FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk, technical and benefit assessment – Application A1324

A1324 – 3-fucosyllactose as a nutritive substance in infant formula products

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Executive summary

Food Standards Australia New Zealand (FSANZ) has assessed an application from Glycom A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit 3-fucosyllactose (3-FL) for use as a nutritive substance in infant formula products up to a maximum permitted amount of 80 mg/100 kJ (2 g/L). The applicant's 3-FL is produced by microbial fermentation from a genetically modified (GM) strain of *Escherichia coli (E. coli)* K-12.

FSANZ has undertaken an assessment of the food technology, safety and beneficial health effects associated with the addition of 3-FL to infant formula products.

The applicant's 3-FL is chemically, structurally and functionally identical to the naturally occurring 3-FL present in human milk. Results from a 2-year accelerated trial indicate the applicant's 3-FL is stable under ambient storage conditions, reflecting the typical stability and storage conditions for an infant formula product. Further, interim results from an ongoing 5-year ambient shelf-life trial also indicate the applicant's 3-FL is stable under ambient storage conditions.

FSANZ's safety assessment did not identify any public health and safety concerns associated with the use of *E. coli* K-12 as a production organism for 3-FL. Characterisation of the GM production strain confirmed that the introduced α-1,3-fucosyltransferase gene is both genetically stable and functional.

3-FL's natural presence in human milk provides a history of safe human exposure. Estimated dietary intakes of 3-FL from infant formula products at the proposed maximum permitted amount are comparable to intakes from naturally occurring 3-FL in human milk.

Overall, the available data indicate that intestinal absorption of human milk oligosaccharides, including 3-FL, is limited. A significant proportion reaches the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces. *In vitro* and *in vivo* studies demonstrated that 3-FL does not pose a concern for genotoxicity. No adverse effects were observed in a 90-day oral toxicity study in neonatal rats at doses up to 4000 mg/kg bw/day, or in older rats at doses ≥ 5900 mg/kg bw/day. There were no adverse effects in neonatal piglets given formula containing up to 2 g/L 3-FL for 21 days.

In three human clinical studies with infants, formula containing 0.24 to 0.8 g/L of 3-FL in combination with other human-identical milk oligosaccharides (HiMO) (with or without probiotics) 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.

Given the absence of any identifiable hazard in toxicological and clinical studies, and noting that estimated dietary intakes of 3-FL from infant formula products are comparable to intakes from human milk, there are no safety concerns from the addition of 3-FL to infant formula products at the proposed maximum permitted amount.

The weight of evidence supports plausible biological mechanisms and the potential for beneficial effects of 3-FL added to infant formula products through an increase in the abundance of *Bifidobacterium* spp. in the infant gut microbiota and anti-pathogenic effects. The inclusion of a wider range of HiMO in infant formula products is likely to support the development of a healthy microbiota.

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1 Introduction

FSANZ received an application from Glycom A/S to amend the Australia New Zealand Food Standards Code (the Code) to permit 3-fucosyllactose (3-FL) for use as a nutritive substance in infant formula products at levels up to 80 mg/100 kJ (2 g/L). The applicant's 3-FL is produced by microbial fermentation from a genetically modified (GM) strain of *Escherichia coli* K-12.

2 Food technology assessment

The objective of the food technology assessment is to determine whether the microbiologically synthesised 3-FL proposed to be added to infant formula products is chemically and structurally identical to that present in human milk and stable under normal conditions of use. The assessment also considered the manufacturing process and the analytical methods and results used to quantify and characterise 3-FL.

FSANZ has assessed recent applications requesting permissions for human-identical milk oligosaccharides (HiMO) for use in infant formula products but not for 3-FL, a structural isomer of 2′-fucosyllactose (2′-FL). Information in this section has built on the assessment of 2′-FL, assessed in 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 2024b).

2.1 Chemical and physical properties

3-FL is part of the human milk oligosaccharide (HMO) fraction found in human milk (see Figure 1) (Du et al. 2024). This 3-FL is produced from a GM *E. coli* K-12 production strain, similar to other HiMO previously assessed by FSANZ. It is a white to off-white amorphous powder or agglomerate and is readily soluble in aqueous solutions but has poor solubility in organic solvents.

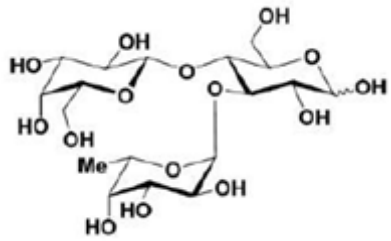

Figure 1 Molecular structure of 3-FL

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.

2.1.1 Equivalence to human milk 3-FL

The application included analytical data provided as Confidential Commercial Information (CCI) to demonstrate that 3-FL obtained from microbial fermentation is chemically and structurally identical to 3-FL naturally present in human milk. The analytical method used to determine this was nuclear magnetic resonance (NMR) spectroscopy where the spectral data from Glycom's 3-FL were compared with human milk derived 3-FL.

The NMR results confirm the 3-FL obtained from microbial fermentation is chemically and structurally identical to that occurring naturally in human milk. The chemical name and

properties of the applicant's 3-FL are provided in Table 1.

Table 1: ***Chemical name and properties of 3-FL***

Property	3-FL
Common name	3-fucosyllactose
IUBMB[1]/IUPAC[2] Chemical name[3]	β -D-Galactopyranosyl-(1-4)-[α-L- fucopyranosyl-(1-3)]- D-glucose
Alternative common names	3-FL; 3FL
Alternative names	3-*O*-Fucosyllactose 3-*O*-L-Fucosyl-D-lactose 3-Fucosidolactose Lewis^x-2g (=glucose analogue of histo-blood group Lewis^x antigen)
IUPAC abbreviation	Gal-(β1-4)-[αFuc-(α1-3)]-Glc
CAS registry number[4]	41312-47-4
Chemical formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol

Abbreviations: Fuc = focosyl; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose
[1] International Union of Biochemistry and Molecular Biology
[2] International Union of Pure and Applied Chemistry
[3] The IUPAC and IUBMB names are the same, based on the joint recommendations from The Joint Commission on Biochemical Nomenclature.
[4] Chemical Abstract Service

2.1.2 Stability of 3-FL under intended conditions of use

The application included analytical data on the stability of the 3-FL ingredient and in a commercially representative infant formula product matrix. This data is CCI.

The 3-FL ingredient was stored under ambient conditions in an ongoing 5-year ambient shelf-life trial (25°C, 60% relative humidity) and a 2-year accelerated shelf-life trial (40°C, 75% relative humidity). The interim results from the 5-year ambient trial and the results from the 2-year accelerated trial showed that 3-FL is stable.

The stability of 3-FL as an ingredient (1 g/100 g powder) in commercially representative infant formula product, packaged in cans and stored at various temperatures and relative humidity for a period of 3 years was also reviewed. The results indicate the 3-FL concentration remains stable over the intended shelf-life of infant formula products.

2.2 Manufacturing process

The applicant provided details of their manufacturing process. Their 3-FL is produced by microbial fermentation of a GM strain of *E. coli* K-12. The production process consists of 2 stages: upstream processing (USP) and downstream processing (DSP). USP includes fermentation while DSP includes purification, isolation and concentration and is summarised in Table 2. The final 3-FL product is subject to batch release based on adherence to

specifications. Details of the raw materials, ingredients in the fermentation medium and processing aids were provided as CCI. Together with the manufacturing process, including filtration and purification steps they do not pose any food safety risks.

According to the applicant, their production process complies with current Good Manufacturing Practice and Hazard Analysis of Critical Control Points principles. Their manufacturing process and food safety management systems also meet relevant food safety systems certification and the International Organisation for Standardisation 9001. This mirrors previous applications for HiMO from the same applicant, assessed in applications A1155 and A1265 (FSANZ 2019, FSANZ 2023a).

Table 2: *Manufacturing process for 3-FL*

Stage 1: Upstream processing	
1	Media preparation
2	Propagation
3	Seed fermentation
4	Main fermentation phases: Growth - batch Production – fed-batch Harvest/storage of culture broth
5	Removal of production microorganism following fermentation
Stage 2: Downstream processing	
6	Purification/Concentration of 3FL
7	Ion removal
8	Decolourisation
9	Purification/Concentration of 3-FL
10	Drying
11	Sampling and Packaging
12	Quality control and batch release

2.3 Specifications

Section 1.1.1—15 of the Code requires that a substance used as a nutritive substance must meet any relevant identity and purity specification in Schedule 3. There is no specification for 3-FL from *E. coli* K-12 in Schedule 3.

The applicant provided a proposed specification for its 3-FL. The specification includes parameters for the identity of 3-FL and impurities. This specification is only applicable to 3-FL sourced from *E. coli* K-12. The applicant provided the methods of analysis used for each parameter.

The limits provided by the applicant for Enterobacteriaceae, yeasts, moulds and residual endotoxins are process hygiene parameters consistent with specifications for other HiMO substances permitted in the Code. Schedule 27 contains limits for *Salmonella* so these are therefore not listed in the specification.

FSANZ is proposing a specification for inclusion in Schedule 3 (see Table 3) based on what the applicant provided and FSANZ's approach for similar specifications for HiMO. The proposed specification aligns with specifications for the applicant's 3-FL in European Union (EU) and United Kingdom (UK) legislation.

The applicant provided certificates of analysis for five independent representative batches of their 3-FL with results consistent across each batch tested. The results demonstrate the product can meet the proposed specification for 3-FL in Table 3.

2.3.1 Impurities

The applicant's product contains a minimum 90% w/w of 3-FL. There are smaller quantities of carbohydrates, including D-lactose, the starting substrate and other related carbohydrates produced during fermentation. These parameters have been included in the specification at Table 3 at the limits the applicant proposed.

Schedule 3 of the Code includes specifications for arsenic, cadmium, mercury and lead (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3. The certificates of analysis for the applicant's 3-FL indicate that those limits were met for the five batches provided. A lower limit for lead of not more than 0.05 mg/kg was proposed by the applicant and is included in the proposed specification compared with the limit of 2 mg/kg in Schedule 3. Similarly, lower limits for lead are included for the 2′-FL specifications in Schedule 3.

The application confirms the production organism is removed during processing and purification steps during production of 3-FL. Qualitative polymerase chain reaction methods were used to confirm that no residual DNA from the production organism remains in the final purified nutritive substance. Additionally, separate testing was conducted to confirm no viable production strain cells were present in the final nutritive substance preparation.

Table 3: *Proposed specification for 3-FL*

Parameter	Specification
Chemical name	β -D-Galactopyranosyl-(1-4)-[α-L-fucopyranosyl-(1-3)]- D-glucose;
Chemical formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol
CAS number	41312-47-4
Description	White to off-white powder or agglomerates
Sum of saccharides (3-fucosyllactose, 3-fucosyllactulose, D-lactose and L-fucose)	not less than 92.0%
3-FL	not less than 90.0%

L-Fucose	not more than 1.0%
D-Lactose	not more than 5.0%
3-Fucosyllactulose	not more than 1.5%
pH (20°C, 5% solution)	3.2 to 7.0
Water	not more than 6.0%
Ash, sulphated	not more than 0.5%
Residual proteins	not more than 0.01%
Lead	not more than 0.05 mg/kg
Microbiological	
Aerobic mesophilic total plate count	not more than 1,000 cfu/g
Enterobacteriaceae	Absent in 10 g
Yeasts	not more than 100 cfu/g
Moulds	not more than 100 cfu/g
Residual endotoxins	not more than 10 EU/mg

2.4 Analytical methods for detection

The applicant has developed internal methods for detecting and quantifying 3-FL using high performance liquid chromatography (HPLC) and high-pressure anion-exchange chromatography (HPAEC) methods.

2.5 Food technology conclusion

From assessment of the data provided in the application, the applicant's 3-FL produced by a microbial fermentation manufacturing process is chemically and structurally identical to the 3-FL naturally occurring in human milk.

A proposed specification (see Table 3) for the use of 3-FL has been prepared by FSANZ for inclusion in Schedule 3 of the Code. The results from analytical testing demonstrate the applicant's product can meet this specification. This specification aligns with current specifications for the applicant's 3-FL in EU and UK legislation.

Interim results from an ongoing 5-year ambient shelf-life trial and the results from a 2-year accelerated study indicate the applicant's 3-FL is stable under ambient storage conditions.

3 Safety assessment

3.1 Genetically modified (GM) production strain assessment

3.1.1 Host organism

Escherichia coli is a facultative anaerobic, Gram-negative, rod-shaped bacteria found in the gut of mammals (Guerra et al. 2019). *E. coli* strains can be pathogenic to humans causing a wide range of diseases, some of which can be fatal (Guerra et al. 2019, Mir and Kudva 2019). However, there are strains of *E. coli* termed safe strains, used in research and industry specifically because of their inability to cause disease in humans. *E. coli* K-12 is one of these strains (Bauer et al. 2008, Browning et al. 2023).

E. coli K-12 was first isolated from a convalescent diphtheria patient in 1922. *E. coli* K-12 is well characterised and its genome has been sequenced and annotated (Riley et al. 2006). It is non-pathogenic, non-toxigenic, and not known to colonise the human gut. FSANZ has previously approved *E. coli* K-12 as a production organism of 2′-FL in applications A1155 and A1265 (FSANZ 2019, FSANZ 2023a). The production strain used in this application has been assessed by European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA) with no safety concerns raised (FDA 2022, EFSA 2023).

Whole genome sequencing (WGS) results for the production strain provided by the applicant confirmed the identity of the production strain as *E. coli* K-12. Microbiological testing of three independent batches provided to FSANZ confirmed the absence of the production organism in the final enzyme preparation.

The FSANZ microbiological risk assessment did not identify any public health and safety concerns associated with the use of *E. coli* K-12 as a production organism for 3-FL.

3.1.2 Gene donor organism

The donor organism's identity was confirmed through CCI data. Endogenous vectors and other genetic material of *Helicobacter pylori* are not relevant because the gene encoding the α-1,3-fucosyltransferase was constructed via synthetic DNA synthesis. The expressed gene product is not associated with any potential toxicity or pathogenic traits of the donor organism.

3.1.3 Characterisation of the GM production organism

3.1.3.1 Development of the GM production strain

To develop the production strain, the α-1,3-fucosyl-transferase gene was codon optimised and chemically synthesised based on the *H. pylori* sequence available in public databases. Data provided by the applicant and analysed by FSANZ confirmed the identity of the α-1,3-fucosyl-transferase enzyme.

The α-1,3-fucosyl-transferase gene was introduced into specific locations in the genome of the host *E. coli* K-12 using homologous recombination.

3.1.3.2 Characterisation of introduced DNA

WGS data provided by the applicant confirmed the presence of the α-1,3-fucosyl-transferase gene at the intended locations in the genome of the production strain. No antibiotic resistance markers are present in the final production strain.

3.1.3.3 Genetic stability and inheritance of the introduced DNA

The applicant provided WGS data which confirmed the inserted gene remained stable over 50 successive generations.

Data was also provided showing that production of 3-FL by the production strain was consistent over a minimum of 50 generations, providing further evidence of the stability and inheritance of the inserted DNA over this period.

3.1.4 Conclusion

The microbiological risk assessment undertaken by FSANZ did not identify any public health and safety concerns associated with the use of *E. coli* K-12 as a production organism for 3-FL. Characterisation of the GM production strain confirmed that the introduced gene was both genetically stable and functional.

On the basis of the data provided, no potential safety concerns were identified in the assessment of the 3-FL production strain.

3.2 Toxicology assessment

3.2.1 Toxicokinetics

The applicant's 3-FL is chemically and structurally identical to 3-FL found in human milk (see section 2.1), and it is not expected that there will be significant differences in pharmacokinetics between the naturally occurring and manufactured forms of this substance.

FSANZ has previously reviewed data on the absorption, distribution, metabolism and excretion of HMO including 3-FL. These data included *in vitro* and *in vivo* studies as well as studies in human infants (FSANZ 2019, FSANZ 2020a). Overall, the available information indicates that only very small amounts of ingested HMO are absorbed, while the majority pass to the large intestine where they are fermented by the intestinal microbiota or excreted unchanged in the faeces.

The pharmacokinetics of 3-FL were also evaluated in a 90-day oral toxicity study in rats not previously reviewed by FSANZ (Pitt et al. 2019) which is described further in section 3.2.2. Quantitation of 3-FL in serum and urine in this study indicated negligible systemic exposure with absorption well below 1% of the daily dose, consistent with the previously reviewed data.

3.2.2 Toxicological studies

As part of Application A1155, FSANZ reviewed a paper reporting on safety studies of a mixture of five HiMO which included 3-FL (Parschat et al. 2020, FSANZ 2020). The mixture was not genotoxic *in vitro* and did not cause adverse effects in a 13-week oral toxicity study in rats. The dose of 3-FL in this study was 232 mg/kg bw/day in males and 286 mg/kg bw/day in females.

The applicant's 3-FL has been evaluated in 14-day and 90-day oral toxicity studies in neonatal rats, a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test. The *in vivo* toxicity studies were adapted by initiating dosing in neonatal animals, in line with recommendations for safety assessment of compounds intended for use by paediatric populations (FDA 2006, EMEA 2008, MHLW 2012, EFSA 2017).

A literature search of PubMed was conducted by FSANZ on 11 March 2025 using the search

terms ‘3-FL AND toxic*’. Two papers reporting results of genotoxicity and toxicity studies of 3-FL produced from another source were identified. A comparison of the available compositional information for the test item used in these studies with the specifications established for the applicant’s 3-FL is shown in Table 4. As the composition of the test item in the studies by Pitt et al. is similar to that of the applicant’s 3-FL specification, these studies provide further evidence on the safety of 3-FL.

Table 4: ***Comparison of specifications for the applicant's 3-FL and the composition of the test item used in studies of 3-FL from an alternative source***

Parameter	Specification for applicant's 3-FL	Test item in studies by Pitt et al. 2019 & Pitt et al. 2024
3-fucosyllactose	≥ 90.0%	94.6%
L-Fucose	≤ 1.0%	1.2% (reported as Fucose)
D-Lactose	≤ 5.0%	1.5% (reported as Lactose)
Glucose/galactose	Not listed	1.3%
3-fucosyllactulose	≤ 1.5%	Not reported
Other carbohydrates	≤ 5.0%	1.4%
Protein	≤ 0.01%	≤ 100 µg/g (≤ 0.01%)
Ash	≤ 0.05%	≤ 0.5%
Water	≤ 6.0%	1.9% (reported as moisture)

3.2.2.1 Short term toxicity

Studies with 3-FL produced by the applicant

14-day dose range-finding study in neonatal rats (Stannard 2021a, Phipps et al 2022) Regulatory status: non-GLP; non-guideline

In a dose range-finding study, groups of 8 male and 8 female neonatal Crl:CD(SD) rats (7 days of age) were administered 0, 3000 or 4000 mg/kg bw/day 3-FL (purity 94.6%) for 14 days. Doses were prepared correcting for the presence of other carbohydrates (total 1.76%) in the test item. The vehicle control was water. Clinical signs and body weight were recorded daily. At the end of the study animals were subjected to a gross macroscopic necropsy.

There were no deaths and no adverse clinical signs. Skin reddening and yellow staining around the anus/perianal region were observed in some treated rats in both dose groups, but as these findings were transient and largely absent by the end of the study they were not considered to be adverse. Treated animals gained similar amounts of body weight as controls. No macroscopic abnormalities were detected at necropsy.

It was concluded that 3-FL was well tolerated and that 4000 mg/kg bw/day would be an acceptable high dose for a 90-day toxicity study in neonatal rats.

90-day oral toxicity study in neonatal rats (Stannard 2021b, Phipps et al. 2022). Regulatory status: GLP; conducted in accordance with OECD TG 408 (2018)

In a 90-day oral toxicity study, 3-FL (purity 94.6%) was administered to Crl:CD(SD) rats (10/sex/group; 7 days of age) by oral gavage at doses of 0, 1000, 2000 or 4000 mg/kg bw/day. Water was used as the vehicle control. A further 5 rats/sex/group in the vehicle control and high dose groups were also dosed daily for 90 days and then kept untreated for 4 weeks to assess the reversibility of any observed effects.

Clinical signs, body weights and food consumption were recorded throughout the study.

Ophthalmology was performed on control and high dose group rats in week 13. Blood samples were collected in week 13 for haematology, coagulation, clinical chemistry and thyroid hormone analysis. Samples for urinalysis were collected in week 13 and at the end of the recovery period.

All animals were subjected to a functional observational battery (FOB) in addition to assessment of grip strength and learning and memory (using the Morris maze). Pre-weaning reflex development, ulna length, sexual maturation and oestrus cycle monitoring were also recorded for all animals. A gross macroscopic necropsy was conducted on all surviving main study animals at the end of the dosing and recovery periods, and selected organ weights were recorded. At the end of the dosing period, a microscopic evaluation was performed on tissues from the vehicle control and high dose groups.

All animals survived up to the end of the study and no treatment-related clinical signs of toxicity were observed. There were no treatment-related changes in body weight, food consumption and ophthalmological examinations. Behaviour of the animals during in-hand and arena observations, Morris maze learning and memory performance, and grip strength were similar across all groups. Pre-weaning development, ulna growth, sexual maturation and oestrus cycles were unaffected by 3-FL administration. There were no treatment-related adverse effects on haematology, coagulation, clinical chemistry, urinalysis, thyroid hormone, organ weights or gross and microscopic pathology.

The no observed adverse effect level (NOAEL) in this study was 4000 mg/kg bw/day, the highest dose tested.

Studies with 3-FL produced by other manufacturers

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

In a 90-day toxicity study, 3-FL (purity 94.6%) was administered in the diet to groups of 10 male and 10 female Crl:CD(SD) rats (age approximately 7 weeks) at concentrations of 0%, 5% or 10% (equal to 0, 3038 and 5975 mg/kg bw/day in males, 0, 3870 and 7270 mg/kg bw/day in females).

There were no treatment-related effects on mortality, clinical signs, neurobehavioural parameters, ophthalmology, body weight, food consumption, haematology, clinical chemistry, urinalysis, organ weights and macroscopic or microscopic histopathological findings. The NOAEL in this study was 10% in the diet (equal to 5975 mg/kg bw/day), the highest concentration tested.

21-day dietary toxicity study in neonatal piglets (Pitt et al. 2024) Regulatory status: GLP; non-guideline

In a neonatal piglet study, Domestic Landrace Crossbred Swine (6/sex/group) were fed a milk replacer containing 3-FL (purity 94.6%) at concentrations of 0, 1 or 2 g/L (equal to 0, 246 and 490 mg/kg bw/day in males, 0, 247 and 494 mg/kg bw/day in females) from postnatal day 2 for 21 days. Clinical signs, body weights and food consumption were recorded daily. Haematology, coagulation and clinical chemistry parameters were assessed on study days 7 and 22. Urine samples were collected at necropsy. At the end of the study organ weights were recorded and tissues were subjected to macroscopic and microscopic pathology.

There were no treatment-related effects on survival, clinical signs, food consumption, food efficiency, haematology, coagulation, clinical chemistry, organ weights and macroscopic or microscopic histopathology. The NOAEL in this study was 2 g/L (equal to 490 mg/kg

bw/day), the highest dose tested.

3.2.2.2 Genotoxicity

Two genotoxicity studies with the applicant's 3-FL were submitted, a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test. In addition, details of 4 genotoxicity studies with 3-FL from another manufacturer are reported in the study by Pitt et al. (2019).

These studies were conducted in accordance with GLP and according to OECD Test Guidelines. The positive controls in these studies produced the expected responses confirming the validity of the test systems.

The results of these studies, as summarised in Table 5, demonstrate that 3-FL does not pose a concern for mutagenicity, clastogenicity and aneugenicity. A statistically significant trend for increasing micronucleus frequency in the presence of metabolic activation was observed in an *in vitro* micronucleus test with 3-FL produced by another manufacturer, which was classed as an equivocal result[1]. The incidences of micronuclei following 3-FL treatment in this study were within the laboratory's historical negative control range and a follow-up *in vivo* mouse micronucleus study was negative. Based on the overall weight of evidence, the data indicate that 3-FL is unlikely to pose a genotoxicity concern *in vivo*.

Table 5: *Genotoxicity studies of 3-FL*

Test	Test object	Concentration	Purity (%)	Results	Reference
Studies with the applicant's 3-FL					
Bacterial reverse mutation test (OECD TG 471 [1997])	*Salmonella typhimurium* strains TA98, TA100, TA1535 & TA1537; *Escherichia coli* strain WP2 *uvrA* (pKM101)	Plate incorporation assay: 0, 5, 15, 50, 150, 500, 1500 or 5000 µg/plate Pre-incubation assay: 0, 50, 150, 500, 1500 or 5000 µg/plate	94.63%	Negative ± S9	(Gilby 2021, Phipps et al. 2022)
In vitro mammalian cell micronucleus test (OECD TG 487 [2016])	Human lymphocytes	0, 500, 1000 or 2000 µg/mL[1]	94.63%	Negative ± S9	(Gilby 2021, Phipps et al. 2022)
Studies with 3-FL produced by other manufacturers					
Bacterial reverse mutation test (OECD TG 471 [1997])	*S. typhimurium* strains TA98, TA100, TA1535 & TA1537; *E.*	0, 333, 667, 1000, 3333 or 5000 µg/plate	94.6%	Negative ± S9	(Pitt et al. 2019)

[1] Results are described as equivocal when the data set will not allow a clear conclusion of a positive or negative response.

	coli strain WP2 *uvrA*				
In vitro mammalian cell micronucleus test (OECD TG 487 [2016])	Chinese hamster ovary (CHO-K1) cells	0, 500, 1000, 2500, 3500 or 5000 µg/mL[2,3]	94.6%	Negative – S9; Equivocal + S9[4]	(Pitt et al. 2019)
In vitro mammalian cell chromosomal aberration test (OECD TG 473 [2016])	Human lymphocytes	0, 1250, 2500 or 5000 µg/mL[2]	94.6%	Negative ± S9	(Pitt et al. 2019)
In vivo mammalian erythrocyte micronucleus test (OECD TG 474 [2016])	CrlCD1(ICR) mice	0, 500, 1000 or 2000 mg/kg bw[5]	94.6%	Negative[6]	(Pitt et al. 2019)

[1] Cells exposed for 3 hours in presence and absence of S9 and for 20 hours without S9
[2] Cells exposed for 4 hours in presence and absence of S9 and for 20 hours without S9
[3] An aneugenic positive control was not included in this study
[4] No statistically significant increases in micronuclei in treated cells and incidences in all treatment groups were within the 95% confidence interval of laboratory historical control data, however a statistically significant trend for higher instances of micronuclei was observed at ≥ 2500 µg/mL in the first assay and at ≥ 1000 µg/mL in a second assay
[5] Animals received a single dose by oral gavage; peripheral blood was collected 48 and 72 h post-dosing
[6] Systemic exposure confirmed by measuring plasma 3-FL (572 and 681 ng/mL, respectively in males and females administered 500 mg/kg bw day)

3.2.3 Studies in humans

FSANZ has previously reviewed two clinical studies of healthy term infants fed formula containing a mixture of five HiMO that included 3-FL (FSANZ 2023a). In both studies the HiMO were 2′-FL, 3-FL, LNT (lacto-N-tetraose), 3′-SL (3′-sialyllactose) and 6′-SL (6′-sialyllactose), with a 3-FL concentration of 0.75 or 0.8 g/L. FSANZ's assessment concluded that in both studies the HiMO-supplemented infant formulas were safe and well tolerated.

For the present evaluation, a further clinical study evaluating the tolerance and safety of infant formula supplemented with 6HiMO including 3-FL was submitted by the applicant.

Clinical study of infant formula supplemented with 3-FL, 2'-FL, DFL, LNT, 3'-SL and 6'-SL (Miranda et al. 2023; Clinical Trial Registry NCT04962594)

In a double-blind, randomised controlled trial conducted across 18 sites in Europe, healthy term infants (age ≤ 14 days) were assigned to receive a control infant formula or an experimental formula containing 3-FL and five other HiMO (2′-FL, difucosyllactose (DFL), LNT, 3′-SL and 6′-SL), as well as *B. infantis* LMG11588 plus *B. lactis* CNCM I-3446 probiotics, for 15 months. The concentration of 3-FL in the experimental formulas was 0.24 g/L in starter infant formula, 0.26 g/L in follow-up formula and 0.29 g/L in growing up milk. A non-randomised parallel reference group of exclusively breastfed (BF) infants was also enrolled. Additional methodological details of this study are summarised in Table A2-2.

There were no significant differences in gastrointestinal tolerance between the experimental formula and control formula groups, assessed using the overall score of the Infant

Gastrointestinal Symptom Questionnaire (IGSQ). Mean IGSQ scores were low in all groups, indicating good gastrointestinal tolerance. Scores by IGSQ domain (stooling, spitting up/vomiting, crying, fussiness, and flatulence) were also similar between groups across timepoints, except at age 1 month, where infants fed experimental formula had a significantly higher stooling domain score compared to breastfed and control formula fed infants.

The incidence of adverse events and serious adverse events was low and similar between the experimental formula and control formula groups. Two adverse events in infants given the experimental formula were considered to be related to the study product, however these were both due to cow's milk protein allergy. There were no significant differences in the incidence or severity of adverse events, or discontinuation due to adverse events, across the three groups.

It was concluded that infant formula supplemented with the 6 HiMO and 2 probiotics was safe and well tolerated.

3.2.4 Post-marketing surveillance

The applicant provided post-marketing surveillance data relating to infant formula products containing 3-FL in combination with up to five other HiMO that have been commercialised in several markets (CCI). No safety concerns were identified from these data.

3.2.5 Allergenicity

The applicant has indicated that proteins are removed during the manufacturing process, and 3-FL is specified to contain ≤ 0.01% protein. Batch analyses indicate that protein levels are well below this limit. Therefore 3-FL is unlikely to pose an allergenicity concern.

3.2.6 Safety assessments by other agencies

EFSA has assessed the safety of 3-FL produced by the applicant. Uses evaluated included infant formula and follow-on formula, food for special medical purposes and food supplements. EFSA concluded that 3-FL is safe under the proposed conditions of use (EFSA 2023).

In the UK, the Advisory Committee on Novel Foods and Processes (ACNFP) reviewed the safety of the applicant's 3-FL to support the Food Standards Agency (FSA) and Food Standards Scotland (FSS) in their evaluation of an application for 3-FL as a novel food. Proposed uses were in dairy products and analogues, bakery wares, foods for special groups, beverages and also as a food supplement. The target population from these uses includes infants (including use in infant formula), children and adults. The ACNFP concluded that 3-FL is safe under the proposed conditions of use and does not pose a safety risk to human health (ACNFP 2024).

The FDA has responded that it has 'no questions' to Glycom's self-assessment that 3-FL produced by microbial fermentation is Generally Recognized as Safe (GRAS) (FDA 2022). FSANZ notes that 'no questions' responses are not in themselves a safety assessment by the US FDA.

3.2.7 Toxicology assessment conclusion

3-FL's presence in human milk provides a history of safe human exposure, and estimated dietary intakes of 3-FL from infant formula products at the proposed maximum permitted amount are comparable to intakes from naturally occurring 3-FL in human milk.

FSANZ has reviewed the available data on absorption, distribution, metabolism and excretion of HMO. Overall, these data indicated that intestinal absorption is limited and a significant proportion reaches the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces. As the applicant's 3-FL is identical to that found in human milk, no differences in pharmacokinetics between the naturally occurring and manufactured forms of this substance are expected. A 90-day oral toxicity study of 3-FL in rats evaluated for the present application found systemic absorption was well below 1% of the administered dose, consistent with these conclusions.

3-FL produced by the applicant was not genotoxic *in vitro*. In a 90-day oral toxicity study of 3-FL in neonatal rats, no adverse effects were observed at doses up to 4000 mg/kg bw/day, the highest dose tested.

Toxicity studies with 3-FL produced by another company are also available. *In vitro* and *in vivo* genotoxicity studies demonstrated that 3-FL is not genotoxic. No adverse effects were observed in a 90-day oral toxicity study in rats at dietary concentrations up to 10% (equal to 5975 mg/kg bw/day in males, 7270 mg/kg bw/day in females). There were also no adverse effects in neonatal piglets given formula containing up to 2 g/L 3-FL for 21 days.

Human clinical studies with infants fed formula containing mixtures of 5 – 6 HiMO, including 3-FL at concentrations ranging from 0.24 – 0.8 g/L, found that the formulas were safe and well tolerated.

The applicant also provided post-marketing surveillance data that found no safety concerns from consumption of infant formula products containing 3-FL in combination with up to five other HiMO.

Proteins are removed during the production process, and 3-FL is unlikely to pose an allergenicity concern.

3.3 Dietary Intake assessment

3.3.1 Objective of the dietary intake assessment

The objective of this dietary intake assessment is to estimate the dietary intake of 3-FL from the proposed use in infant formula, follow-on formula and special medical purpose products for infants (SMPPi).

3.3.2 Approach to estimating dietary intakes of 3-FL

Dietary intake assessments require data on the concentrations of the chemical of interest in the foods requested, including any naturally-occurring sources and any current permissions for additions to food; and consumption data for the foods that have been collected through a national nutrition survey. As there are no national consumption data for Australian children younger than two years of age, the dietary intakes of 3-FL were estimated based on model diets for infants aged 3 months and 9 months. Dietary intakes from human milk were also considered for comparative purposes.

A summary of the general FSANZ approach to conducting the dietary intake assessment for this application is in Appendix 1. 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 2024c).

3.3.3 Consumption data used

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

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

Model diets were used for the population groups aged 3 months and 9 months, to represent the consumption of infant formula or follow-on formula (where appropriate) and human milk for these groups. How the model diets were constructed, and the justification for not including a model diet for infants consuming SMPPi is in Appendix 1.

3.3.4 Proposed concentrations of 3-FL in infant formula products

The application seeks permission to add 3-FL to infant formula products (as prepared or ready-to-feed) at a maximum use level of 2 g/L. The food categories requested in the application proposed to contain 3-FL and the maximum use level in infant formula products are listed in Table 6. The maximum use levels provided by the applicant were used in the FSANZ dietary intake assessment.

The minimum energy content of 2510 kJ/L currently permitted for infant formula and follow-on formula in the Code (section 2.9.1—5) was used to convert the proposed 2 g/L amount to 80 mg/100 kJ. This approach based on mg/100 kJ would mean that the actual amount of 3-FL in infant formula products could vary depending on the energy content of the formula. In particular, a formula with a higher energy content per 100 mL may contain more 3-FL than a formula with a lower energy content. However, where a formula has a higher energy content, less formula would need to be consumed to meet infant energy requirements. Conversely, more would need to be consumed to meet infant energy requirements for a formula with a lower energy content. As such, the respective dietary intakes for 3-FL would be similar for formulas with varying energy contents as the amount of formula consumed is regulated by infant energy needs.

Table 6: ***Proposed maximum use levels of 3-FL in infant formula products***

Food (as prepared or ready-to-feed)	**Maximum use level of 3-FL (g/L)**	**Maximum infant formula and follow-on formula concentration (g/kg)**
Infant formula	2.0	1.9
Follow-on formula	2.0	1.9
Special Medical Purpose Products for infants	2.0	1.9

Note: 1 litre of infant formula, follow-on formula and SMPPi is equal to 1,050 grams.

3.3.5 Concentrations of 3-FL in human milk

The applicant reported the mean of means and maximum mean of concentrations of 3-FL in samples of mature human milk (excluding colostrum) from the scoping literature review prepared for the EFSA safety evaluation of HiMO as novel foods (Malih et al. 2024). FSANZ

reviewed original data published since 2021 included in the scoping literature review against FSANZ's inclusion criteria (lactation period, term birth, units) 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.

3-FL is unique among other HMO in that it is present in the breastmilk of all women regardless of their secretor status and the concentration level increases throughout lactation (Soyyılmaz et al. 2021). Table 7 lists the concentration of 3-FL in both mature and late human milk.

Table 7: ***Concentration (g/L) of 3-FL in mature human milk from Soyyılmaz et al. 2021, used in the FSANZ dietary intake assessment***

Lactation period	**15 to 90 Days**		**90+ Days**	
Use in infant model	**3 month old**		**9 month old**	
Concentration level	Mean[1]	High[2]	Mean[1]	High[2]
3-FL (g/L)	0.72	1.9	0.92	2.57

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

3.3.6 Concentrations of 3-FL in domestic mammalian milk

Infant formula products are made with domestic mammalian milk bases, particularly cows' milk. Several authors have reported oligosaccharide concentrations (including 3-FL) are significantly lower in bovine milk compared with human milk. Urashima et al. (2013) reported bovine colostrum collected immediately post-partum contained 1 g/L total oligosaccharides, and this concentration rapidly decreases after 48 hours.

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

3.3.7 Assumptions and limitations of the dietary intake assessment

The aim of the dietary intake assessment was to make the most realistic estimation of dietary intake of 3-FL 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:

- unless otherwise specified, all foods within a category contain 3-FL at the concentrations specified in Table 6 for infant formula and follow-on formula, and Table 7 for human milk
- 1 litre of infant formula or follow-on formula equals 1,050 grams
- 1 litre of human milk equals 1,040 grams
- infants aged 3 months are exclusively infant formula fed
- infants aged 9 months consume follow-on formula

- there is no contribution to 3-FL intake through foods and beverages other than from infant formula, follow-on formula and cows' milk (noting that cow's milk was not included in the dietary intake assessment in this case)
- there is no contribution to 3-FL intake through the use of complementary or other medicines.

3.3.8 Estimated dietary intakes of 3-FL

3.3.8.1 3-FL from human milk

When it is assumed that infants aged <12 months are consuming human milk (and no infant formula or follow-on formula), the estimated mean and P90 dietary intakes of 3-FL from mature and late human milk have been calculated and shown in Tables 8 and 9 respectively, using the concentration values from Table 7.

The estimated dietary intake of 3-FL for 3 month old infants consuming mature milk are 0.083 - 0.17 g/kg/bw/day in mean concentration and 0.22 – 0.44 g/kg/bw/day at a high concentration. For 9 month old infants consuming late milk, the estimated dietary intakes of 3-FL are 0.051 – 0.1 g/kg/bw/day in mean concentration and 0.14 – 0.29 g/kg/bw/day at a high concentration.

Table 8: ***Estimated dietary intake of 3-FL from mean concentrations in mature and late human milk***

	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	2195	2937
100% of energy requirements	kJ/day	2195	n/a
50% of energy requirements	kJ/day	n/a	1469
Mean dietary intake	g/kg bw/day	0.083	0.051
P90 dietary intake	g/kg bw/day	0.17	0.1

Table 9: ***Estimated dietary intake of 3-FL from a high concentration in mature and late human milk***

	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	2195	2937
100% of energy requirements	kJ/day	2195	n/a
50% of energy requirements	kJ/day	n/a	1469

Mean dietary intake	g/kg bw/day	0.22	0.14
P90 dietary intake	g/kg bw/day	0.44	0.29

3.3.8.2 3-FL from infant formula products

The estimated dietary intake of 3-FL for 3 month old infants consuming infant formula products is 0.25 g/kg/bw/day at the mean and 0.49 g/kg/bw/day for a high intake (Table 10). For 9 month old infants the estimated dietary intake of 3-FL are 0.12 g/kg/bw/day at the mean and 0.24 g/kg/bw/day for a high intake (Table 10).

Table 10: ***Estimated dietary intakes assuming maximum proposed concentration of 3-FL in infant formula and follow on formula of 2 g/L***

	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	2195	2937
100% of energy requirements	kJ/day	2195	n/a
50% of energy requirements	kJ/day	n/a	1469
Mean dietary intake	g/kg bw/day	0.25	0.12
P90 dietary intake	g/kg bw/day	0.49	0.24

3.3.9 Conclusion

Mean estimated dietary intakes of 3-FL from infant formula products (0.12-0.25 g/kg bw/day) were comparable to mean estimated dietary intakes from mature human milk (0.051-0.22 g/kg bw/day; for both 3- and 9-month-olds and mean and high concentrations). High (90th percentile) estimated dietary intakes from infant formula products (0.24-0.49 g/kg bw/day) were comparable to high intakes from mature human milk (0.1-0.44 g/kg bw/day; for both 3- and 9-month-olds and mean and high concentrations). The upper end of the range of 3-FL intakes is marginally higher from infant formula products compared to that from human milk. This is not of concern considering that manufacturers may use lower concentrations than the maximum permitted amount which is the level modelled in the intake estimates. There will also be variation in actual intakes from both human milk and infant formula products due to additional factors such as naturally occurring concentrations of 3-FL, variation in human milk and/or formula intakes, increases in body weight from infant growth, and other dietary sources of 3-FL.

3.4 Nutrition assessment

3.4.1 Approach for the nutrition assessment

The objective of the nutrition assessment is to determine the effect (if any) of the addition of 3-FL to infant formula products on infant growth at a maximum permitted amount of 80 mg/100 kJ, equivalent to 2 g/L.

3.4.2 Effect of 3-FL on infant growth

3.4.2.1 Previous FSANZ assessments

In Application A1265, FSANZ assessed the effects of a HiMO blend containing 3-FL, 2′-FL, LNT, 6′-SL and 3′-SL on infant growth in two human clinical studies (Parschat et al. 2021, Lasekan et al. 2022). The studies compared weight gain per day from enrolment to 4 months of age in infants fed a HiMO formula or a non-HiMO control formula as the primary outcome. The concentration of 3-FL in the HiMO formula was 0.75 – 0.8 g/L (Table A2-1). Both studies found no clinically relevant differences in mean body weight gain between groups (differences in mean body weight gain were within the non-inferiority margin of ±3 g/day). That is, a group fed the HiMO-containing formulas that showed a difference in weight gain compared to a control formula or breastfed was not deemed to show inferior or excessive growth if within this margin.

3.4.2.2 Current assessment

The applicant provided two clinical studies that have been previously assessed by FSANZ as described above. In addition one confidential unpublished clinical study was provided to support their application (Miranda et al. 2023). FSANZ conducted a literature search[2] in PubMed on 19 February 2025 to identify any additional relevant studies. The search returned 67 studies. No additional studies were identified that were relevant to include in the assessment.

The three studies included in the body of evidence tested the effect of infant formula with 3-FL as a blend with other HiMO on growth. A summary of the study parameters for the three studies is shown in Table A2-2.

In a multicentre European double-blind randomised control trial, Miranda et al. (2023) investigated the effects of a blend of 3-FL, 2′-FL, DFL, LNT, 3′-SL and 6′-SL and 2 probiotics (*B. lactis* and *B. infantis*) in healthy term infants from enrolment (aged up to 14 days) to 15 months of age on infant growth. Formula fed infants were randomised to receive control formula of partially hydrolysed 100% whey-based formula, or experimental formula (control formula with HiMO/probiotic blend). The concentration of 3-FL in the experimental formula was 0.24, 0.26 and 0.29 g/L in the formula for ages 0-6 months, 6-12 months and 12-15 months respectively (Table A2-1). A breastfed reference group was also included.

The primary endpoint was weight gain (g/day) from enrolment to 4 months of age, with a non-inferiority margin of ±3 g/day. The difference in mean weight gain per day from enrolment to 4 months was non-inferior in the experimental formula versus control formula fed infants.

Several other growth endpoints were measured up to 15 months. At all timepoints, the experimental and control formula groups had similar z-scores (weight-for-age, length-for-age, head circumference-for-age, BMI-for-age, and weight-for-length), with the majority of infants tracking within ± 0.5 standard deviations of the World Health Organization (WHO) median z-scores. Further details of the study were assessed by FSANZ but cannot be reported as they are confidential commercial information.

3.4.3 Conclusion

The applicant has requested the addition of 3-FL up to 2 g/L to infant formula products which

[2] Search terms: “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”

is within the range found in human milk (section 3.3.5).

Three clinical trials in infants were included in the body of evidence that tested the effect of 3-FL up to 0.8 g/L in combination with other HiMO (with or without probiotics) on infant growth. No studies were identified that tested the effect of 3-FL alone. None of the available studies found a difference in growth compared to control formula. FSANZ is unable to make any conclusions on the effect, if any, of 3-FL on growth at concentrations above 0.8 g/L due to a lack of available evidence.

However, based on the lack of adverse effects on growth in clinical studies and the limited oral absorption of 3-FL as discussed in section 3.2.1, there is no evidence to indicate a nutritional concern with the addition of 3-FL to infant formula products at concentrations corresponding to those typically observed in human milk.

4 Beneficial effects assessment

The objective of this assessment is to review reported benefits of the addition of 3-FL to infant formula products on the development of the gut microbiota, in terms of composition (bifidogenic effect) and anti-pathogenic effects for a formula-fed infant.

The stated purpose for adding 3-FL to infant formula products is to better reflect the oligosaccharide composition of human milk. The addition of 3-FL to infant formula products is stated by the applicant to confer functional benefits to infants, consistent with the HMO fraction of human milk, with two specified beneficial effects: (1) a bifidogenic effect; and (2) an anti-infective effect against pathogens.

Modulations of the gut microbiome have the potential to vary widely from individual to individual (Gibson et al. 2017). This is due to the unique microbial ecology of individuals and a variety of host and environmental factors that may influence the effect of a dietary intervention. Moreover, microbial utilisation of 3-FL can only occur if the appropriate bacteria are a component of the gut microbiome. As such, it is difficult to definitively and reproducibly demonstrate causality of an effect associated with the addition of 3-FL to infant formula products. A weight of evidence approach (FSANZ 2020c) informed the assessment of the physiological, biochemical and/or functional effects of 3-FL and their link to specific benefits.

FSANZ assessed 31 studies provided by the applicant to support these proposed beneficial effects. To ensure a comprehensive assessment was carried out a literature search was performed on 07 March 2025 in the Core Collection of the Web of Science, identifying 51 papers, 7 of which had already been provided by the applicant. 19 studies meet the inclusion criteria and 56 studies were excluded because they were not relevant.

4.1 Bifidogenic effect

FSANZ has previously described the critical role of intestinal microbiota, focusing on *Bifidobacterium,* in health and development of infants (FSANZ 2019, FSANZ 2020b). Briefly, the development of intestinal microflora in breastfed infants is characterised by a bifidogenic effect, where bifidobacteria proliferate and dominate the gut microbiota. This development is influenced by factors such as delivery method, diet, geographical origin, and antibiotic use. Studies indicate that breastfed infants have a more homogeneous gut microbiota dominated by bifidobacteria compared to formula-fed infants. Key species include *B. longum* subsp. *infantis*, *B. breve, and B. bifidum* in infants, while *B. adolescentis* is more common in adults. Research shows that maternal secretor status affects the abundance of bifidobacteria, with higher levels in infants from secretor mothers. The introduction of solids and weaning reduces bifidobacteria as other bacteria become more dominant. Overall, breastfeeding

significantly promotes a bifidobacteria-dominant gut microbiota in infants.

4.1.1 *In vitro* studies

The ability of *Bifidobacteria* to utilise 3-FL has been demonstrated in numerous *in vitro* studies (Yu et al. 2013a, Yu et al. 2013b, Garrido et al. 2015, Cheng et al. 2020, Salli et al. 2021). Garrido et al. (2015) found that all *B. infantis* strains tested grew well on 3-FL alone, demonstrating 3-FL was consistently and efficiently utilised. *B. bifidum* strains were more variable with some growing well, while others showed very limited or no growth. Salli et al. (2021) reported strong growth with 3-FL for strains of *Bifidobacterium longum subsp. infantis*, *B. bifidum*, and *B. pseudocatenulatum*. Weak to no growth was seen for *B. breve, B. adolescentis*, *B. animalis subsp. lactis*, *B. catenulatum*, *B. longum* subsp. *longum*. These results support that the ability of *Bifidobacteria* to utilise 3-FL is strain specific.

4.1.2 *Ex vivo* studies

Two *ex vivo* studies using infant faecal samples were identified (Kong et al., 2021 and Salli et al., 2023). Kong et al. (2021) performed *in vitro* fermentation studies with HiMO 3-FL and LNT2 using pooled faecal microbiota from four 12-week-old exclusively breastfed infants. The 3-FL use was monitored via chromatography with microbial community changes assessed via 16S rRNA sequencing. The microbial community shifts were observed for 3-FL were the *Bifidobacterium* relative abundance increased by about 10% after 36h. *B. longum* subsp. *longum* remained stable during the fermentation and *B. bifidum* did not increase. The authors suggested this may indicate that *B. longum* do not ferment 3-FL similar to other findings (Cheng et al. 2020, Salli et al. 2021). Salli et al. (2023) used a 4-stage semi-continuous colon simulator model to study the effects of HiMO including 3-FL on the infant intestinal microbiota. Faecal samples from three infants under four months of age were used as the faecal inoculum for the colon simulated system. The authors concluded that the addition of HiMO increased those species that utilise HiMO, albeit differentially between individual simulations.

4.1.3 Animal studies

Two animal studies which included 3-FL were identified. In both studies very low, or no Bifidobacteria were detected.

Holst et al. (2022) used a mouse model to investigate how HiMO 3-FL (*n*=16; further split into 3-FL and 3-FL- washout groups), LNT (*n*=8), and 6′-SL (*n*=8) affect microbial diversity in in a complex mammalian gut environment. 16 mice were administered drinking water supplemented with or without 50 g/L of 3-FL, a daily intake ≈ 0.25 g/animal. No bifidogenic effect of 3-FL was observed likely due to the very low absence of HMO-adapted infant-type strains in the mouse model. The authors suggested an increased abundance of *Bacteroidaceae* strains was due to a lack of direct competition with bifidobacterial species. No adverse effects were observed, and the mice tolerated the 3-FL supplemented water well. The study demonstrated that while 3-FL might promote growth of other human relevant bacteria in a mouse model, the bifidogenic potential is context-dependent and likely most effective primarily when specialist HMO adapted *Bifidobacterium* species are present.

Pitt et al (2024) in a 21-day safety study of 3-FL in piglets (see 3.2.2.1 for details) reported an absence of *Bifidobacterium* species in the gut microflora. The 3-FL treatment groups were found to have enriched microflora for non-bifidogenic bacteria indicating that other bacteria can metabolise HMOs.

4.1.4 Human studies including 3-FL in combination with other HiMO

Two intervention infant clinical trials (Holst et al., 2023 as well as Picaud et al., 2023 and Picard et al. 2024 – see Table A2-2 for study details) were considered. The interpretation of the studies is confounded by the use of multiple-HiMOs in addition to 3-FL: 5 HiMOs for Holst study and 6 HiMOs for Picaud study with the inclusion of two probiotics, *B. lactis* CNCM I-3446 and B. *infantis* LMG115.

4.2 Anti-pathogenic effects

FSANZ has previously described the anti-infective effect of human milk (FSANZ 2019, FSANZ 2020b). Exclusive breastfeeding for the first 3–6 months significantly reduces gastrointestinal infections in full-term infants, and when followed by continued partial breastfeeding has been associated with a lower risk of gastrointestinal infections in infants. This protective effect may be primarily due to the immune-supportive components found in human milk. These include HMO and glycoproteins such as lactoferrin and mucins, which act as decoy receptors to prevent pathogens from binding to the infant's intestinal lining. Additionally, secretory IgA, the predominant antibody in human milk, binds to pathogens in the gut mucosa, disrupting infection processes without causing significant inflammation.

Coppa et al. (2006) explored the antiadhesive properties of HMO, including 3-FL tested at a concentration of 0.5 g/L, against three diarrhoeal pathogens: *E. coli* O119, *Vibrio cholerae*, and *Salmonella fyris*, using a Caco-2 epithelial colon cancer cell model. The proposed anti-pathogenic mechanism involves competitive inhibition, where 3-FL mimics glycan structures on the host cell surface, binding bacterial adhesins and preventing pathogen attachment to intestinal epithelial receptors. The authors highlighted that multiple HMO likely work together to enhance binding diversity and pathogen blocking potential.

Weichert et al. (2013) tested HiMOs for their inhibitory potential of pathogen adhesion in the human intestinal cancer cell line Caco-2 and the human respiratory cancer cell line A459. Enteric pathogens in this study include enteropathogenic *E. coli*, and *S. fyris* along with primarily respiratory pathogen *Pseudomonas aeruginosa*. The authors suggested the anti-adhesive mechanism of 3-FL is likely facilitated by its structural mimicry of host cell surface glycans and may prevent adhesion by *P. aeruginosa* through interacting with specific lectins (PA-IIL). These findings support the potential of 3-FL having a plausible biological mechanism to bind to pathogens reducing adhesion.

Weichert et al. (2016) analysed the ability of 3-FL to block GII.10 norovirus virus like particles (VLPs) from binding to histo-blood group antigens (HBGAs) needed for viral attachment and infection. VLPs of norovirus GII.10 strain were pre-incubated with 3-FL before being exposed to substrates containing HBGAs: porcine gastric mucin (PGM), A-type human saliva, and B-type human saliva. X-ray crystallography confirmed that 3-FL bound directly to the HBGA-binding pocket on the norovirus capsid. The structural data showed that the fucose part of 3-FL formed a series of conserved hydrogen bonds with residues in the viral P domain, similar to natural HBGA ligands. Although the central and terminal sugars in 3-FL were oriented differently from traditional HBGAs, the fucose occupied the same position. This suggests that 3-FL acts as a molecular mimic, capable of competitively blocking viral adhesion.

4.3 Beneficial effects conclusions

The link between breastfeeding and higher levels of *bifidobacteria* in the infant gastrointestinal tract (GIT) is reasonably well established from numerous observational studies (FSANZ 2019, FSANZ 2020b). The results are variable, but most conclude that the proportion of *Bifidobacterium* and *Lactobacillus* is significantly lower for formula-fed infants. There is broad scientific consensus that a *Bifidobacterium*-enriched microbiota has functional

benefits in the normal growth and development of infants. However, it is recognised that, in most cases, the precise molecular mechanisms underlying such beneficial effects are still to be fully characterised.

The literature has shown consistent evidence of the strain specific ability of *Bifidobacterium* spp. to metabolise 3-FL, however the direct link between levels of 3-FL in human milk and levels of *Bifidobacteria* in the infant gastrointestinal tract is not well established. FSANZ has reviewed evidence for 3-FL's bifidogenic effect but note the assessment is limited by a lack of infant trials solely investigating 3-FL that would provide a larger weight of evidence. However, as for other HiMO, plausible biological mechanisms by which 3-FL can be utilised by beneficial bacteria to influence the composition of the gastrointestinal microbiome have been established. The *in vitro* studies demonstrate credible mechanisms by which 3-FL may contribute to a *Bifidobacterium*-enriched microbiota closer to that observed in breastfed infants when compared to infant formula without 3-FL. Human studies suggest that 3-FL as part of a HiMO blend may play a role in moving the microbiome of infant formula fed infants more closely to that of breastfed infants, however the magnitude of any effect of 3-FL cannot be determined from those studies.

The evidence for the anti-infective effect for specific pathogens is limited and for ethical reasons, human trials do not exist to test if 3-FL inhibits pathogen-binding and subsequent infection rates in infants. Evidence from the *in vitro* studies demonstrate plausible biological mechanisms by which 3-FL can directly and indirectly inhibit pathogens. Although repeated studies on specific pathogens are rare, consistent results were seen for 3-FL ability to bind bacterial pathogens and norovirus virus like particles in *in vitro* studies.

Overall, the weight of evidence supports plausible biological mechanisms and the potential for beneficial effects of 3-FL added to infant formula products through an increase in the abundance of *Bifidobacterium* spp. in the infant gut microbiota, and anti-pathogenic effects. The inclusion of a wider range of HiMO in infant formula products is likely to support the development of a healthy microbiota.

5 Conclusions

Information reviewed in the food technology assessment demonstrates 3-FL is chemically and structurally identical to the naturally occurring forms of these substances in human milk. 3-FL was shown to be stable in infant formula products under the intended conditions of use. Multi-batch analyses showed 3-FL can meet the proposed specifications.

The FSANZ microbiological risk assessment did not identify any public health and safety concerns associated with the use of *E. coli* K-12 as a production organism for 3-FL. Characterisation of the GM production strain confirmed that the introduced gene was genetically stable and functional.

3-FL is naturally present in human milk, providing a history of safe human exposure. The estimated dietary intakes of 3-FL from infant formula products with a proposed maximum permitted amount of 2 g/L are comparable to intakes from naturally occurring 3-FL in human milk.

Post-marketing surveillance data have also found no safety concerns from consumption of infant formula containing 3-FL in combination with up to five other HiMO.

Given the absence of any identifiable hazard in toxicological and clinical studies, and noting that estimated dietary intakes of 3-FL from infant formula products are comparable to intakes from human milk, there are no safety concerns from the addition of 3-FL to infant formula products at the proposed maximum permitted amount.

The weight of evidence supports plausible biological mechanisms and the potential for beneficial effects of 3-FL added to infant formula products through an increase in the abundance of *Bifidobacterium* spp. in the infant gut microbiota, and anti-pathogenic effects. The inclusion of a wider range of HiMO in infant formula products is likely to support the development of a healthy microbiota.

6 References

ACNFP (2024) ACNFP advice on the safety of 3-fucosyllactose (3-FL) as a novel food. Available online at: https://acnfp.food.gov.uk/ACNFPAdviceonthesafetyof3-fucosyllactose(3-FL)asaNovelFood

Bauer AP, Ludwig W, Schleifer K-H (2008) A novel DNA microarray design for accurate and straightforward identification of Escherichia coli safety and laboratory strains. Systematic and Applied Microbiology, 31(1):50-61.
doi:10.1016/j.syapm.2008.01.001

Browning DF, Hobman JL, Busby SJW (2023) Laboratory strains of Escherichia coli K-12: things are seldom what they seem. Microbial Genomics, 9(2).
doi:10.1099/mgen.0.000922

Butte N, Cobb K, Dwyer J, Graney L, Heird W, Rickard K (2004) The Start Healthy Feeding Guidelines for Infants and Toddlers. Journal of the American Dietetic Association, 104(3):442-454.
doi:10.1016/j.jada.2004.01.027

Cheng L, Kiewiet MBG, Logtenberg MJ, Groeneveld A, Nauta A, Schols HA, Walvoort MTC, Harmsen HJM, de Vos P (2020) Effects of Different Human Milk Oligosaccharides on Growth of Bifidobacteria in Monoculture and Co-culture With Faecalibacterium prausnitzii. Frontiers in Microbiology, 11:569700.
doi:10.3389/fmicb.2020.569700

Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, Orazio G (2006) Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. Pediatric Research, 59(3):377-382.
doi:10.1203/01.pdr.0000200805.45593.17

Derya SM, Spiegel H, Hanisch F-G, Morozov V, Schroten H, Jennewein S, Parschat K (2020) Biotechnologically produced fucosylated oligosaccharides inhibit the binding of human noroviruses to their natural receptors. Journal of Biotechnology, 318:31-38.
doi:10.1016/j.jbiotec.2020.05.001

Dewey K (2003). Guiding principles for complementary feeding of the breastfed child. K. Dewey. Washington, D.C, Pan American Health Organization.

Du Z, Li Z, Guang C, Zhu Y, Mu W (2024) Recent advances of 3-fucosyllactose in health effects and production. Arch Microbiol, 206(9):378.
doi:10.1007/s00203-024-04104-2

EFSA (2017) Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age. EFSA Journal, 15(5):e04849.
doi:10.2903/j.efsa.2017.4849

EFSA (2023) Safety of 3-fucosyllactose (3-FL) produced by a derivative strain of Escherichia coli K-12 DH1 as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 21(6):e08026.
doi:10.2903/j.efsa.2023.8026

EMEA (2008) Guidelines on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications. Available online at: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-need-nonclinical-

testing-juvenile-animals-pharmaceuticals-paediatricindications_en.pdf

FDA (2006) Guidance for industry: nonclinical safety evaluation of pediatric drug products. Available online at: https://www.fda.gov/media/119658/download

FDA (2022) GRN No. 1037: 3-fucosyllactose. Available online at: https://www.fda.gov/media/165249/download

FSANZ (2016) AUSNUT 2011-13 Food nutrient database. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/science-data/food-composition-databases/ausnut/ausnut-datafiles

FSANZ (2019) Application A1155 - 2′-FL and LNnT in infant formula and other products. Supporting Document 1 at Second Call for Submissions. Safety, technical and health effects assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155_SD1_Risk%20assessment%20-%202nd%20CFS.pdf

FSANZ (2020a) Review of Application A1155 – 2′-FL and LNnT in infant formula and other products. Supporting Document 1. Review of new toxicology, clinical safety, growth and development studies. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/code/applications/Documents/A1155%20Review%20SD1%20Safety%20and%20growth%20.pdf

FSANZ (2020b) Review of Application A1155 – 2′-FL and LNnT in infant formula and other products. Supporting Document 4. IEAG Summary of conclusions. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155%20Review%20SD4%20IEAG%20Summary%20of%20Conclusions.docx.pdf

FSANZ (2020c) Review of Application A1155 – 2′-FL and LNnT in infant formula and other products. Supporting Document 2. Assessment of beneficial effects. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155%20Review%20SD2%20Beneficial%20effects.pdf

FSANZ (2021) Application A1190 - 2′-FL in infant formula and other products. Supporting Document 1 at Approval. Risk and safety assessment. Available online at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1190_SD1%20at%20Approval.pdf

FSANZ (2022a) Application A1233 - 2′-FL from new GM source for infant formula. Supporting Document 1 at Approval. Food technology and safety assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.govt.nz/sites/default/files/food-standards-code/applications/Documents/A1233%20SD1%20at%20approval.pdf

FSANZ (2022b) Application A1251 - 2′-FL combined with galacto-oligosaccharides and/or insulin-type fructans in infant formula products. Supporting Document 1 – Risk and technical assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1251%20SD1.pdf

FSANZ (2023a) Application A1265 - 2′-FL DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt

for use as nutritive substances in infant formula products. Supporting Document 1 at Approval. Risk assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/2023-11/A1265%20Approval%20Report.pdf

FSANZ (2023b) Application A1277 - 2′-FL from GM Escherichia coli K-12 (gene donor: Helicobacter enhydrae) in infant formula products. Supporting Document 1. Risk and technical assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/2023-11/A1277%20SD1.pdf

FSANZ (2024a) A1283 - 2′-FL from GM Corynebacterium glutamicum in infant formula products. Supporting Document 1. Risk and technical assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/2024-02/A1283%20SD1.pdf

FSANZ (2024b) A1308 - 2′-FL from GM Escherichia coli W in infant formula products. Supporting Document 1. Risk and technical assessment. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/sites/default/files/2024-11/A1308%20SD1_0.pdf

FSANZ (2024c) Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes. Food Standards Australia New Zealand, Canberra. Available online at: https://www.foodstandards.gov.au/publications/principles-and-practices-of-dietary-exposure-assessment

Garrido D, Ruiz-Moyano S, Lemay DG, Sela DA, German JB, Mills DA (2015) Comparative transcriptomics reveals key differences in the response to milk oligosaccharides of infant gut-associated bifidobacteria. Scientific Reports, 5(1):13517. doi:10.1038/srep13517

Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD, Verbeke K, Reid G (2017) The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology & Hepatology, 14(8):491-502. doi:10.1038/nrgastro.2017.75

Gilby B (2021). 3-Fucosyllactose (3-FL): bacterial reverse mutation test. Unpublished, Covance Laboratories Limited.

Guerra S, Lechinski de Paula C, Daza Bolaños CA, Hernandes R, Ribeiro M (2019) Virulence factors of Escherichia coli: an overview of animal and human infections with emphasis in bovine mastitis. Semina: Ciências Agrárias, 40:2087. doi:10.5433/1679-0359.2019v40n5p2087

Hitchcock NE, Gracey M, Gilmour AI, Owles EN, Lifschitz C (1987) Nutrition and Growth in Infancy and Early Childhood. Journal of Pediatric Gastroenterology and Nutrition, 6(5). doi:10.1097/00005176-198709000-00039

Holst AQ, Jois H, Laursen MF, Sommer MOA, Licht TR, Bahl MI (2022) Human milk oligosaccharides induce acute yet reversible compositional changes in the gut microbiota of conventional mice linked to a reduction of butyrate levels. Microlife, 3:uqac006. doi:10.1093/femsml/uqac006

Holst AQ, Myers P, Rodríguez-García P, Hermes GDA, Melsaether C, Baker A, Jensen SR, Parschat K (2023) Infant Formula Supplemented with Five Human Milk Oligosaccharides

Shifts the Fecal Microbiome of Formula-Fed Infants Closer to That of Breastfed Infants. Nutrients, 15(14).
doi:10.3390/nu15143087

Kong C, Akkerman R, Klostermann CE, Beukema M, Oerlemans MMP, Schols HA, de Vos P (2021) Distinct fermentation of human milk oligosaccharides 3-FL and LNT2 and GOS/inulin by infant gut microbiota and impact on adhesion of Lactobacillus plantarum WCFS1 to gut epithelial cells. Food Funct, 12(24):12513-12525.
doi:10.1039/d1fo02563e

Lasekan J, Choe Y, Dvoretskiy S, Devitt A, Zhang S, Mackey A, Wulf K, Buck R, Steele C, Johnson M, Baggs G (2022) Growth and Gastrointestinal Tolerance in Healthy Term Infants Fed Milk-Based Infant Formula Supplemented with Five Human Milk Oligosaccharides (HMOs): A Randomized Multicenter Trial. Nutrients, 14(13).
doi:10.3390/nu14132625

Malih N, Konieczna J, Fernandez MN, Fiol-deRoque MA, Zamanillo-Campos R, Ricci-Cabello I (2024) Preparatory work for the safety evaluation by EFSA of Human-identical Milk Oligosaccharides as Novel Foods. EFSA Supporting Publications, 21(7).
doi:10.2903/sp.efsa.2024.EN-8955

MHLW (2012) Guideline on the nonclinical safety study in juvenile animals for pediatric drugs. Available online at: https://www.pref.kagawa.lg.jp/documents/7476/161_241015.pdf

Mir RA, Kudva IT (2019) Antibiotic-resistant Shiga toxin-producing Escherichia coli: An overview of prevalence and intervention strategies. Zoonoses Public Health, 66(1):1-13.
doi:10.1111/zph.12533

Miranda J, Claris O, Gil Campos M, Salamanca I, Cornette L, Alliet P, Cornette L, Alliet P, Léké A, Castanet M, Piloquet H, de Halleux V, Mitanchez D, Vandenplas Y, Maton P, Jochum F, Olbertz D, Negre S, Lahiry A, Silva Zolezzi I, Hays NP, Sprenger N, Picaud J (2023) Infant formula with a specific age-adapted synbiotic blend of six human milk oligosaccharides and B. infantis LMG11588 plus B. lactis CNCM I-3446 supports healthy, age-appropriate growth and is well-tolerated: a staged analysis of a double-blind, randomized, controlled trial [abstract]. In: Abstracts of the 15th Excellence in Pediatrics; Paris (France); Cogent Public Health, 10(1):2275942.
doi:10.1080/27707571.2023.2275942

Moon S, Lee KW, Park M, Moon J, Park SH, Kim S, Hwang J, Yoon J-W, Jeon S-M, Kim J-S, Jeon Y-J, Kweon D-H (2024) 3-Fucosyllactose-mediated modulation of immune response against virus infection. International Journal of Antimicrobial Agents, 64(1):107187.
doi:10.1016/j.ijantimicag.2024.107187

Parschat K, Melsaether C, Jäpelt KR, Jennewein S (2021) Clinical Evaluation of 16-Week Supplementation with 5HMO-Mix in Healthy-Term Human Infants to Determine Tolerability, Safety, and Effect on Growth. Nutrients, 13(8).
doi:10.3390/nu13082871

Phipps KR, Lozon D, Stannard DR, Gilby B, Baldwin N, Miks MH, Lau A, Rohrig CH (2022) Neonatal subchronic toxicity and in vitro genotoxicity studies of the human-identical milk oligosaccharide 3-fucosyllactose. Journal of Applied Toxicology, 42(10):1671-1687.
doi:10.1002/jat.4335

Picaud JC, Moll JM, Miranda J, Claris O, Gil-Campos M, Salamanca I, Cornette L, Alliet P, Léké A, Castanet M, Piloquet H, de Halleux V, Mitanchez D, Vandenplas Y, Maton P,

Jochum F, Olbertz D, Negre S, Lahiry A, Rodríguez-García P, Silva Zolezzi I, Hays NP, Sprenger N (2023) Gut microbiota and probiotic establishment in healthy term infants fed an infant formula with a specific age-adapted synbiotic blend of six human milk oligosaccharides and B. infantis LMG11588 plus B. lactis CNCM I-3446: a staged analysis of a double-blind, randomized, controlled tria l[abstract]. In: Abstracts of the 15th Excellence in Pediatrics; Paris (France); Cogent Public Health, 10(1):2275942.
doi:10.1080/27707571.2023.2275942

Picaud JC, Moll JM, Miranda J, Claris O, Palomino Fernández L, Salamanca de la Cueva I, et al. (2024). Gut microbiota establishment in infants fed formulas with an age-adapted synbiotic blend of six human milk oligosaccharides, B. infantis LMG11588, and B. lactis CNCM I-3446: a staged analysis of a double-blind, randomized trial [abstract]. In: Abstracts of the 2024 American Academy of Pediatrics National Conference & Exhibition; Orlando, Florida (USA); Available at: https://aapexperience24.eventscribe.net/

Pitt J, Bond J, Roper J, Tenning P, Mukherjea R, Evans K, Saarinen MT, Anglenius H, Hirvonen J, Hasselwander O, Lim A (2024) A 21-day safety evaluation of biotechnologically produced 3-fucosyllactose (3-FL) in neonatal farm piglets to support use in infant formulas. Food and Chemical Toxicology, 187:114592.
doi:10.1016/j.fct.2024.114592

Pitt J, Chan M, Gibson C, Hasselwander O, Lim A, Mukerji P, Mukherjea R, Myhre A, Sarela P, Tenning P, Himmelstein MW, Roper JM (2019) Safety assessment of the biotechnologically produced human-identical milk oligosaccharide 3-Fucosyllactose (3-FL). Food and Chemical Toxicology, 134:110818.
doi:10.1016/j.fct.2019.110818

Riley M, Abe T, Arnaud MB, Berlyn MKB, Blattner FR, Chaudhuri RR, Glasner JD, Horiuchi T, Keseler IM, Kosuge T, Mori H, Perna NT, Plunkett G, III, Rudd KE, Serres MH, Thomas GH, Thomson NR, Wishart D, Wanner BL (2006) Escherichia coli K-12: a cooperatively developed annotation snapshot—2005. Nucleic Acids Research, 34(1):1-9.
doi:10.1093/nar/gkj405

Salli K, Hirvonen J, Anglenius H, Hibberd AA, Ahonen I, Saarinen MT, Maukonen J, Ouwehand AC (2023) The Effect of Human Milk Oligosaccharides and Bifidobacterium longum subspecies infantis Bi-26 on Simulated Infant Gut Microbiome and Metabolites. Microorganisms, 11(6):1553.
doi:10.3390/microorganisms11061553

Salli K, Hirvonen J, Siitonen J, Ahonen I, Anglenius H, Maukonen J (2021) Selective Utilization of the Human Milk Oligosaccharides 2′-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose by Various Probiotic and Pathogenic Bacteria. Journal of Agricultural and Food Chemistry, 69(1):170-182.
doi:10.1021/acs.jafc.0c06041

Soyyılmaz B, Mikš MH, Röhrig CH, Matwiejuk M, Meszaros-Matwiejuk A, Vigsnæs LK (2021) The Mean of Milk: A Review of Human Milk Oligosaccharide Concentrations throughout Lactation. Nutrients, 13(8):2737.
doi:10.3390/nu13082737

Stannard D (2021a). 3-Fucosyllactose (3-FL): 14-day dose range finding study in the neonatal Crl:CD(SD) rat by oral (gavage) administration. Unpublished, Covance Laboratories Limited.

Stannard D (2021b). 3-Fucosyllactose (3-FL): 90-day toxicity study in the neonatal

Crl:CD(SD) rat by oral (gavage) administration followed by a 4-week recovery period. Unpublished, Covance Laboratories Limited.

Urashima T, Taufik E, Fukuda K, Asakuma S (2013) Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Bioscience, Biotechnology, and Biochemistry, 77(3):455-466.
doi:10.1271/bbb.120810

Weichert S, Jennewein S, Hüfner E, Weiss C, Borkowski J, Putze J, Schroten H (2013) Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutrition Research Reviews, 33(10):831-838.
doi:10.1016/j.nutres.2013.07.009

Weichert S, Koromyslova A, Singh BK, Hansman S, Jennewein S, Schroten H, Hansman GS (2016) Structural Basis for Norovirus Inhibition by Human Milk Oligosaccharides. Journal of Virology, 90(9):4843-4848.
doi:10.1128/jvi.03223-15

World Health Organization (1985). Guidelines for the study of dietary intakes of chemical contaminants / text prepared under the Joint FAO/WHO Food Contamination Monitoring Programme, in collaboration with the Joint FAO/WHO Food Standards Programme. Geneva, World Health Organization.

World Health Organization (2006) WHO child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development. Available online at: https://www.who.int/publications/i/item/924154693X

World Health Organization & Food and Agriculture Organization of the United Nations & United Nations University (2004). Human energy requirements: report of a joint FAO/WHO/UNU Expert Consultation, Rome 17-24 October 2001. Rome, Food and Agriculture Organization of the United Nations

Yu W, Li Y, Liu D, Wang Y, Li J, Du Y, Gao GF, Li Z, Xu Y, Wei J (2023) Evaluation and Mechanistic Investigation of Human Milk Oligosaccharide against SARS-CoV-2. Journal of Agricultural and Food Chemistry, 71(43):16102-16113.
doi:10.1021/acs.jafc.3c04275

Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, Heidtman M, Newburg DS (2013a) The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology, 23(2):169-177.
doi:10.1093/glycob/cws138

Yu ZT, Chen C, Newburg DS (2013b) Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. Glycobiology, 23(11):1281-1292.
doi:10.1093/glycob/cwt065

Appendix 1

How were the estimated dietary intakes calculated?

As there are no data available from the 2011-12 National Nutrition and Physical Activity Survey or the New Zealand National Children's Nutrition Survey for children aged less than 2 years, model diets were constructed to estimate dietary 3-FL intake for the target groups of children aged 3 months and 9 months.

As the 3 month and 9 month old infant model diets are based on mean food consumption amounts only, a distribution of food consumption was not available and hence, a distribution of 3-FL dietary intake was not able to be produced. Therefore, the 90th percentile dietary intakes were estimated using the calculation shown in Equation 1.

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

90th percentile exposure = mean exposure x 2*

* (World Health Organization 1985)

The energy contents of infant formula, follow-on formula, SMPPi and human milk are required for the calculation of the amount of infant formula / follow-on formula / SMPPi / human milk in the model diets for 3 month and 9 month old infants. AUSNUT 2011-12 is the latest nutrient data set published for Australian foods. In this dataset, the energy content of *Infant formula, 6-12 months, prepared with water* is 264 kJ/100 g and *Milk, human/breast, mature, fluid* is 286 kJ/100 g (FSANZ 2016). A set of model diets were developed using the AUSNUT energy contents for infant formula / follow-on formula / human milk in the calculation of infant formula, follow-on formula and human milk consumption for 3 month and 9 month old infants.

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. From an examination of a range of products currently on the market, 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, the range of energy contents is 269 – 415 kJ/100 g. 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 3-FL is anticipated to be similar to that outlined in the dietary intake assessment results. 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 3-FL is anticipated to be similar to or lower than that outlined in the dietary intake assessment results.

Infants aged 3 months

The recommended energy intake for a 3month-old boy (343 kJ/kg bw/day) (World Health Organization & Food and Agriculture Organization of the United Nations & United Nations University 2004) and the 50th percentile weight (6.4 kg) (World Health Organization 2006) for the same age and sex were used as the basis for the model diet. Boys' weights were used because boys tend to be heavier than girls at the same age and therefore have higher overall

energy and food requirements. The entire energy requirement in the 3 month old infant diet is derived from infant formula or human milk, depending on the assessment. The body weight of 6.4 kg was used to estimate dietary intakes for 3 month old infants on a body weight basis.

Infants aged 9 months

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) (World Health Organization & Food and Agriculture Organization of the United Nations & United Nations University 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. 1987, Dewey 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.

Appendix 2

Study parameters for human intervention studies.

Three human intervention studies that included 3-FL as an HiMO blend were provided by the applicant (Parschat et al. 2021, Lasekan et al. 2022, Miranda et al. 2023).

The composition of the HiMO blend tested in each study is shown in Table A2-1. Study parameters are summarised in Table A2-2. The applicant is requesting a maximum permitted level of 3-FL in infant formula products of 80 mg/100 kJ (2 g/L).

Table A2-1: ***Comparison of HiMO content in test formulas and the requested permission for 3-FL in g/L***

HiMO	Parschat et al. 2021	Lasekan et al. 2022	Miranda et al. 2023		
			0 to 6 months	6 to 12 months	12 to 15 months
3-FL	**0.75**	**0.8**	**0.24**	**0.26**	**0.29**
2′-FL	2.99	3	0.87	0.26	0.21
DFL	-	-	0.12	0.04	0.03
LNT	1.5	1.5	0.29	0.15	0.08
6′-SL	0.28	0.3	0.15	0.05	0.04
3′-SL	0.23	0.2	0.11	0.11	0.11
Total	5.75	5.8	1.78	0.87	0.76

3-fucosyllactose (3-FL), 2′-fucosyllactose (2′-FL), difucosyllactose (DFL), lacto-N-tetraose (LNT), 6′-sialyllactose (6′-SL), 3′-sialyllactose (3′-SL)

Table A2-2: ***Summary of human intervention trials***

Study design	IF composition	Study population and allocation	Outcomes	Results
Lasekan et al. (2021) United States (multisite)				
Randomised, double-blind, controlled parallel trial. Randomisation method: computer-generated using a dynamic minimization algorithm with a random component. Randomisation was stratified by sex. The study was registered at clinicaltrials.gov (# NCT04105686). Conflict of interest: study was funded by Abbott Nutrition and all authors are current employees of the study sponsor.	Control formula (CF): standard commercial milk-based formula that met US FDA requirements Experimental formula (EF): CF with HiMO mixture. See Table A2-1 for composition HiMO source: not provided	Healthy full term infants ≤ 14 days fed CFor EF from enrolment to 119 days of age (approx. 4 months). Enrolled: 366 HiMO formula: 130 non-HiMO formula: 129 Human milk reference group (non-randomised): 104 Completed at 4 months: 222 HiMO formula: 76 non-HiMO formula: 78 Human milk reference group (non-randomised): 85 The predominant reason for exit was lost to follow-up with 10.3%, 12.5%, and 2.9% in the CF, EF and human milk-fed group, respectively. In response to the COVID-19 pandemic, the protocol was amended to allow for alternative anthropometric measurements and extend the study visit window	***Growth:**** Primary outcome: weight gain per day (D) from D14 to D119. Secondary outcomes were weight gain per day, length gain per day, head circumference (HC) gain per day. Mean weight for age, mean length for age and mean HC for age were plotted on the WHO growth charts. ***Safety/Tolerance:*** Records collected by parents: spit up and vomit with feeding, stool characteristics (frequency, consistency, colour), infant feeding and stool pattern questionnaires, infant behaviour questionnaires, formula satisfaction questionnaires. Physician confirmed non-serious and serious adverse effects. ***Microbiology:*** Not investigated	No significant differences were observed among the three groups for weight gain per day from 14 to 119 days (D) of age (all $p \geq 0.337$). There was no significant difference in weight gain per day from D14 to D119 between EF and CF and the group fed CF (EF: 29.4 ± 0.7 g/day and CF: 29.9 ± 0.7 g/day, p = 0.348). The sensitivity analysis confirmed the result of non-inferiority of EF relative to CF (using a margin of 3 g/day). There was no difference among the three groups for weight gain per day at D14-D28, D28-D42, D42-D56 and D56-D84, with one exception: males in the EF had significantly greater weight gain than males in the HM group at D84-D119. There were also no statistically significant differences between all three groups on secondary growth outcome measures (head circumference and length gain per day).
Parschat et al. (2021) and Holst et al. 2023 Multicentre (Germany, Italy and Spain)				
Randomised, double-blind, controlled, multi-centre, parallel nutritional noninferiority study undertaken in Germany, Italy and Spain	Control formula: "Basic infant formula" manufactured by Töpfer (Dietmannsried) to meet EU regulations	Healthy full term infants ≤ 14 days fed control or test formulas from enrolment to 112 days. Voluntary 8-week follow-up period.	***Growth*:*** Primary outcome: weight gain per day over 4 month period (to 112 days). Secondary outcomes included changes in weight, length, and HC, their increments, and their	Mean weights at 4 months of 6578 ± 697.6 g for the HiMO-group versus 6557.3 ± 672.8 g for the non-HiMO group. The primary endpoint noninferiority of the 5HiMO-mix was observed in the full analysis set

Study design	IF composition	Study population and allocation	Outcomes	Results
Randomisation method: a randomisation list was generated by an independent statistician in a 1:1 ratio in blocks of four and stratified by sex and site. The study was registered at clinicaltrials.gov (# NCT03513744). Conflict of interest: study was funded by Chr. Hansen HiMO GmbH, Rheinbreitbach, Germany and all authors are employees of Chr. Hansen or the former affiliation Jennewein Biotechnologie.	EF: CF with HiMO mixture (partial replacement of carbohydrate in control formula). See Table A2-1 for composition HiMO source: The 5HiMO-mix was produced by Chr. Hansen HMO GmbH, Rheinbreitbach, Germany.	Enrolled: 341 HiMO formula: 113 non-HMO formula: 112 Human milk reference group (non-randomised): 116 Completed at 4 months: 265 HiMO formula: 97 non-HiMO formula: 101 Human milk reference group (non-randomised): 102	respective WHO growth standard z-scores (weight-for-age, length-for-age, HC-for-age, and weight-for-length). ***Safety/Tolerance:*** Records collected by parents: stool frequency and consistency, digestive tolerance (regurgitation, vomiting, flatulence), behaviour (fussiness, crying, awakening at night). Adverse events: any medical occurrence during the intervention period; concomitant medication, medical treatment or healthcare utilisation were recorded. ***Microbiology:*** Faecal microbiological composition, Bifidobacteria abundance	(FAS) ($p < 0.001$) and the per protocol (PP) ($p < 0.001$) versus IF. There were no significant differences in mean weight, length, or head circumferences between the two intervention groups during the study (data for length and head circumference not shown). No significant differences in weight-for-age z-scores were observed between the 5HiMO-mix and IF groups at any time point between day 14 and 112 in the FAS.
Miranda et al. 2023, Picaud et al. 2023 & Picaud et al. 2024 (unpublished) Multicentre (Belgium, Spain, France, Germany)				
Double-blind, randomised, controlled multi-centre intervention study. Fifteen month intervention period. Randomisation method: infants exclusively consuming and tolerating cow's milk infant formula were randomised to the CF or EF groups. A non-randomised parallel reference group of exclusively breastfed (BF) infants was also enrolled. The study was registered at clinicaltrials.gov (#NCT04962594). Additional study details were provided in the Application were commercial confidential information	CF: Partially-hydrolysed bovine whey protein-based infant formula, follow on formula and growing up milk. EFs were supplemented with the 6 manufactured HiMO (**3-FL**, 2′-FL, DFL, LNT, 3′-SL, and 6′-SL) and *B. infantis* LMG11588 plus *B. lactis* CNCM I-3446 probiotics. See Table A2-1 for HiMO composition	Healthy term infants aged < 14 days. Enrolled: 318 HiMO formula: 119 Non-HiMO formula: 117 Human milk reference group (non-randomised): 82 Completed at 4 months: 313 HiMO formula: 118 non-HiMO formula: 114 Human milk reference group (non-randomised): 81	***Growth:*** Primary outcome: weight gain (g/day) from enrolment to 4 months of age. Secondary outcome to demonstrate the Bifidogenic effect of a combination of 6 HiMO and 2 probiotics. Additional endpoints evaluated at different stages of feeding included weight-for-age, length-for-age, HC for-age, BMI-for-age and weight-for-length z score, stooling patterns. ***Safety/Tolerance:*** Records collected by parents: gastrointestinal tolerance, symptoms and behaviour. Adverse events and medication use. ***Microbiology:*** *Bifidobacterium* abundance, faecal pH, faecal metabolic profile, faecal markers of immune and gut health, and blood markers of immune health.	Weight gain at four months was non-inferior in EF vs CF in both the per PP and FAS. At all measured timepoints, similar weight-for-age, length-for-age, head circumference-for-age, BMI-for-age and weight-for-length z scores were observed, with the majority of infants falling within the normal range compared to WHO median z-scores. Further details cannot be provided as they are confidential commercial information.