/kg bw/day and 0.11 g/kg bw/day for high concentrations. Estimated mean and 90th percentile intakes of 6′-SL sodium salt from follow-on formula were 0.040 g/kg bw/day and 0.081 g/kg bw/day.

Table 10: Estimated dietary intakes of 6'-SL sodium salt from infant formula products and human milk for infants aged 3 months and 9 months

		Estimated intakes (g/kg bw/day)	
		3 months	**9 months**
Human milk, mean concentration	**Mean dietary intake**	0.046	0.017
	P90 dietary intake	0.093	0.033
Human milk, high concentration	**Mean dietary intake**	0.085	0.056
	P90 dietary intake	0.17	0.11
Infant formula products, 0.7 g/L	**Mean dietary intake**	0.084	0.040
	P90 dietary intake	0.17	0.081



Note: Bars represent the range of estimated intakes for infants with mean to 90th percentile consumption of human milk or infant formula.

Figure 5: Estimated dietary intakes of 6'-SL sodium salt from infant formula products and human milk for infants aged 3 months and 9 months

3.3.5 Conclusion

Estimated dietary intakes of 2′-FL from infant formula products at both the requested concentrations (3.0 and 3.64 g/L) for 3- and 9-month-old infants are similar to those for infants consuming human milk with high concentrations of this HMO. Infants consuming high amounts of human milk or infant formula products would have intakes generally higher than those consuming human milk with mean concentrations of this HMO.

Estimated dietary intakes of 3-FL at 0.9 g/L in infant formula products are comparable to that for human milk with mean concentration of this HMO for both 3- and 9-month-old infants. At the higher requested concentration of 1.2 g/L and high levels of consumption, intakes for 3-month-old infants consuming infant formula products exceed those for infants consuming human milk with mean concentrations of this HMO but fall below those consuming human milk with high concentrations.

Estimated dietary intakes of LNT at 1.82 g/L in infant formula products are above those for both 3- and 9-month-old infants consuming human milk at mean concentrations of this HMO, however are similar to the range of intakes based on high concentrations in human milk, with the upper end of the range of intakes from formula being slightly above the range from human milk. The intakes from infant formula products are up to 0.07 g/kg bw per day above those estimated based on high concentrations in human milk, which would be within normal daily variation in dietary intakes.

Estimated dietary intakes of 3′-SL sodium salt for 3-month-old infants consuming infant formula with 0.28 g/L are within the range of dietary intakes for infants consuming human milk with mean and high concentrations of 3′-SL sodium salt. For 9-month-old infants, those consuming infant formula at the requested concentration have intakes of 3′-SL sodium salt similar to those consuming human milk with high concentrations.

Estimated dietary intakes of 6′-SL sodium salt for 3-month-old infants are similar for those consuming infant formula products at the requested concentration and those consuming human milk with high concentrations of this HMO. For 9-month-old infants, intakes for those consuming infant formula products at the requested concentration are slightly below those for infants consuming human milk at high concentrations at similar consumption levels. Intakes from infant formula products exceed those from human milk with mean concentrations for this age group.

3.4 Nutrition assessment

3.4.1 Objectives of the nutrition assessment

The objective of the nutrition assessment is to determine the effect on infant growth (if any) from the addition of the HiMO (2′-FL, 3-FL, LNT, 3′-SL sodium salt, 6′-SL sodium salt) to infant formula products. The 5 HiMO are currently permitted for use in the Code as per Standard 2.9.1 however the current application requests different concentrations to those currently permitted for each HiMO. In most cases the applicant is requesting higher concentrations than current permissions, with the exception of 3-FL (Table A2.1).

3.4.2 Previous FSANZ assessments of HiMO

2′-FL

FSANZ has previously assessed the effect of 2′-FL in infant formula products on infant growth in 8 assessments: A1155, A1190, A1233, A1251, A1265, A1277, A1283, and A1308

(FSANZ 2020, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024a, FSANZ 2025a).

FSANZ considered evidence from 21 clinical trials[2] and cohort studies that measured the effects of 2′-FL either alone or in combination with other HiMO or oligosaccharides on infant growth. It was concluded that the addition of 2′-FL to infant formula products is unlikely to affect normal infant growth at concentrations normally found in human milk. Schedule 29 currently permits 2′-FL at concentrations of 2.4 g/L (96 mg/100 kJ).

3-FL

FSANZ considered the effect of 3-FL at concentrations up to 0.8 g/L in infant formula products on infant growth in Application A1324 (FSANZ 2025b). Three studies were considered in the body of evidence including two published studies (Parschat et al. 2021; Lasekan et al. 2022) and one confidential study (Miranda et al. 2023). The two published studies were also pivotal in the assessment of 3′-SL, 6′-SL and LNT (see below).

Parschat et al. (2021) undertook a randomised, controlled parallel trial to evaluate the safety and tolerability of the requested HiMO mix (at different concentrations) and its effect on infant growth. Over the 16 week intervention period healthy full-term infants consumed control formula (n=112) or formula containing the 5 HiMO (2.99 g/L 2′-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3′-SL, 0.28 g/L 6′-SL) *ad libitum* (n=113). The study included a breastfed reference group (n=116). Further details of the study are in Table A2.2.

The two-sided 95% confidence interval for mean daily body weight increase in the HiMO fed group was -0.7 to 2.4 g/day in the full data set[3] and -0.8 to 2.3 g/day in the per-protocol dataset. Statistical analysis was undertaken for non-inferiority only, rather than the clinically relevant difference of ± 3 g/day (American Academy of Pediatrics 1988). Compared to control formula, growth in the HiMO formula group was non-inferior in both datasets ($p < 0.001$), with the lower bound above the non-inferiority margin of 3 g/day in both populations. However, growth parameters were ± 3 g/day for both datasets. The authors reported no significant differences in mean weight, length, or head circumferences between the two infant formula fed groups at each timepoint in the full data set, with data for weight provided. Data for the per-protocol cohort was not provided. Mean weight-for-age, length-for-age and head circumference-for-age z-scores were within the normal range, defined as up to two standard deviations below and up to one standard deviation above the median WHO growth standards (WHO 2008).

Lasekan et al. (2022) also measured the effect of infant formula containing the 5 requested HiMO (3.0 g/L 2′-FL, 0.8 g/L 3-FL, 1.5 g/L LNT, 0.2 g/L 3′-SL sodium salt and 0.3 g/L 6′-SL sodium salt; total concentration 5.75 g/L) on infant growth (n=130) compared to a control group (n=129), in a randomised, controlled, double-blind multi-centre parallel feeding trial of healthy infants. The study also contained a non-randomised breastfed reference group (n=104). Further details of the study are in Table A2.2.

No significant difference in weight gain or length gain per day was observed from day 14 to 119 between infant formula groups, or between the three groups in the intention-to-treat or

2 Marriage et al. 2015; Kajzer et al. 2016; Puccio et al. 2017; Sprenger et al. 2017; Reverri et al. 2018; Larsson et al. 2019; Storm et al. 2019; Berger et al. 2020; Lagström et al. 2020; Leung et al. 2020; Román et al. 2020; Vandenplas et al. 2020; Parschat et al. 2021; Ramirez-Farias et al. 2021; Alliet et al. 2022; Cohen 2022; Gold et al. 2022; Lasekan et al. 2022; Vandenplas et al. 2022; Wallingford et al. 2022; Lazarini et al. 2024

3 Full data set was defined as all the randomised subjects who consumed at least one formula feed and with at least one tolerability and growth measurement after baseline reading.

protocol-evaluable[4] populations (p > 0.05; Table A2.2). The authors reported a significant difference in head circumference gain per day in males between the EF and CF groups (p = 0.043). EF was non-inferior to CF in weight gain per day (not less than 3 g/day). One limitation of the study is the measurement of one-sided non-inferiority in weight gain per day compared to control formula.

Similar findings were reported in a confidential study by Miranda et al. (2023).

Following the assessment of the evidence including the limited oral absorption of 3-FL, FSANZ concluded that there was no evidence to indicate a nutritional concern with the addition of 3-FL to infant formula products at concentrations normally found in human milk.

3′-SL, 6′-SL and LNT

FSANZ assessed the effects of LNT, 3′-SL sodium salt and 6′-SL sodium salt (as well as 2′-FL/DFL) in infant formula products on infant growth in Application A1265 (FSANZ 2023a). The assessment considered three studies, two of which were discussed above (Parschat et al. 2021; Lasekan et al. 2022) and a confidential study - Cohen et al. (2022).

Based on the available evidence it was concluded that LNT, 3′-SL sodium salt and 6′-SL sodium salt (as well as 2′-FL/DFL) added to infant formula products at concentrations normally found in human milk are unlikely to pose a risk to normal growth of infants.

3.4.3 Current assessment

The applicant provided two studies that measured the effect of a mixture of the 5 HiMO on infant growth which have been discussed above and summarised in Table A2.2 (Parschat et al. 2021; Lasekan et al. 2022).

In addition, the applicant provided several studies that were previously assessed by FSANZ (Marriage et al. 2015; Goehring et al. 2016; Kajzer et al. 2016; Puccio et al. 2017; Storm et al. 2019; Berger et al. 2020; Ramirez- Vandenplas et al. 2020; Farias et al. 2021; Vandenplas et al. 2022) or did not measure growth (Dogra et al. 2021; Nowak-Wegrzyn et al. 2019).

FSANZ undertook several searches to identify relevant literature published since the previous relevant HiMO applications were assessed by FSANZ. Details of search terms, dates and outcomes are provided in Table A2.3. No new relevant studies were identified to include in the body of evidence.

3.4.4 Discussion

The objective of the nutrition assessment is to determine the effect (if any) of infant formula products containing the proposed combination and concentration of HiMO (3 g/L 2′-FL in infant formula, 3.64 g/L 2′-FL in follow-on formula, 0.9 g/L 3-FL in infant formula, 1.2 g/L 3-FL in follow-on formula, 1.82 g/L LNT, 0.28 g/L 3′-SL sodium salt, 0.7 g/L 6′-SL sodium salt in infant formula products) on infant growth (Table A2.1).

FSANZ has previously considered the effect of the 5 requested HiMO either individually or a subset of the 5 HiMO, on infant growth in 10 applications (Table A2.3). Two studies considered the effect of the 5 HiMO together on infant growth, but not at the requested concentrations (Parschat et al. 2021; Lasekan et al. 2022). Based on the available evidence

[4] The authors did not define the protocol-evaluable population and how it differed to the standard per-protocol population.

and due to the limited absorption of HiMO it was concluded that when added at levels normally found in human milk, the 5 HiMO are not likely to affect normal growth in infants.

Table A2.1 outlines the current permissions for the addition of the 5 HiMO in Schedule 29, and the concentrations requested in this application. In the case of 2′-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt, requested permissions are greater than current permissions.

FSANZ considered the evidence provided by the applicant and undertook literature searches however no new relevant studies were added to the body of evidence.

2′-FL

2′-FL is currently permitted in infant formula products at concentrations up to 2.4 g/L in infant formula products. The applicant has requested permission for a concentration of 3 g/L in infant formula and a concentration of 3.64 g/L to be permitted in follow-on formula. FSANZ previously reported that 2′-FL is found in human milk at concentrations of up to 4.28 g/L in milk for infants up to 90 days of age, and up to 4.27 g/L in milk for infants aged over 90 days (Table 4, Section 3.3.2.4). Therefore, based on the available evidence FSANZ considers that 2′-FL added to infant formula products at the requested concentrations is unlikely to affect normal infant growth.

3-FL

3-FL is currently permitted in infant formula products at concentrations up to 2 g/L. The applicant has requested permission for a concentration lower than the currently permitted concentration, 0.9 g/L in infant formula and 1.2 g/L in follow-on formula. Based on the previous assessment of the evidence and an absence of new studies, FSANZ maintains the conclusion that 3-FL added to infant formula products at concentrations found in human milk is unlikely to affect normal infant growth.

LNT

LNT is currently permitted in infant formula products at concentrations up to 0.8 g/L. The applicant has requested permission for a concentration of 1.82 g/L in infant formula products. FSANZ previously considered the evidence for the effects on infant growth in Application A1265. The concentration of LNT in human milk for infants aged up to 90 days is up to 1.6 g/L, and up to 1.37 g/L in milk for infants aged over 90 days (Table 4, Section 3.3.2.4). Therefore, FSANZ can conclude that LNT added to infant formula at concentrations up to 1.6 g/L and at concentrations up to 1.37 g/L in follow-on formula are unlikely to affect normal infant growth but cannot determine the effect on growth at the requested concentration of 1.82 g/L.

3′-SL sodium salt

3′-SL sodium salt is currently permitted in infant formula products at concentrations up to 0.2 g/L. The applicant has requested permission for a concentration of 0.28 g/L in infant formula products. FSANZ previously considered evidence from studies that measured the effect of 3′-SL at concentrations up to 0.23 g/L and did not identify any concerns regarding infant growth. The mean concentration of 3′-SL in human milk was previously reported as up to 0.7 g/L in milk for infants aged 15-90 days, and up to 0.3 g/L in milk for infants over 90 days of age (Table 4, Section 3.3.2.4). Therefore, based on the available evidence, its concentration in human milk and the limited absorption of HiMO, FSANZ concludes that the addition of 3′-SL sodium salt to infant formula products up to a concentration of 0.28 g/L is unlikely to affect normal infant growth.

6′-SL sodium salt

6-SL sodium salt is currently permitted in infant formula products at concentrations up to 0.4 g/L. The applicant has requested permission for a concentration of 0.7 g/L in infant formula products. FSANZ previously considered the evidence for the addition of 6′-SL at

concentrations of up to 0.3 g/L (Table A2.1). The mean concentration of 6′-SL in human milk for infants up to 90 days old is up to 0.74 g/L and up to 1 g/L in milk for infants over 90 days of age (Table 4, Section 3.3.2.4). Therefore, based on the available evidence, its concentration in human milk and the limited absorption of HiMO, FSANZ concludes that the addition of 6-SL sodium salt to infant formula products up to a concentration of 0.7 g/L is unlikely to affect normal infant growth.

3.4.5 Conclusion

The applicant is requesting permission for the addition of HiMO (2′-FL, 3-FL, LNT, 3′-SL sodium salt, 6′-SL sodium salt) to infant formula products at concentrations that differ to those already permitted in the Code. FSANZ has previously assessed the effect of the 5 individual HiMO in several previous applications; in four cases at lower concentrations than that requested by the current application.

Following a review of the available evidence, the limited absorption of HiMO and the concentration of the requested HiMO in human milk, FSANZ considers that the requested concentrations of 2′-FL (3 g/L in infant formula, 3.6 g/L in follow-on formula), 3-FL (0.9 g/L in infant formula, 1.2 g/L in follow-on formula), 3′-SL sodium salt (0.28 g/L in infant formula products), and 6′-SL sodium salt (0.7 g/L in infant formula products), are unlikely to affect normal infant growth. FSANZ considers that the addition of LNT at concentrations up to 1.6 g/L in infant formula and up to 1.37 g/L in follow-on formula are unlikely to affect normal infant growth but is unable to determine the effect at the requested concentrations of 1.82 g/L in infant formula products.

4 Beneficial effects assessment

The objective of this benefit assessment is to review reported health effects associated with the addition of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt to infant formula products. The assessment focuses on gut microbiota development in formula-fed infants, specifically regarding bifidogenic composition, anti-pathogenic activity, and immunomodulation effects.

FSANZ has completed assessments reviewing the health benefits of these HiMO in applications A1155, A1190, A1233, A1251, A1265, A1277, A1283, A1308 and A1324 (FSANZ 2019, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024a, FSANZ 2025a, FSANZ 2025b). These assessments found 2′-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt support plausible biological mechanisms for anti-pathogenic, immunomodulatory and bifidogenic effects, while 3-FL supports plausible mechanisms for anti-pathogenic and bifidogenic effects.

The applicant is requesting higher concentrations for 2′-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt than currently permitted under the Code. These proposed concentrations align with international regulations and remain below levels observed in human milk (Soyyılmaz et al. 2021, EFSA et al. 2024, Malih et al. 2024). Additionally, the applicant seeks recognition of an immunomodulatory effect for 3-FL.

4.1 Literature search

A literature review, covering the period 2022-2025, searched for articles relevant to bifidogenic, anti-pathogenic, and immunomodulatory effects of 2′-FL, LNT, 3′-SL and 6′-SL. This date range covered the period after application A1265, the last major literature review FSANZ did as part of an assessment for these HiMO. The literature review found 12 studies relevant to the investigated benefits. Two studies investigated the beneficial effect of a single HiMO addition while the remaining 10 investigated HiMO mixtures. The aim of the literature

review was to investigate whether the benefits established in the previous FSANZ assessments were still supported.

The studies identified supported the previous FSANZ conclusion that infant formula products fortified with 2′-FL, LNT, 3′-SL sodium salt or 6′-SL sodium salt provided plausible biological mechanisms for anti-pathogenic, immunomodulatory and bifidogenic effects (Bosheva et al. 2022, Lasekan et al. 2022, Hill and Buck 2023, Holst et al. 2023, Lindner et al. 2023, Tuplin et al. 2023, Guo et al. 2024, Kim et al. 2024, Liu et al. 2024, Sanchez-Gallardo et al. 2024, Walsh et al. 2024, Chen et al. 2025).

A literature review, performed as part of A1324, searched for articles relevant to bifidogenic and anti-pathogenic effects of 3-FL. Another literature review, performed on 14 October 2025, searched for studies relevant to bifidogenic, anti-pathogenic and immunomodulatory effects of 3-FL. After excluding the studies that were identified as relevant in A1324, 3 relevant studies remained. All 3 studies investigated immunomodulatory effects associated with the addition of 3-FL.

4.2 Higher concentrations of 2′-FL, LNT, 3′-SL sodium salt or 6′-SL sodium salt

Several studies were identified that investigated HiMO concentrations comparable to those proposed in this application (Hester et al. 2013, Yu et al. 2013, Parschat et al. 2021, Lasekan et al. 2022, Holst et al. 2023, Lindner et al. 2023, Kim et al. 2024, Walsh et al. 2024). Three of these studies were clinical trials (Parschat et al. 2021, Laseken et al. 2022, Holst et al. 2023). Holst et al. (2023) reported that infants fed a mixture of 5 HiMO exhibited a faecal microbiome more closely resembling that of breastfed infants, with a lower prevalence of opportunistic pathogenic species in the early weeks of life compared to a non-HiMO control. Parschat et al. (2021) and Laseken et al. (2022) explored outcomes beyond the scope of this assessment but demonstrated the ability of infants to tolerate HiMO at higher concentrations. This tolerance is expected, as the concentrations under review remain below those naturally found in human milk.

Studies comparing the effects of varying HiMO concentrations were also identified in the literature review. Bosheva et al. (2022), Linder et al. (2023) and Tuplin et al. (2023), assessed bifidogenic outcomes. Bosheva et al. (2022) and Linder et al. (2023) found that both high and low concentrations of 2′-FL, LNT, 3′-SL and 6′-SL produced similar effects on the abundance of *Bifidobacterium*. Whereas Tuplin et al. (2023), using a rat model, observed significant benefits only at higher concentrations of 2′-FL and 3-FL. Lindner et al. (2023) also reported comparable pathogen reduction across high and low concentrations of 2′-FL in faecal batch fermentation models.

Immunomodulatory effects at varying concentrations of HiMO were reported by Bosheva et al. (2022), Hill and Buck (2023), Tuplin et al. (2023), Kim et al. (2024), Liu et al. (2024) and Walsh et al. (2024). Kim et al. (2024), Walsh et al. (2024), and Liu et al. (2024) found that all tested concentrations of 2′-FL, 3′-SL and 6′-SL provided benefits, with higher concentrations yielding greater cytokines abundance or broader intestinal effects. Whereas Bosheva et al. (2022), Hill and Buck (2023), and Tuplin et al. (2023) observed beneficial immunomodulatory effects only at higher concentrations of 2′-FL, LNT, 3′-SL and 6′-SL. These observations suggest that HiMO can confer health benefits across a range of concentrations.

4.3 Immunomodulation effects of 3-FL

FSANZ has previously outlined the immunomodulatory role of human milk in neonatal development (FSANZ 2019, FSANZ 2023a). Briefly, the gastrointestinal immune system of neonates is immature and human milk, in addition to nutrition from other sources, is understood to support immune homeostasis and promote antigen tolerance. Bioactive compounds in human milk are important for the development of appropriate immune

responses and antigenic memory. Among these compounds, 3-FL is believed to contribute to these immunomodulatory effects (Kim et al. 2023, Zuurveld et al. 2024, Boll et al. 2024).

Kim et al. (2023) investigated the effects of HiMO, including 3-FL, on intestinal barrier function post inflammation. Using Caco-2 cell and mouse models, they found pretreatment with 3-FL reduced pro-inflammatory cytokine expression and decreased inflammatory cell infiltration, and preserved tight junction integrity, thus maintaining gut homeostasis. These findings support the potential of 3-FL having a plausible biological mechanism to reduce inflammation and maintain the tight junction.

Boll et al. (2024) examined individual HiMO effects on the intestinal epithelium and immune cell responses. In inflamed Caco-2 cells, 3-FL stabilised the cell barrier and tight junctions, but only when present on both sides of the barrier. In activated dendritic cells and macrophages, 3-FL enhanced T helper 1 cell and T helper 17 cell responses while reducing T helper 2 cell responses. An abundance of T helper 2 cell responses may promote the risk of allergies later in life (McFadden et al. 2015).

Zuurveld et al. (2023) explored HiMO effects in cell models representing intestinal barrier and immune functioning. Exposure to 3-FL, increased both pro- and anti-inflammatory cytokine expression and reduced T helper 2 cell activity. However, unlike Kim et al. (2023) and Boll et al. (2024), they found that 3-FL did not prevent tight junction disruption in T84 cells. This discrepancy may reflect differences in cell models and 3-FL concentrations across studies.

4.4 Beneficial effects conclusion

The literature has shown evidence of interactions between the immune system and 3-FL. While this evidence is limited, the *in vitro* studies summarised in this assessment demonstrate plausible biological mechanisms by which 3-FL may contribute to the immunomodulation of infants. This supports the hypothesis that the addition of 3-FL to infant formula products is likely to provide a wider range of immunomodulatory activity.

The HiMO concentrations assessed in this application are below the natural concentrations of HMO in human milk. Based on this, and previous FSANZ microbiological assessments supported by current literature, FSANZ has concluded there are no microbiological public health and safety concerns. The associated health benefits from the use of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt as nutritive substances in infant formula products are:

- an anti-pathogenic effect
- immunomodulation
- development of the gut microbiome through support of *Bifidobacteria* spp. growth.

5 Conclusion

FSANZ has undertaken an assessment of the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt (alone or in combination) to infant formula products.

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

FSANZ's microbiological risk assessment did not identify any public health and safety concerns associated with the use of *E. coli* BL21 as the production organism. The production strains are all genetically and phenotypically stable.

No public health or safety concerns with the applicant's 5HMO-Mix were identified. The five HiMO in the applicant's 5HMO-Mix have all previously been assessed and approved by FSANZ. Intestinal absorption of HiMO is very limited. Results of genotoxicity assays are consistently negative. No adverse effects of the 5HMO-Mix were observed in a 13-week dietary toxicity study in rats or a 21-day neonatal piglet study, and no treatment-related adverse events were observed in infants consuming a formula containing the 5HMO-Mix for four months. The requested maximum levels of the 5 HiMO are already approved in the EU, with no reports of adverse effects in that population.

Using a model diet approach, estimated dietary intakes of 2′-FL, 3-FL, 3′-SL and 6′-SL from infant formula products at the requested concentrations are similar to or lower than estimated intakes from human milk for 3- and 9-month-old infants. For LNT, estimated dietary intakes from infant formula products at the requested concentration are higher than estimated intakes from human milk for 3- and 9-month-old infants, however would be within normal daily variation in dietary intakes.

Following a review of the available evidence and limited absorption of HiMO, FSANZ concluded that the addition of 4 HiMO (2′-FL, 3-FL, 3′-SL sodium salt and 6′-SL sodium salt) in infant formula products at the requested concentrations which are within the range of HMO in human milk, is unlikely to affect normal infant growth. The addition of LNT at concentrations up to 1.6 g/L in infant formula and up to 1.37 g/L in follow-on formula is not likely to affect infant growth. The requested concentration for LNT (1.82 g/L) is greater than that found in human milk and its effect on infant growth at these concentrations cannot be determined.

Based on previous FSANZ microbiological assessments supported by current literature and the weight of evidence that supports plausible biological mechanisms by which 3-FL may contribute to the immunomodulation of infants. FSANZ has concluded there are no microbiological public health and safety concerns. The associated health benefits of 2′-FL, 3-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt as nutritive substances in infant formula products are (1) an anti-pathogenic effect, (2) immunomodulation, and (3) development of the gut microbiome through support of *Bifidobacteria* spp. growth.

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Appendix 1: Toxicology data

Kinetics and Metabolism

No new information was submitted or located that would alter the conclusions previously reached by FSANZ concerning the metabolism of any of the HiMO specified in this application.

Toxicity data for individual HiMO

Studies of individual HiMO cited in the application had either been previously reviewed by FSANZ or were not considered to be relevant because they were conducted in adult humans rather than the target populations for this application.

Toxicity data for the applicant's 5HMO-Mix

1. Animal studies

Thirteen-week dietary toxicity study of 5HMO-mix in CD rats (Parschat et al. 2020). Regulatory status: GLP; conducted in accordance with OECD Test Guideline 408

The test article for this study consisted of 47.1% 2′-FL, 16.0% 3-FL, 23.7% LNT, 3′-SL (4.1% dry weight), 4.0% 6′-SL, and 5.1% other minor carbohydrates, all on a dry weight basis. There was one treatment group and one negative control group, each comprising 10 rats/sex. Rats were approximately 65 days old at the start of the study. Based on a seven-day oral tolerance study in rats of the same strain, the test article was fed at 0 or 10% w/w in standard rat feed. Rats were individually housed under standard conditions of environment and husbandry. Feed and water were provided *ad libitum*.

Parameters recorded included survival, daily cage-side clinical observations, body weights and bodyweight changes, and consumption of feed and water. Detailed clinical observations were made pre-study and weekly during the in-life phase. Ophthalmological examinations were made pre-study and within a week of scheduled termination. In the final week of the in-life phase, rats were subject to neurological assessments including sensory reactivity, grip strength and locomotor activity. Samples of blood and urine were collected in the 24 hours prior to termination. On the final day of the in-life phase, rats were anaesthetised and killed. Rats were subject to gross necropsy, fresh organ weights were recorded, smears of fresh bone marrow were prepared, and a comprehensive list of organs and tissues was preserved for histopathological examination.

Mean intakes of 5HMO-Mix were 5.67 g/kg bw/day for males and 6.97 g/kg bw/day for females. Mean intakes of the individual HiMO were calculated and are shown in Table A1:1. No treatment-related adverse effects were found in this study.

Table 21:1: Mean intakes of individual HiMO in rats

HiMO	Mean intake for male rats (g/kg bw/day)	Mean intake for female rats (g/kg bw/day)
2′-FL	2.67	3.28
3-FL	0.91	1.12
LNT	1.34	1.65
3′-SL sodium salt	0.23	0.29
6′-SL sodium salt	0.23	0.28

Twenty-one day tolerance study of 5HMO-mix in neonatal piglets (Hanlon, 2020). Regulatory status: GLP; no guideline cited.

The test article for this study was the applicant's 5HMO-Mix containing 49.1% 2′-FL, 10.4% 3-FL, 19.9% LNT, 3.5% 3′-SL, and 4.2% 6′-SL on a dry weight basis. The vehicle and control article was Land O'Lakes Specialty Milk Replacer. The study was initiated when Yorkshire-cross piglets were two days old, after they had been with their dams for 48 hours to ensure adequate colostrum intake. Piglets were individually housed and consumed the milk replacer on a voluntary basis from a feed bowl. Piglets were assigned to one of three groups, 6/sex/group, and provided with milk replacer containing 0, 5.75 or 8.0 g/L 5HMO-Mix. Endpoints determined during the study included survival, clinical observations, bodyweights, feed consumption, clinical pathology (haematology, clinical chemistry, and coagulation parameters from blood collected on Days 7 and 21; urinalysis of urine collected at necropsy on Day 22), gross necropsy findings, organ weights and histopathology findings. Feed efficiency and compound consumption were calculated.

The calculated intakes of 5HMO-Mix at the high dose of 8.0 g/L were 3.6 and 3.7 g/kg bw/day in males and females, respectively, corresponding to intakes in both sexes of approximately 1.8 g 2′-FL/kg bw/day, 0.4 g 3-FL/kg bw/day, 0.7 g LNT/kg bw/day, 0.1 g 3′-SL/kg bw/day, and 0.2 g 6′-SL/kg bw/day.

All piglets in the control and 5.75 g/L groups survived to the end of the study. One male piglet in the 8.0 g/L group was euthanized on Day 7 due to poor body condition and recumbency, and *E.coli* was found on faecal culture. The morbidity of the piglet was attributed to an incidental infection most likely contracted at the breeding farm from which the piglets were purchased. There were no treatment-related effects on bodyweight, milk replacer consumption, feed efficiency, clinical pathology parameters, gross necropsy findings or histopathological findings. Decreased rectum weights in both sexes in the 8.0 g/L group were not considered adverse because there were no microscopic correlates.

2. Genotoxicity studies

Genotoxicity assays conducted with the applicant's 5HMO-Mix were reported by Parschat et al. (2020). The studies were conducted under GLP, and in accordance with relevant guidelines, as listed in Table A1:23A1:2. No evidence of genotoxicity was found in these studies.

Table A1:23: Genotoxicity studies of 5HMO-Mix

Test	*Test system*	*Concentration*	*Results*
Bacterial reverse mutation assay (OECD TG 471)	*Salmonella enteridis* var. Typhimurium test strains TA98, TA100, TA102, TA1535 and TA1537.	Experiment I[1]: 5.0, 10.0, 31.6, 100, 316 or 600 mg/plate Experiment II[2]: 5.0, 10.0, 31.6, 100, 316 or 600 mg/plate	Negative ± S9
***In Vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (OECD TG 487)**	Cultured human peripheral blood lymphocytes[3]	4+16 hour -S9: 7.5, 15, 30, and 60 mg/mL 4+16 hour +S9: 7.5, 15, 30, and 60 mg/mL 20 hour -S9: 7.5, 15, 30, and 60 mg/mL	Negative ± S9

[1] Conducted in triplicate by the plate incorporation method.
[2] Conducted in triplicate by the preincubation method.
[3] Lymphocytes obtained from young, healthy, nonsmoking donors.

3. Human tolerance studies with 5HMO-Mix

A multi-centre, randomised, double-blinded, controlled, parallel group clinical study was conducted for a duration of 4 months to evaluate the safety and tolerability of the 5HMO-Mix that is the subject of this application, and was reported by Parschat et al. (2021). The study has previously been reviewed by FSANZ under several other applications for approval of HMO. Briefly, healthy term infants ≤14 days of age were randomised to receive exclusive feeding with an infant formula containing 5HMO-Mix (n=113) or a control infant formula (n=112) as a reference control for 4 months. A group of exclusively breastfed infants was also included as a reference control (n=116). The 5HMO-Mix provided 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. There were no statistically significant differences in body weight, length, or head circumference gain between the two formula groups, and formula supplemented with 5HMO-Mix was comparable to the control formula with respect to mean daily body weight gain. The total incidence of adverse events (AEs) and serious AEs were comparable across all three groups. Overall, the addition of 5HMO-Mix into infant formula was safe and well-tolerated.

Appendix 2: Nutrition data

Table A2.1 Current and requested HiMO concentrations and concentrations used in key studies in evidence base

HiMO	Current IFP Permission mg/100 kJ (g/L[1])	Requested Permission mg/100 kJ (g/L)	Parschat et al. 2021 g/L	Lasekan et al. 2022 g/L
2′-FL	96 (2.4)	IF: 119.52 (3.0) FoF: 145.02 (3.64)	2.99	3.0
3-FL	80 (2)	IF: 35.86 (0.9) FoF: 47.81 (1.20)	0.75	0.8
LNT	32 (0.8)	IF and FoF: 72.51 (1.82)	1.5	1.5
3′-SL sodium salt	8 (0.2)	IF and FoF: 11.16 (0.28)	0.23	0.2
6′-SL sodium salt	16 (0.4)	IF and FoF: 27.89 (0.7)	0.28	0.3

IFP: infant formula products including infant formula and follow-on formula; IF: infant formula; FoF: follow-on formula

[1]Permitted concentrations of HiMO in infant formula and follow-on formula are defined in Schedule 29 in mg/100kJ. FSANZ has previously converted permitted concentrations to g/L for comparison purposes, based on the minimum energy content of infant formula products of 2510 kJ/L, noting that formulas with greater energy content will have slightly greater HiMO concentrations

Table A2.2 Key published studies used for growth assessment of 3-FL, LNT, 3′-SL,6′-SL

Study design	IF composition	Study population and allocation	Outcomes	Key Results
Lasekan et al. (2022) United States (multisite)				
Randomised, controlled double-blind parallel growth and tolerance study mostly undertaken during Covid-19 pandemic lockdown at 34 sites Randomisation: computer-generated using a dynamic minimization algorithm with a random component, stratified by sex	CF: standard cow's milk formula EF: CF with added 5 HiMO blend (Table A2.1)	Healthy term infants aged ≤ 14 days at enrolment fed EF or CF *ad libitum* to 119 days. HM reference group (non-randomised): 104 (n=3 not randomised because non-eligible) EF: Enrolled n=130, ITT n=128, PE 72 CF: Enrolled n=129, ITT n=126, PE 77 HM: Enrolled n=104, ITT n=102, PE 73	Primary outcome: weight gain per day from day 14 to 119. Secondary outcomes: weight, interval weight gain per day, length, length gain per day, head circumference, head circumference gain per day. Mean weight for age, mean length for age and mean head circumference for age were plotted on the WHO growth charts. At day 14, 28, 42, 56, 84, 119 +- 3 days Analysis was undertaken for ITT: intention to treat and "protocol-evaluable" which was not defined Due to COVID-19, anthropometric measurement protocol was adjusted.	Clinical trial registration no. NCT04105686 **Weight gain per day** ITT: EF: 29.5 ± 0.7, CF:29.9 ± 0.6 g/day; p =0.271 PE: EF: 29.4 ± 0.7, CF 29.9 ± 0.7 g/day; p =0.348 **Length gain per day** ITT: EF: 0.11 ± 0.002, CF 0.108 ± 0.02 cm/day; p =0.708 PE: EF: 0.11 ± 0.002, CF 0.109 ± 0.003 cm/day; p =0.89 **Head circumference gain per day** ITT: EF: 0.043 ± 0.003, CF 0.038 ± 0.002 cm/day; p= 0.043 PE: EF: 0.059 ± 0.002, CF 0.058 ± 0.001 cm/day; p < 0.05 Study was funded by Abbott Nutrition, and all authors are current employees of the study sponsor.
Parschat et al. (2021) Multicentre (Germany, Italy and Spain)				
Randomised, double-blind, controlled, multi-centre, parallel noninferiority study Randomisation: randomisation list generated by an independent statistician in a 1:1 ratio in blocks	CF: Töpfer (Dietmannsried) 2163 kJ/100 g EF: CF with added HiMO mix (Chr. Hansen HMO GmbH) 5.75 g/L (2.99 g/L 2'-FL, 0.75 g/L 3-FL,	Healthy term infants ≤ 14 days fed EF or CF *ad libitum* from enrolment to 112 days. Enrolled: EF: 113; CF: 112; HM (non-randomised): 116	***Growth:*** Primary outcome: weight gain per day over 4 months period (to 112 days). Secondary outcomes included change in weight, length, and head circumference, their increments, and their respective WHO growth standard z-scores (weight-for-age,	Clinical trial registration no. NCT03513744 Full data set: 95% confidence interval mean body weight increase in HiMO group -0.7 to 2.4 g/day Per protocol data set: 95% confidence interval mean body weight increase in HiMO group -0.8 to 2.3 g/day

Study design	IF composition	Study population and allocation	Outcomes	Key Results
of four and stratified by sex and site.	1.5 g/L LNT, 0.23 g/L 3'-SL, and 0.28 g/L 6'-SL): The 5 HiMO mix partially replaced maltodextrin in EF. No further details provided	Completed: EF: 86 (76%); CF: 91 (81%); HM: 88 (76%) Loss to follow-up: EF:8; CF:1; BM:6 Errors were noted in reporting of reasons for drop-out	length-for-age, head circumference-for-age, and weight-for-length). Due to COVID-19, anthropometric measurements were undertaken via telephone	Study was funded and authors were employees of Chr. Hansen HMO GmbH (or Jennewein Biotechnologie)

Table A2.3 Previous FSANZ HiMO applications and literature search data

HiMO	Previous Application/s	Date of search for current assessment	Search terms	Search results/no. relevant
2′-FL	A1308 (2′-FL) A1283 (2′-FL) A1277 (2′-FL) A1265 (2′-FL, 3′-SL, 6′-SL, DFL, LNT) A1251 (2′-FL, GoS/ ITF) A1233 (2′-FL) A1190 (2′-FL) A1155 (2′-FL, LNnT)	23/7/25-12/12/3000	"2'FL or 2'-FL or 2'-fucosyllactose or 2'fucosyllactose" and "milk or breast or formula" and "anthropometric or weight or growth or development" and "child or infant or baby or maternal"	11 studies; not relevant

3-FL	A1324 (3-FL)	19/2/2025-12/12/3000	“3FL or 3-FL or 3-fucosyllactose or 3fucosyllactose or 3′FL or 3′-FL or 3′-fucosyllactose” and “milk or breast or formula” and “anthropometric or weight or growth or development” and “child or infant or baby or maternal”	10 studies; not relevant
3′-SL, 6′-SL, LNT	A1265 (2′-FL/DFL 3′-SL, 6′-SL, LNT)	22/11/2022-12/12/3000	“lacto-N- tetraose or LNT or lacto N tetraose or lacto-N tetraose or lacto N-tetraose or 3’-sialyllactose or 3’ sialyllactose or 3’-SL or 3SL or 3’SL or 6’-sialyllactose or 6’ sialyllactose or 6’-SL or 6SL or 6’SL” and “milk or breast or formula” and “anthropometric or weight or growth or development” and “child or infant or baby or maternal”	93 studies; not relevant