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

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163-21

Supporting document 1

Risk and safety assessment – Application A1190

2′-FL in infant formula and other products

Executive summary

Genetically modified (GM) *Escherichia coli* BL21 strains have been developed for the production of the oligosaccharide 2′-fucosyllactose (2′-FL) by fermentation. 2′-FL is one of the oligosaccharides that can be found in human milk, for the majority of women. Limited evidence suggests 2′-FL may be a bioactive compound with functional benefits in infants. The applicant has requested this oligosaccharide be added to infant formula products (IFP), which include: infant formula, follow-on formula, and infant formula products for special dietary use; and formulated supplementary foods for young children (FSFYC), with the aim of better reflecting the oligosaccharide composition of human milk. The maximum amount of 2′-FL requested to be used in these products is 2 g/L. The maximum permitted level in the Australia New Zealand Food Standards Code (the Code) is 96 mg/100 kJ, which equates to 2.4 g/L. As 2′-FL is already permitted in the Code, the purpose of this assessment is to determine the risk and safety of 2′-FL, produced by a new production strain, with its own specifications.

In conducting the assessment of microbial-sourced 2′-FL, FSANZ addressed questions of the safety and any risks posed by the 2′-FL product. The assessments included:

- a dietary intake assessment, analysing the intake levels from the proposed products and comparison to levels found in human milk.

- a review of the manufacturing process, including a safety assessment of the *E. coli* source organisms.

- a toxicology assessment to identify any potential hazards in the product.

- a benefits assessment, covering nutrition and health benefits.

A literature review undertaken as part of the dietary intake assessment determined the level being requested is within the normal range of 2′-FL reported in human milk (0.6–7.8 g/L), produced by the majority of women. Evidence suggest 70–80% women have the ability to make 2′-FL. The estimated dietary intakes of 2′-FL based on a concentration of 2.4 g/L for infants up to 12 months ranged between 0.1–0.33 g/kg bw/day at the mean and 0.2–0.66 g/kg bw/day at the 90th percentile, and for children 2–3 years from 0.077–0.15 g/kg bw/day at the mean and 0.15–0.31 g/kg bw/day at the 90th percentile.

2′-FL sourced from microbial fermentation was shown to be chemically and structurally identical to the naturally occurring oligosaccharide in human milk. The final product was shown to be free of fermentation-derived contaminants. The purity and other constituents of the final product have been identified and listed in the specification for the product. The shelf-life and specifications are appropriate for addition to IFP and FSFYC.

The examination of the source organisms concluded there were no safety concerns. The host organism is a well characterised bacterium with a recognised safe history of use for the production of industrial compounds and human therapeutics. It is neither pathogenic nor toxigenic. The production strains were confirmed to contain the inserted DNA, which was shown to be inherited across several generations.

FSANZ has previously determined that there are no safety concerns associated with the addition of 2′-FL to IFP and FSFYC at concentrations up to 2.4 g/L. A number of new studies evaluated as a part of this application did not indicate a reason to change this conclusion.

2′-FL was not genotoxic *in vitro* or *in vivo*. No adverse effects were observed in multiple subchronic oral toxicity studies in neonatal rats at doses up to 5000 mg/kg bw/day, or in older rats at doses > 7000 mg/kg bw/day. Three-week studies with neonatal piglets administered formula containing 2′-FL at concentrations up to 4 g/L also found no adverse effects. In human studies, infant formula supplemented with 2′-FL was well tolerated with no significant increases in adverse events. 2′-FL was also well tolerated in studies with children and adults.

Protein was not detected in the 2′-FL product, therefore 2′-FL is unlikely to pose an allergenicity concern.

The nutritional assessment concluded the addition of 2′-FL to IFP is not expected to affect the growth profiles of infants. Combined with the limited gastrointestinal absorption of 2′-FL, there is no evidence to indicate a nutritional concern at concentrations that are typically observed in human milk.

Assessment of the proposed beneficial role of 2′-FL concluded there was limited but sufficient evidence to support a bifidogenic effect. 2′-FL dietary supplementation results in increased relative abundance of bifodobacteria in the gastrointestinal microbiome. This observation is supported by *in vitro* studies demonstrating preferential utilisation of 2′-FL by specific strains of bifidobacteria, providing a selective growth advantage over other commensal and pathogenic bacteria. A feeding study performed in adults showed that this effect is not restricted to infants.

Another proposed beneficial role of 2′-FL was the prevention of infection by pathogenic strains of *Campylobacter jejuni*. The mechanism of action by which 2′-FL can prevent infection and illness caused by invasive strains of *Campylobacter jejuni* was established *in vitro*. It was shown that 2′-FL acted as an inhibitor of the attachment of *C. jejuni* to the intestinal H2 antigen binding receptor. The plausibility of this anti-infective health effect occurring in infants was demonstrated in mice supplemented with 2′-FL and challenged with invasive *C. jejuni*. Animals showed decreased intestinal infection, invasiveness and disease severity. A human study showing decreased incidence of *Campylobacter*-associated diarrhoea in infants and young children of mothers with a higher proportion of 2′-FL in their milk, provides additional epidemiological evidence of 2′-FL having an anti-infective health effect.

2′-FL is naturally present in human milk in a range of concentrations, providing a history of safe human exposure. FSANZ concludes there are no safety concerns associated with the addition of 2′-FL to IFP and FSFYC at the requested level of 2 g/L, or at higher estimated dietary intakes based on the permitted level in the Code (2.4 g/L).

Glossary of terms

2′-FL	2′-fucosyllactose also known as 2′-O-fucosyllactose
2′-FL$_{chem}$	2′-fucosyllactose produced by chemical synthesis
2′-FL$_{micro}$	2′-fucosyllactose produced by microbial fermentation
2′-FL$_{human}$	2′-fucosyllactose naturally occurring in human milk
BMI	body mass index
bw	body weight
DFL	difucosyllactose
FOS	fructooligosaccharide
FSFYC	formulated supplementary foods for young children (or 'toddler milk')
GLP	good laboratory practice
g	gram
GM	genetically modified
GOS	galactooligosaccharide
IFP	infant formula products
kg	kilogram
L	litre
LNnT	lacto-N-neotetraose
LOD	limit of detection
mg	milligram
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
scFOS	Short-chain fructooligosaccharide

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1 Introduction

FSANZ has received an application from Jennewein Biotechnologie GmbH[1] to amend the Australia New Zealand Food Standards Code (the Code) to include an oligosaccharide found in human milk[2] known as 2′-fucosyllactose (2′-FL), derived from a genetically modified (GM) *Escherichia coli* BL21. The applicant has requested the addition of 2′-FL to infant formula products (IFP), which include: infant formula, follow-on formula, and infant formula products for special dietary use; and formulated supplementary foods for young children (FSFYC or 'toddler milk'). While the applicant has requested a maximum of 2 g/L 2′-FL to be used in these products, the maximum level permitted in the Code is 96 mg/100 kJ, which is equivalent to 2.4 g/L. As 2′-FL is already permitted in the Code, the purpose of this assessment is to determine the risk and safety of 2′-FL, produced by a new production strain, with its own specifications.

The stated purpose for adding 2′-FL to IFP and FSFYC is to better reflect the oligosaccharide composition of human milk. The applicant states that 2′-FL confers functional benefits to infants and toddlers.

The primary risk assessment question to be addressed is whether addition of 2′-FL would pose a public health and safety risk to the target population when added to IFP or FSFYC.

2 Food technology assessment

The food technology assessment provides information on the chemical identification, physicochemical properties and specifications for 2′-FL. The assessment primarily aimed to address whether the microbiologically-synthesised 2′-FL is chemically and structurally identical to the naturally occurring oligosaccharide present in human milk. The assessment also considered the manufacturing process and the validity of analytical methods used to characterise 2′-FL during production and as a component of IFP and FSFYC.

2.1 Chemical properties

2.1.1 Chemical names, properties, and structures

2′-FL is a naturally occurring oligosaccharide found in human milk. For research and commercial purposes, it has been isolated from human milk or produced using chemical synthesis or microbial fermentation. This application relates specifically to the production and isolation of 2′-FL by microbial fermentation. Throughout this assessment, the differently produced forms of 2′-FL will be identified using the following subscripts:

- 2′-FL$_{human}$ - 2′-FL in human milk
- 2′-FL$_{chem}$ - 2′-FL produced by chemical synthesis

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[1] After receipt of this application from Jennewein Biotechnologie, Jennewein was acquired by Chr. Hansen Holding A/S. The applicant is now Chr. Hansen.

[2] Human milk will be the term used to indicate breastmilk

- 2′-FL$_{micro}$ - 2′-FL produced by microbial fermentation.

The nomenclature and chemical properties for 2′-FL are listed in Table 2.1.

Table 2.1: *Nomenclature and chemical properties of 2'-FL*

Property	2′-FL
Chemical name	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Common name	2′-*O*-fucocsyllactose 2′-fucocsyllactose 2′-fucosyl-D-lactose
Alternative names[1]	α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc Fuc-α-1,2-Gal-β-1,4-Glc 2′-FL
CAS registry number	41263-94-9
Chemical formula	$C_{18}H_{32}O_{15}$
Molecular mass	488.44 g / mol

[1] *Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose*
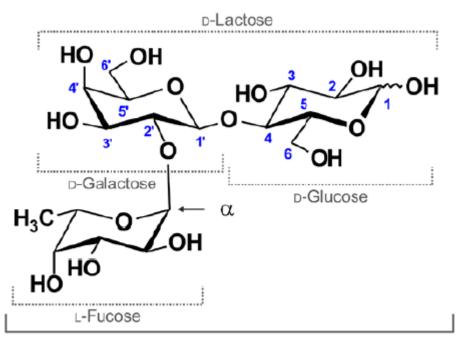
The applicant's 2′-FL$_{micro}$ is available both as a powder and liquid concentrate and will be marketed as an ingredient to be added to IFP and FSFYC. The general physical properties for the powder and concentrate are listed in Table 2.2.

Table 2.2: *Physical properties of 2'-FL*

Property	Powder	Concentrate
Solubility[1] in water	min. 500 g/L (25ºC)	N/A
Stability (25ºC/ 65% humidity)	2 years	6 months
Form	Fine, hygroscopic spray-dried powder	Liquid; clear solution
Colour	White to ivory	Clear, colourless to slightly yellow
Minimum purity	90%	90%

[1] *For comparison, the solubility of lactose = 195 g/L.*

2′-FL is the most abundant oligosaccharide in human milk and has a simple structure. It is a fucosylated, neutral trisaccharide consisting of the monosaccharides L-fucose, D-galactose, and D-glucose (Figure 2.1). It can also be described as the disaccharide D-lactose at the reducing end and the monosaccharide L-fucose at the terminal galactose in alpha (1→2) linkage to form the trisaccharide.

Figure 2.1 *Molecular Structure of 2'-FL (Image from: EFSA NDA Panel 2015[3])*

2.1.2 Structural identification

A range of methods were used to confirm the identity of 2′-FL$_{micro}$ and determine its structural and chemical equivalence to a commercially available 2′-FL$_{human}$ reference. Methods included nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography and mass spectrometry (LC-MS/MS), multiple reaction monitoring (MRM) mass spectrometry, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) and optical rotation. These methods are all accepted techniques for identifying and characterising oligosaccharides (Ruhaak and Lebrilla 2012; Tonon et al. 2019a; Xu et al. 2017). The applicant has provided sufficient explanation of how these methods were used.

The analyses performed on several batches of 2′-FL$_{micro}$ confirmed its identity and it's structural and chemical equivalence to 2′-FL$_{human}$. The data also showed 2′-FL$_{micro}$ was the main constituent of the final product, with a purity of 90%.

Importance of confirming structural equivalence of 2'-FL based on source

2′-FL$_{chem}$ was used in studies submitted by the applicant as supporting evidence for the safety and health benefits of 2'-FL. Structural data demonstrating the equivalence of the applicants 2′-FL$_{micro}$ and 2′-FL$_{chem}$ was not provided. FSANZ has previously reviewed

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[3] efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2015.4184

structural analyses for 2′-FL derived from all three sources and confirmed that 2′-FL$_{chem}$ is chemically and structurally identical to the naturally occurring substances in human milk and 2′-FL$_{micro}$ (FSANZ 2019).

2.2 Analytical methods for detection

There are no internationally recognised methods for the analysis of 2′-FL. However there is a large body of information in the scientific literature on the separation, quantitation and characterisation of milk oligosaccharides (Grabarics et al. 2017). Appropriate analytical detection methods were developed by the applicant and these were provided to FSANZ. The information provided included:

- detailed methods to determine carbohydrate composition using LC-MS/MS, high performance liquid chromatography (HPLC) and HPAEC-PAD. These methods are well established techniques for the analysis of oligosaccharides with sensitivity to ensure that degradation products and contaminants can be detected

- details including reagents, reference materials and standards, solution preparation, run parameters and procedures, evaluation, calculations, and typical chromatograms

- description of analytical procedures to assay for carbohydrate impurities listed in the specification (see Section 2.3.2)

- quantification of 2′-FL in infant formula (conducted by a 3rd party laboratory).

Batch analyses were conducted by the applicant or by an accredited analytical laboratory. The analyses demonstrated that 2′-FL$_{micro}$ can be manufactured and assayed to a purity that would be consistent with the specifications proposed by the applicant (see Section 2.4).

2.3 Manufacturing processes

2.3.1 Fermentation and purification

2′-FL$_{micro}$ is manufactured via batch fermentation, using glycerol as the primary carbon source. The production organism is a GM strain of *E. coli* BL21(DE3) (See Section 3). Microbial fermentation is a well established method for the production of food and food grade ingredients.

After the fermentation process is complete, bacterial cells are separated from the medium and the 2′-FL is purified from the fermentation medium using a variety of purification steps involving sequential filtration and chromatographic procedures (Figure 2.2).

The applicant provided full details of their manufacturing and purification processes, including identification of the raw materials and processing aids, the production organisms, methodology for the purification and isolation steps, and quality controls. Detailed information cannot be specified in this assessment as it is considered confidential commercial information (CCI).
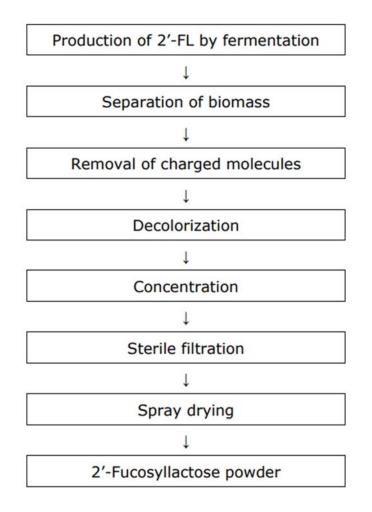
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┌─────────────────────────────────────────┐
│   Production of 2'-FL by fermentation    │
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│          Separation of biomass           │
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│        Removal of charged molecules       │
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┌─────────────────────────────────────────┐
│         2'-Fucosyllactose powder         │
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Figure 2.2 *Purification process for 2'-FL$_{micro}$*

2.3.2 Fermentation-derived contaminants

The final 2′-FL$_{micro}$ product was analysed to determine if there were any fermentation-related contaminants. This included bacterial DNA, protein and microbial toxins, such as *E. coli*-derived endotoxin and fungal-derived aflatoxin, which could indicate contamination of the fermentation system. Bacterial DNA was measured using a quantitative PCR method with an LOD < 9 x 10^5 ng/µl DNA. Protein was analysed using a quantitative modified Bradford assay, with an LOD = 100 µg/g. The microbial toxins endotoxin and aflatoxin were measured using internationally recognised methods. The results from these analyses showed that there was no bacterial DNA, protein or microbial toxins in the final product.

During the fermentation process, it is expected that other carbohydrates will be produced or will remain in the fermentation medium. The presence of these other carbohydrates was determined by chromatographic and mass spectrometry techniques. These analyses identified a mixture of carbohydrates including: 3′-fucosyllactose, difucosyllactose, lactose, fucose and galactose. These are naturally occurring carbohydrates found in human milk (Asakuma et al. 2008; Thurl et al. 1996). Fucosyl-galactose, a naturally occurring breakdown product of 2′-FL was also detected and quantified. Results from multiple batch analyses of the final isolated 2′-FL$_{micro}$ demonstrate that amounts of these carbohydrates were consistently within the specifications proposed by the applicant.

2.3.3 Stability

Stability studies conducted on at least two batches of 2′-FL$_{micro}$ powder and liquid concentrate demonstrated a shelf life of two years or six months respectively, when stored under standard conditions (25°C/60% humidity). Additional studies performed on powder demonstrated that under accelerated storage conditions (40°C/75% humidity), 2′-FL$_{micro}$ is stable for at least six months.

2′-FL$_{micro}$ will be added to IFP and FSFYC either via wet blending with subsequent spray drying or dry-blending, where the 2′-FL$_{micro}$ is added to other previously spray-dried ingredients.

2′-FL$_{micro}$ is stable when incorporated into IFP and the reconstitution process of dry IFP has no significant effect on 2′-FL levels. An analysis of IFP containing 2′-FL$_{micro}$ powder was submitted by the applicant as confidential commercial information. The data demonstrated that 2′-FL concentrations remained stable for up to 12 months in liquid formula and show no signs of degradation at 24 months in powdered formula, when stored under ambient conditions. In additional stability studies, 2′-FL$_{micro}$ was demonstrated to be stable in three representative batches of powdered IFP for at least 60 minutes after reconstitution.

2.4 Product specifications

The applicant has established a specification for 2′-FL$_{micro}$. A relevant specification for 2′-FL is listed in Schedule 3 under section S3—2(2) of the Code (gazetted 25 March 2021 A1155; FSANZ 2019).The applicant's specification prescribes maximum amounts for carbohydrate by-products (see Section 2.3.2) and impurities related to the microorganisms used in the fermentation process that differ from those listed in Schedule 3 under section S3—2(2). The chemical, physical and microbiological specifications are presented in Table 2.3.

Submitted certificates of analysis for several batches of 2′-FL$_{micro}$ demonstrated that both the powdered product and liquid concentrate meet the specifications proposed by the applicant. Results were consistent across all batches tested.

Where there is no relevant specification under section S3—2 or S3—3, there are additional requirements for a substance under section S3—4 relating to heavy metals. The certificates of analysis demonstrate that 2′-FL$_{micro}$ meets S3—4 requirements for lead, arsenic, cadmium and mercury.

Table 2.3 *Product specifications proposed for 2′-FL*

| Parameter | Specification | | Method |
	Powder	Liquid concentrate	
Chemical			
Solids Content	N/A	45% w/v (± 5% w/v) dry matter in water	Dry weight after freeze-drying
Water content	≤ 9.0%	N/A	Karl-Fischer titration
Protein content	≤ 100 µg/g	≤ 100 µg/g FDM	Nanoquant (modified Bradford)
Total ash	≤ 0.5%	≤ 0.5% FDM	ASU L 06.00-4 (a)

	Specification		
Parameter	**Powder**	**Liquid concentrate**	**Method**
2'-Fucosyllactose	≥ 90% (Area)	≥ 90% (Area)	HPAEC-PAD
Lactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
3-Fucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Difucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Fucosyl-Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Glucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Fucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Heavy Metals			
Lead	≤ 0.02 mg/kg	≤ 0.02 mg/kg FDM	ASU L 00.00-19/3 (a)
Arsenic	≤ 0.2 mg/kg	≤ 0.2 mg/kg FDM	ASU L 12.00-06 (a)
Cadmium	≤ 0.1 mg/kg	≤ 0.1 mg/kg FDM	ASU L 00.00-19/3 (a)
Mercury	≤ 0.5 mg/kg	≤ 0.5 mg/kg FDM	ASU 00.00-19/4 (a)
Microbiological Parameters			
Standard Plate Count	≤ 10000 cfu/g	≤ 5000 cfu/g	ISO 4833-2
Coliform / Enterobacteriaceae	Absent in 11 g	absent in 22 ml	ISO 4832 / ISO 21528-2
Cronobacter sakazakii	Absent in 100 g	absent in 200 ml	ISO/TS 22964
Salmonella	Absent in 100 g	absent in 200 ml	ISO 6579
Endotoxins	≤ 10 EU/mg	≤ 10 EU/mg	Ph. Eur. 2.6.14
Aflatoxin M1	≤ 0.025 µg/kg	≤ 0.025 µg/kg	DIN EN ISO 14501
GMO detection	Negative	Negative	qPCR

Abbreviations: ASU = Official collection of determination methods according to § 64 LFGB; cfu = colony-forming units; DIN EN ISO 14501 = German Institute for Standardization Milk and milk powder - Determination of aflatoxin M1 content - Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography; FDM – Freeze-dried matter; HPAEC-PAD = Highperformance anion-exchange chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; ISO/TS = International Organization for Standardization Technical Specifications; LFGB = German Code on food and feed; N/A = Not Available; Ph. Eur = European Pharmacopoeia; qPCR = quantitative polymerase chain reaction.

2.5 Key findings of the food technology assessment

2′-FL$_{micro}$ subject to this application is chemically and structurally identical to naturally occurring 2′-FL$_{human}$. The final product was shown to be free of fermentation-derived contaminants. 2′-FL$_{micro}$ is stable for up to 104 weeks, which is within the expected shelf life for IFP and FSFYC. The applicant has proposed specifications to be included in Schedule 3 for the use of 2′-FL$_{micro}$ in IFP and no safety concerns were identified in the production process and characterisation of 2-FL$_{micro}$.

3 Safety assessment of the genetically modified production strain

The objectives of this safety assessment are to identify and evaluate any potential safety concerns that may arise from the use of a GM production system, generated for the large scale production of 2'-FL by fermentation. Specifically the safety assessment focuses on the:

- history of use of the source organisms, and
- characterisation of the genetic modification(s).

3.1 History of use

3.1.1 Host organism

Escherichia coli BL21(DE3) is a common strain used for recombinant protein production. BL21 is derived from the well characterised *E. coli* B strain, first used in the bacteriophage studies by Luria and Delbrück in the 1940s (Delbrück and Luria 1942; Daegelen et al. 2009). The DE3 substrain has specifically been engineered to allow expression of recombinant proteins under the control of a T7 promoter (Studier and Moffatt 1986).

BL21(DE3) is a well characterised strain, with a fully published genomic sequence and is devoid of extrachromosomal DNA such as plasmids (Daegelen et al. 2009; Jeong et al. 2009; Jeong et al. 2015; Studier et al. 2009). This strain does not cause disease in immunocompromised individuals nor poses a hazard to humans or the environment[4]. The host strain was purchased from a commercial supplier and whole genome sequence analyses confirmed taxonomy of the BL21(DE3) strain.

3.1.2 Gene donor organism(s)

The α-1,2-fucosyltransferase gene was sourced from *E. coli* O126 (Engels and Elling, 2014). This organism is a diarrhoeagenic agent belonging to the enteropathogenic *E. coli* O serogroup (Jensen et al. 2014), associated with human disease, where the disease is rarely serious and there is availability of preventative or therapeutic interventions[3]. The gene sequence used in this process does not pose a hazard to humans or the environment.

3.1.3 Other genetic material donors

Genetic material was also sourced from a range of other organisms, to mediate genetic modifications to support the production of 2'-FL. The applicant provided a full description of these organisms[5]. No risks were identified in the assessment of the gene donors.

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[4] Biosafety in Microbiological and Biomedical Laboratories (BMBL) www.cdc.gov/labs/BMBL.html

[5] Due to the requirements of confidential commercial information, specific information cannot be provided.

3.2 Characterisation of the genetic modification(s)

3.2.1 Preparation of the 2′-FL production system

The 2′-FL being assessed in this application is produced via fermentation using a production system that includes multiple production strains[5], that can be used separately or in combination.

To achieve the genetic modifications used to make the production strains, several expression cassettes were generated containing the genes of interest flanked by well characterised promoter and terminator sequences[5]. Selection of positive transformants was achieved using genes encoding standard selectable markers, co-localised on the expression cassettes or co-transformed. Transformation of expression cassettes was performed by electroporation.

3.2.2 Characterisation of the genetically modified organisms

Characterisation of introduced DNA

Data was provided from genomic DNA sequence analyses. This data enabled identification of both the insertion sites and the determination of insert copy numbers. No safety concerns were identified.

Genetic stability and inheritance of the introduced DNA

The applicant provided data from a genotypic analysis, showing no changes in the gene sequences for six introduced genes, over 100+ generations. At the phenotypic level, production of 2′-FL was compared from clones cultured for 100+ generations to an original isolate. The expressed levels were consistent for all samples. The data confirms the inheritance and genetic stability of the introduced DNA.

3.3 Key findings of the GM assessment

The *E. coli* BL21(DE3) host organism has a long history of use for the production of recombinant proteins and poses no risks to humans. Analyses of the gene donors also confirmed there were no safety concerns.

Characterisation of the production strains confirmed integration of the introduced genes into the genome and that the inserted DNA was both genetically stable and functional.

On the basis of the data provided in the present application, and other available information, no potential safety concerns were identified in the assessment of the 2′-FL production strains.

4 Toxicology assessment

4.1 Previous FSANZ safety assessments of 2′-FL

FSANZ has previously assessed an extensive toxicological database on 2′-FL as part of application A1155 (FSANZ 2019 and A1155 Review Supporting Document 1[6]).

In addition to data derived using Glycom's 2′-FL$_{micro}$, publicly available studies using Glycom's 2′-FL$_{chem}$, Chr. Hansen's 2′-FL$_{micro}$ and Friesland Campina's 2′-FL$_{micro}$ were also evaluated. As noted in Section 2, evidence has been provided demonstrating that 2′-FL$_{micro}$ subject to this application is chemically and structurally identical to naturally occurring 2′-FL$_{human}$. Similarly, the assessment of 2′-FL$_{micro}$ produced by Glycom confirmed its chemical and structural identity to 2′-FL$_{human}$ (FSANZ 2019). The substances produced by other manufacturers are also reported to be identical to that in human milk. Studies with all these forms of 2′-FL are therefore considered to be relevant to the evaluation.

Studies evaluated included:

- Information on the absorption, distribution, metabolism and excretion of oligosaccharides found in human milk, including 2′-FL
- In vitro genotoxicity studies of 2′-FL plus an in vivo micronucleus assay
- In vitro genotoxicity studies of 2′-FL in combination with difucosyllactose (DFL) or with four other oligosaccharides found in human milk
- Short-term toxicity studies of 2′-FL in rats, including studies with neonatal rats
- A short-term toxicity study of 2′-FL in neonatal piglets
- A short-term toxicity study of 2′-FL in combination with DFL in neonatal rats
- A short-term toxicity rodent study of 2′-FL in combination with four other oligosaccharides found in human milk
- Human clinical studies in infants, children and adults.

The assessment concluded that there are no safety concerns associated with the addition of 2′-FL to IFP or FSFYC.

4.2 Newly available data

The applicant provided new studies as part of their submission:

- an *in vitro* micronucleus assay with 2′-FL$_{micro}$,
- the full report of a toxicity study in neonatal piglets (only a published version was previously available), and
- human clinical studies of tolerance and hypoallergenicity.

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[6]
www.foodstandards.gov.au/code/applications/Documents/A1155%20Review%20SD1%20Safety%20and%20growth%20.pdf

Additional studies identified by FSANZ in a literature search conducted in PubMed on 29th April 2021 using the search term '2′-fucosyllactose' have also been reviewed.

4.2.1 Toxicological studies with 2′-FL

Short-term toxicity studies conducted with the applicant's 2'-FL$_{micro}$

Three-week oral toxicity study with 2'-FL$_{micro}$ in neonatal piglets (Hanlon and Thorsrud 2014) Regulatory status: GLP; conducted in compliance with ICH M3(R2) (2010) and the European Medicines Agency (EMEA) guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications (2008)

The test article used in this study had a purity of 97.9% 2′-FL. A total of 27 male and 21 female two-day old Domestic Yorkshire Crossbred Swine were received by the test facility. Due to the imbalance in the numbers of male and female piglets available at the start of the study, animals were assigned to treatment groups so as to ensure that the control and high dose groups had an equal distribution of males and females. Piglets received liquid diets containing 0 (6 males and 6 females), 200 (8 males and 4 females), 500 (7 males and 5 females) or 2000 (6 males and 6 females) mg/L 2′-FL for three weeks. Prior to receipt, piglets were given injections of an iron supplement and a broad spectrum antibiotic, with additional injections provided at intervals during the study. The test vehicle and control was a commercially available milk replacer formula. Feeds were administered orally via a feeding container.

Animals were monitored twice daily for mortality, morbidity, injury and food availability, and a detailed clinical examination was performed twice weekly. Food consumption was measured daily. Body weights were measured daily during the first week and every other day for the remainder of the study. Blood samples were collected from all animals on study days 7 and 21 for haematology and clinical analyses. Urine samples were collected at terminal gross necropsy on study day 22, and a pH measurement was obtained from the caecum and colon contents. Organ weights were recorded and microscopic evaluations were performed on selected organs and tissues.

All animals survived to scheduled necropsy and no definitive test article-related changes in clinical observations were recorded during the course of the study. Doses of 2′-FL at dietary concentrations of 0, 200, 500 and 2000 mg/L were calculated to be 0, 29.37, 72.22 and 291.74 mg/kg bw/day, respectively in males and 29.30, 74.31 and 298.99 mg/kg bw/day, respectively in females. Watery faeces were noted in 0/6 males and 0/6 females in the control group, 2/8 males and 2/4 females in the 200 mg/L group, 1/7 males and 2/5 females in the 500 mg/L group and 3/6 males and 2/6 females given 2000 mg/L 2′-FL. This was not considered to be test article-related based on the lack of dose-response and the absence of any associated pathological findings.

There were no differences in body weight gain, food consumption and food efficiency between the control and treatment groups. One male and two females in the 2000 mg/L group had a lack of appetite on one day of the study, and one female in the 200 mg/L dose showed a lack of appetite for two days during the study. As there was no dose response and no impact on growth or feed intake, this observation was not considered to be adverse.

No significant differences in pH of the caecum and colon contents were observed. There were no test article-related effects on haematology, coagulation, clinical chemistry or urinalysis parameters.

No treatment related macroscopic changes or alterations in organ weights (absolute and relative to body and brain weights) were observed. Some animals in all groups had variable, minimal to mild, focal, acute inflammation within the keratinised portion of the nonglandular stomach and variable thickness of the keratinised portion. In 1/6 males and 1/6 females at 2000 mg/L and 1/5 females at 500 mg/L an increased magnitude of inflammation within the keratinised portion of the stratified squamous epithelium was observed (Table 4.2). In addition, a focal loss/thinning (erosion) of the keratinized portion of the stratified squamous epithelium in the male at 2000 mg/L was associated with the regions of inflammation. The underlying squamous epithelium remained intact without ulceration, however, and there were no correlative stomach macroscopic findings.

Table 4.2: Summary of non-glandular stomach microscopic observations in the three-week dietary study in piglets

	2′-FL concentration (mg/L)			
	0	**200**	**500**	**2000**
Males				
Within normal limits	1/6	2/8	5/7	3/6
Acute inflammation, minimal	5/6	6/8	2/7	2/6
Acute inflammation, mild	0/6	0/8	0/7	0/6
Subacute/chronic inflammation, mild	0/6	0/8	0/7	0/6
Subacute/chronic inflammation, moderate	0/6	0/8	0/7	1/6
Females				
Within normal limits	4/6	2/4	2/5	3/6
Acute inflammation, minimal	1/6	1/4	2/5	2/6
Acute inflammation, mild	1/6	1/4	0/5	0/6

	2′-FL concentration (mg/L)			
Subacute/chronic inflammation, mild	0/6	0/4	1/5	0/6
Subacute/chronic inflammation, moderate	0/6	0/4	0/5	1/6

Because the majority of control animals and animals at all doses had variable, minimal to mild, focal acute inflammation within the keratinized portion of the nonglandular stomach and variable thickness of the keratinized portion, the increased magnitude of inflammation and/or erosion in a small number of animals at the high dose was not considered to be clearly treatment related. The study authors noted that erosion/ulceration and hyperkeratosis of the nonglandular portion of the stomach are commonly observed in swine and are thought to be related to diet, gastric acidity and/or presence of commensal bacteria. In addition, erosions and/or inflammation of the nonglandular stomach have been documented in historical control data for piglets of the same age at the test facility (Mahadevan et al. 2014). No other microscopic changes were observed.

It was concluded that dietary exposure to 2′-FL at concentrations up to 2000 mg/L was well tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth and development. The NOAEL for 2′-FL in this study was 2000 mg/L, equivalent to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females.

Short-term toxicity studies conducted with other 2′-FL preparations

90-day oral toxicity study with a mixture of Glycom's 2′-FL and lacto-N-fucopentaose I in neonatal rats (Phipps et al. 2020) Regulatory status: GLP; conducted in compliance with OECD Test Guideline (TG) 408 (2018)

The test item in this study was a mixture of lacto-N-fucopentaose I (LNFP-I; 59.4% w/w), 2′-FL (31.5% w/w) and other oligosaccharides (4.6% w/w), produced through microbial fermentation. The total LNFP-I/2′-FL content (excluding the other oligosaccharides) was 91.0% w/w. Water was used as the vehicle control. Neonatal Sprague-Dawley rats (10/sex/group) were administered 0, 1100, 3330 or 5550 mg/kg bw/day LNFP-I/2′-FL mixture by oral gavage from postnatal day (PND) 7 for 90 days, equal to 0, 1000, 3000 or 5000 mg/kg bw/day LNFP-I/2′-FL, respectively. An additional reference control group was administered 5000 mg/kg bw/day oligofructose (FOS) for 90 days. An additional 5 rats/sex/group in the vehicle control, high dose and FOS groups were also dosed for 90 days then retained undosed for a 4-week recovery period. Clinical signs were monitored twice daily and detailed physical examinations were performed daily from PND 7 – 20 and weekly from PND 21 onwards. Ophthalmoscopy was performed on all animals in the vehicle control, FOS and high dose LNFP-I/2′-FL groups during the last week of dosing. Body weights were recorded daily for the first two weeks of dosing and twice weekly thereafter. Food consumption was recorded twice weekly from weaning on PND 21. Animals were monitored for timing of sexual maturation (balano-preputial separation for males and vaginal opening for females), eye opening and air righting reflex. Ulna length was measured fortnightly from PND 14 (Day 8 of dosing). Wet vaginal smears were collected from all females at necropsy to determine the stage of estrus. A functional observational battery test was conducted on all animals during Week 11 of dosing and spatial learning and memory was assessed using the Morris water maze during Week 12. Auditory and visual function (startle response and pupil

closure response, respectively) were examined on PND 20 (Day 16 of dosing). Blood and urine samples were collected from fasted main study animals for haematology, coagulation, clinical chemistry, thyroid hormone and urinalysis parameters just prior to scheduled necropsy. Additional blood and urine samples were collected from recovery animals prior to necropsy for clinical chemistry and urinalysis. Animals were killed on the day after receiving the final dose (PND 98) or at the end of the recovery period (PND 126) and subjected to a full macroscopic necropsy including measurement of organ weights. Organs and tissues were collected from all animals and a histopathological examination was performed on those from the vehicle control and 5000 mg/kg bw/day LNFP-I/2′-FL groups as well as any early decedents.

Nine animals died or were killed due to body weight loss or poor clinical condition between days 7 – 15 of dosing. One of these deaths (high-dose male) was attributed to a dosing error. The cause of death could not be determined for the remaining eight animals, as there were no macroscopic or microscopic changes at necropsy. As five of these deaths were in the FOS group, two were in the mid-dose group and one was in the high-dose group they were considered unrelated to administration of LNFP-I/2′-FL. No test item-related clinical signs or opthalmoscopic findings were observed during the study. Mean body weights and food consumption were similar in all groups. No treatment-related differences in the age or body weight at which animals attained sexual maturation, surface and air-righting reflexes were reported. No treatment related differences in the pupil reflex and startle response tests or mean ulna growth were observed between LNFP-I/2′-FL groups and vehicle controls. No treatment-related effects on neurobehavioural observations were observed in the functional observational battery and Morris maze tests. Estrus cycles were unaffected by treatment with the test item. No treatment-related changes in haematology, coagulation, clinical chemistry and urinalysis parameters and organ weights (relative to body weight) were observed. While a number of statistically significant changes were observed in these parameters they were not associated with a dose-response and were generally within historical control ranges for the test facility. Increases in T3 relative to vehicle controls were observed for high-dose males and females, but there was no clear dose-response in either sex, values were similar to those of the respective FOS-treated animals and there were no associated changes in T4 or TSH levels, cholesterol measures, or pathological changes in thyroid tissues. The study authors noted that a high carbohydrate intake has been associated with higher T3 levels in humans (Danforth et al. 1979; Kopp 2004) and suggested that the high oligosaccharide intake could have caused the changes in T3 levels, or alternatively they could have been incidental changes. Regardless, the increase in T3 was not considered to be an adverse effect. No adverse macroscopic or microscopic changes in organs and tissues were observed.

The NOAEL of LNFP-I/2′-FL in this study was 5000 mg/kg bw/day, equal to 1730 mg/kg bw/day 2′-FL, the highest dose tested.

Three-week study with the applicant's 2'-FL$_{micro}$ in combination with four other oligosaccharides in neonatal piglets (Hanlon 2020) Regulatory status: GLP

The test item in this study was a mixture of five oligosaccharides produced by fermentation by the applicant. The test substance was a dry powder blend of 2′-FL (49.1% dry weight), 3-fucosyllactose (3-FL; 10.4%), lacto-N-tetraose (LNT; 19.9%), 3′-sialyllactose (3′-SL; 3.5%), 6′-sialyllactose (6′-SL; 87.2%) and other carbohydrates. Groups of piglets (6/sex/group) were fed milk replacer containing 0, 5.75 or 8.0 g/L test item, equal to 0, 2556 or 3574 mg/kg bw/day in males and 0, 2604 or 3660 mg/kg bw in females, six times per day for 21 days. Cage side observations were conducted twice daily and detailed clinical observations were performed twice weekly. Body weights and food consumption were monitored daily. Blood

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samples were collected on study days 7 and 21 for haematology, coagulation and clinical chemistry analysis, and urinalysis was conducted on study day 22. At necropsy tissues were examined macroscopically, organ weights recorded and a range of tissues were collected for histopathological examination.

One male piglet in the 8.0 g/L group was killed for humane reasons on study day 7. All other animals survived until the end of the study period and there were no significant differences in body weight or food consumption between the control and treatment groups. Clinical observations of the animal that was killed included yellow discoloured faeces, thin body condition, unkempt appearance, generalised muscle wasting and lateral recumbency. Other animals in all three groups also exhibited yellow discoloured faeces which resolved following treatment with an antibiotic. Clinical chemistry and microscopic analysis of the animal that was killed showed a number of findings that were not seen in any other piglets in any of the control or treatment groups. A faecal culture from this animal found the presence of *E. coli*, and it was concluded that the decreased physical condition that led to killing of the animal was due to a bacterial infection that was likely obtained at the farm prior to study enrolment.

No treatment related changes in haematology, coagulation, clinical chemistry, or urinalysis parambers were observed. There were no treatment-related changes in organ weights, except for increased large intestine (caecum and colon) weights in males given 8.0 g/L test item which were considered an adaptive response to the oligosaccharides present in the diet. No treatment related macroscopic or microscopic changes in organs or tissues were observed.

The NOAEL for the test item in this study was 8.0 g/L in the diet (~4 g/L 2′-FL), equal to 3574 mg/kg bw/day for males (1755 mg/kg 2′-FL) and 3660 mg/kg bw/day for females (1797 mg/kg bw/day 2′-FL), the highest concentration tested.

Genotoxicity studies conducted with the applicant's 2'-FL$_{micro}$

In vitro micronucleus assay with human lymphocytes (FDA 2020) Regulatory status: GLP; conducted in compliance with OECD TG 487 (2014)

The test item in this study had a purity of 94.1% (Batch no. 2FL-2013-43-2632) and the vehicle control was dimethylsulfoxide (DMSO). The assay was conducted using cultured human peripheral lymphocytes obtained from healthy, non-smoking volunteers aged approximately 18 – 35 years of age with no known recent exposures to genotoxic chemicals or radiation. Proliferation of lymphocytes was initiated by the addition of phytohaemagglutinin to the culture medium 48 hours prior to exposure to the test item. Concentrations of 0, 500, 1000, 2000 or 5000 µg/mL 2′-FL were used in the main study, based on the results of a dose range finding test in which no cytotoxicity or precipitation was observed. Positive control clastogens were mitomycin C or cyclophosphamide in the absence or presence of metabolic activation, respectively. Colchicine was used as a positive control for aneugenicity in the absence of metabolic activation. Two micronucleus tests were performed. In experiment I, cells were exposed to the test item for 4 hours in the presence or absence of metabolic activation (S9), washed and then cultured in the presence of cytochalasin B for a further 20 hours. In experiment II, cells were exposed to the test item for 20 hours without metabolic activation, then washed and cultured in the presence of cytochalsin B for an additional 20 hours. At the end of the studies, cells were harvested and slides were prepared. Assays were conducted in duplicate with 1000 binucleated cells in each replicate scored for micronuclei. Evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or Replication Index (RI).

No significant increase in the frequency of binucleated cells with micronuclei was observed following treatment with 2′-FL in the presence or absence of metabolic activation, and no dose-related increases were observed. In all experiments the incidence of micronuclei in 2′-FL treated cells was within the range of the laboratory's historical vehicle control data. The positive controls induced significant increases in the number of binucleated cells with micronuclei, confirming the validity of the test system.

It was concluded that 2′-FL did not cause chromosomal damage in human lymphocytes under the conditions of this study.

Genotoxicity studies conducted with other 2'-FL preparations

Bacterial reverse mutation assay with a mixture of Glycom's 2'-FL and LNFP-I (Phipps et al. 2020). Regulatory status: GLP; in compliance with OECD Guideline 471 (1997)

The test article for this study was a mixture of LNFP-I (59.4% w/w), 2′-FL (31.5% w/w) and other carbohydrates (4.6% w/w), produced through microbial fermentation. The total LNFP-I/2′-FL content (excluding the other oligosaccharides) was 91.0% w/w. The solvent and negative control article was water. Test systems for this assay were *Salmonella typhimurium* strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* strain WP2 *uvr*A (pKM101). For assays conducted without S9 mix for metabolic activation, positive control articles were 2-nitrofluorene for TA98, sodium azide for TA 1535 and TA 100, 9-aminoacridine for TA1537, and 4-nitroquinoline-1-oxide for WP2*uvr*A. For assays conducted in the presence of S9, positive control articles were benzo(a)pyrene for TA98 and TA1537 and 2-aminoanthracene for TA100, TA1535 and WP2*uvr*A. Assays were conducted in triplicate using the plate incorporation and preincubation methods. Test article concentrations were 5.55 - 5550 µg/plate in the plate incorporation assay and 55.5 – 5550 µg/plate in the preincubation assay, equal to LNFP-I/2′-FL concentrations of 5 – 5000 and 50 – 5000 µg/plate, respectively. The test article showed no evidence of precipitation or toxicity, and there was no significant increase in revertant colonies compared with vehicle controls, with or without S9 mix, and values were within the laboratory's historical control range. A significant increase in revertant colonies was observed in the presence of positive control articles, confirming the validity of the assays. It was concluded that LNFP-I/2′-FL was not mutagenic under the conditions of this study.

In vitro mammalian cell micronucleus assay with a mixture of Glycom's 2'-FL and LNFP-I (Phipps et al. 2020). Regulatory status: GLP; in compliance with OECD Guideline 471 (1997)

The test article was a mixture of LNFP-I (59.4% w/w), 2′-FL (31.5% w/w/) and other carbohydrates (4.6% w/w), produced through microbial fermentation. The solvent and negative control was water. The test system comprised human peripheral blood lymphocytes collected from non-smoking donors. Cells were exposed to 0.56 – 2220 µg/mL of the test item, equal to 0.5 – 2000 µg/mL LNFP-I/2′-FL for 3 hours in the absence and presence of metabolic activation (S9) and for 20 hours in the absence of S9 only. Positive controls were mitomycin C and colchicine in the absence of S9 and cyclophosphamide in the presence of S9. At the end of the 3 hour exposures, the culture medium was replaced and cells were treated with the cytokinesis inhibitor cytochalasin B before being incubated for an additional 17 hours. For cell cultures undergoing long-term exposure, cytochalasin B was added at the beginning of treatment. At the end of the 20 hour incubation period cells were fixed on slides and stained. Assays were conducted in duplicate with 1000 binucleated cells in each replicate scored for micronuclei. Cytotoxicity was evaluated based on the CBPI.

No evidence of cytotoxicity was observed at any concentration of LNFP-I/2′-FL. No significant increases in the frequency of micronucleated cells were observed following treatment with LNFP-I/2′-FL in the presence or absence of metabolic activation. The incidence of micronuclei in LNFP-I/2′-FL treated cells was within the range of the laboratory's historical vehicle control data, with the exception of cultures exposed to 500 μg/mL LNFP-I/2′-FL for 3 hours in the presence of S9. In this group the mean value slightly exceeded the upper 95% confidence limit (10.5 versus 9.2, respectively). This assay was repeated and all micronucleus frequencies for LNFP-I/2′-FL and vehicle control groups were within the historical negative control range. All positive controls produced the expected increase in the frequency of micronucleated cells, with the exception of mitomycin C in the short-term test in the absence of S9. This assay was also repeated and in the repeat test a significant increase in micronucleated cells was observed with mitomycin C, confirming the validity of the test system. It was concluded that LNFP-I/2′-FL was not clastogenic or aneugenic under the conditions of this study.

4.2.2 Human studies with 2′-FL

Clinical studies in infants and young children

Clinical study of infants fed extensively hydrolysed infant formula containing 2'-FL (Ramirez-Farias et al. 2021)

In a non-randomised, single-group, multicentre study, infants (aged 0 – 60 days) with suspected food protein allergy, persistent feeding intolerance or presenting conditions where an extensively hydrolysed formula (eHF) was deemed appropriate were fed a hypoallergenic eHF containing 0.2 g/L 2′-FL for 60 ± 5 days. The primary outcome was maintenance of weight for age z-score during the study. Information on weight, length, head circumference, formula intake, tolerance measures, clinical symptoms was collected at intervals during the study. Safety monitoring consisted of the assessment of adverse events and serious adverse events.

Forty-eight infants were enrolled in the study, 47 were included in the intention-to-treat (ITT) group and 36 fully completed the study and were included in the protocol evaluable cohort. Weight for age z-scores showed a significant improvement from study day 1 to study day 60. There was no indication of adverse effects of the study formula on tolerance measures such as mean rank stool consistency, stool colour, average volume of formula intake and percent feedings with spit-up/vomit 1 hour after feeding. Several infants were experiencing persisting symptoms at enrolment such as diarrhoea, constipation, blood in stool, vomiting, spit-up/gagging/reflux and rash or eczema. After 60 days of consuming the eHF containing 2′-FL, all persisting symptoms either remained the same, improved or resolved. Adverse events occurred in 15 (32%) of the infants in the ITT cohort. The most commonly reported adverse events were seborrheic dermatitis (five infants), gastrointestinal reflux disease (three infants) and infantile spit-up (two infants. Most adverse events were mild in severity and considered unrelated to the test formula. The study authors concluded that eHF containing 0.2 g/L 2′-FL was safe and well tolerated.

Clinical study of toddlers consuming young child formula containing 2'-FL (Leung et al. 2020)

In a randomised, controlled, double blind, parallel-group trial, healthy Chinese children aged 1 – 2.5 years were assigned to consume two 200 mL/day servings of one of four different young child formulas for six months. Children received either a standard milk formula or one of three trial formulas containing varying quantities of milk fat, immunoglobulins, lactoferrin and transforming growth factor-β (TGF-β). Two of the formulas also contained 3 g/L 2′-FL.

Primary outcomes were incidence of upper respiratory tract infection and duration of gastrointestinal infections. Secondary outcomes included anthropometric data (z-scores for weight for age, height for age and weight for height) recorded every two months and details of adverse events. Severity and likelihood of the relationship between adverse events and the intervention were scored by a paediatrician and reviewed by an independent data safety monitoring board (DSMB).

The final ITT group comprised 456 children (114/group), and per-protocol analysis was possible in 85 – 89% of subjects. No significant differences in anthropometric measures were observed in children given the test formulas compared with those given control formula. All four groups had similar frequencies of adverse events and serious adverse events. None of the adverse events reported were considered to be related to the interventions. It was concluded that young child formula containing 3 g/L 2′-FL was safe and well tolerated by toddlers.

Clinical studies in adults

Trial of 2'-FL and LNnT in adults with irritable bowel syndrome (Palsson et al. 2020)

A publication provides details of a prospective multi-centre, open label, single arm clinical trial of the efficacy and safety of a 4:1 mixture of 2′-FL and LNnT in adults with irritable bowel syndrome (IBS). Limited details of this study reported in an abstract were previously considered by FSANZ as part of A1155 Review[6]. A total of 317 subjects (70.7% females; mean age 44.0 years, range 18-93 years) recruited from 17 sites around the US were asked to orally consume 5 g of a 4:1 mixture of 2′-FL and LNnT daily for 12 weeks. Bowel habits, IBS symptoms and quality of life were assessed at baseline and every four weeks during the intervention. Safety was assessed by collecting and monitoring adverse events throughout the course of the trial.

The full 12 week intervention was completed by 245 patients. Intention-toTreat (ITT) analysis found a significant improvement in the percentage of bowel movements with abnormal stool consistency, IBS symptom severity and health-related quality of life. Patients generally reported that the intervention was well tolerated. In total 46 patients (14.5% of the ITT sample) reported 87 adverse events, and 8 patients (2.5%) discontinued participating due to adverse events. In total 65 adverse events reported by 33 patients were considered to be possibly or probably related to the intervention on review. The majority of adverse events were mild and related to the gastrointestinal tract; the most commonly reported symptom was passing gas, followed by abdominal distention and abdominal pain. One serious adverse event (brief hospitalisation due to colitis) occurred during the trial, however on review the study's medical safety officer concluded that this was unrelated to the intervention.

The study authors concluded that supplementation with 2′-FL and LNnT improved IBS symptoms and quality of life without substantial side effects, and that the oligosaccharides mix tested is safe for adults with IBS.

Post-marketing surveillance

At the time of the original application, the applicant indicated that based on an infant formula supplier's post-market surveillance of products containing the applicant's 2′-FL, no indications of issues had been observed or reported. The applicant has confirmed that they remain unaware of any reports of issues related to products containing their 2′-FL.

4.2.3 Allergenicity and intolerance

Batch analysis of the $2'$-FL_{micro} ingredient indicates a lack of detectable proteins (Section 2.3). The protein content listed in the specification is < 100 µg/g (0.01%), while batch analyses have found protein levels to be < 10 µg/g (0.001%). Therefore $2'$-FL is unlikely to pose an allergenicity concern. Consistent with this conclusion, a study by Nowak-Wegrzyn et al. (2019), reviewed as part of the A1155 Review[6], found infant formula containing 1.0 g/L $2'$-FL (source unspecified) was hypoallergenic in infants with cow's milk protein allergy.

The specification for the applicants $2'$-FL indicates it may contain residual lactose, at a concentration of up to 5% (Table 2.3). Batch analysis found lactose was present at lower levels than this, up to 0.5% total sugars or 1.5% AUC. Lactose may also be produced from $2'$-FL in the gastrointestinal tract, and is the main carbohydrate present in human and mammalian milk.

4.2.4 Assessments by other regulatory agencies

The Netherlands Committee on Safety Assessment of Novel Foods completed an evaluation of $2'$-FL_{micro} produced by the applicant in 2016 (NFU 2016). This assessment concluded that the evidence presented adequately demonstrated that $2'$-FL can be safely used as an ingredient in infant formula and follow-on formula at the proposed use level of up to 2 g/L.

The US Food and Drug Administration (FDA) has responded with 'no questions' to the applicants self assessment that its $2'$-FL_{micro} is Generally Recognized as Safe (GRAS) when used as an ingredient in infant and toddler formula at concentrations up to 2.0 g/L[7]. The USFDA has also issued 'no questions' responses to other manufacturers' self-assessed GRAS notifications (GRN) for $2'$-FL produced by Glycom ($2'$-FL_{chem}[9] and $2'$-FL_{micro}[10]), Friesland Campina[11], DuPont[12] and BASF[13].

The European Food Safety Authority (EFSA) assessed $2'$-FL_{chem} produced by Glycom as a novel food ingredient in 2015, and concluded it was safe for use alone or in combination with LNnT when added to infant formula, follow-on formula and young-child formula at concentrations up to 1.2 g/L $2'$-FL ad 0.6 g/L LNnT at a ratio of 2:1 (EFSA 2015). The Food Safety Authority of Ireland (FSAI) has issued an opinion concluding that Glycom's $2'$-FL_{micro} is substantially equivalent to the previously approved chemically synthesised form, and therefore raises no safety concerns (FSAI 2016).

EFSA has also concluded that a mixture of $2'$-FL/DFL_{micro} produced by Glycom is safe for addition to a variety of foods, including infant and follow-on formula, foods for infants and young children, foods for special medical purposes and food supplements under the proposed use conditions.

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[7] GRAS Notice (GRN) 571; [9] GRN546; [10] GRN650 ($2'$-FL) & GRN815 ($2'$-FL/DFL mixture); [11] GRN735; [12] GRN749 and GRN897; [13] GRN852. For further information about this GRNs, visit the FDA GRAS Notices website www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices.

4.3 Key findings of the toxicology assessment

2′-FL has previously been assessed by FSANZ (FSANZ 2019; A1155 Review[6]). Data on 2′-FL produced from a variety of sources, including the applicant's $2'\text{-FL}_{micro}$, were considered as part of the evaluation. The assessment concluded that there were no safety concerns associated with the addition of 2′-FL to IFP and FSFYC at concentrations up to 2.4 g/L.

Toxicological and clinical studies on oligosaccharides found in human milk submitted by the applicant or published since FSANZ's earlier assessments did not indicate a need to amend the conclusions of the previous evaluation.

The full study report of the three-week dietary toxicity study in neonatal piglets with the applicant's $2'\text{-FL}_{micro}$ did not indicate a need to amend the previous conclusion that there were no adverse effects at formula concentrations up to 2.0 g/L 2′-FL, the highest concentration tested. In another three-week study in neonatal piglets fed formula containing a mixture of five oligosaccharides including 2′-FL, no adverse effects were observed at concentrations up to 8.0 g/L (equal to ~ 4.0 g/L 2′-FL). A 90-day oral toxicity study with a mixture of 2′-FL and LNFP-I in neonatal rats also found no adverse effects at the highest dose tested, 5000 mg/kg bw/day LNFP-I/2′-FL, equal to 1730 mg/kg bw/day 2′-FL.

An *in vitro* micronucleus assay with the applicant's $2'\text{-FL}_{micro}$, plus *in vitro* mutagenicity and micronucleus tests with a mixture of 2′-FL and LNFP-I, confirmed that 2′-FL is not genotoxic.

Newly available information from human clinical studies found that eHF infant formula containing 2′-FL at 0.2 g/L was well tolerated by infants with suspected food protein allergy, feeding intolerance or other underlying health conditions. No intervention-related adverse events were observed in study of healthy toddlers consuming young child formula containing 3 g/L 2′-FL for six months. Consumption of a 4:1 mixture of 2′-FL and LNnT by adults with IBS was also well tolerated, with the most common side effects being mild gastrointestinal symptoms experienced by a small proportion of study participants.

The applicant has also indicated that post-marketing surveillance by an infant formula supplier has not identified any reports of issues with products containing the applicant's 2′-FL.

$2'\text{-FL}_{micro}$ does not contain detectable proteins and is therefore unlikely to pose an allergenicity concern. A double blind, placebo controlled food challenge study previously reviewed by FSANZ demonstrated that infant formula containing 2′-FL was hypoallergenic in children with cow's milk protein allergy, consistent with this conclusion.

Based on the available toxicological and clinical evidence, and taking into account that the proposed maximum concentration of $2'\text{-FL}_{micro}$ (2.4 g/L) is within the range of naturally occurring levels in human milk from the majority of women (0.6 – 7.8 g/L), there are no safety concerns associated with the addition of 2′-FL to IFP and FSFYC at concentrations up to 2.4 g/L 2′-FL.

5 Nutrition assessment

5.1 Previous FSANZ assessment

For application A1155, FSANZ assessed three infant cohort studies (Sprenger et al. 2017a; Larsson et al. 2019; Lagström et al. 2020) and five clinical trials in infants (Marriage et al.

2015; Kajzer et al. 2016; Puccio et al. 2017; Storm et al. 2019; Román et al. 2020), investigating the potential effects of 2′-FL on growth (FSANZ 2019; A1155 Review[6]). The conclusion from assessment of these studies was that the addition of 2′-FL to IFP is not expected to change the growth profiles of infants and young children, at the concentrations typically observed in human milk.

5.2 Current assessment

The applicant provided a review of studies on infant formula containing added 2'-FL (Reverri et al. 2018) and a further three studies were identified following a literature search (Berger et al. 2020; Leung et al. 2020; Ramirez-Farias et al. 2021).

Reverri et al. (2018) summarised infant feeding studies based on three cohorts, of which two were addressed in application A1155 (FSANZ 2019; A1155 Review[6]). Reverri et al. (2018) also described an unpublished infant study with partially hydrolysed whey-based formula containing 2'-FL, however there was no control group. Infant growth was reported to be normal, however no data were provided. Reverri et al. (2018) provided additional information for a study described in an abstract (Kajzer et al. 2016), however FSANZ considered this study to be of inadequate duration (6 weeks) for the assessment of any effects on body weight and other anthropometric parameters.

Berger et al. (2020) reported a study investigating potential effects of 2′-FL on cognitive development relative to maternal obesity and feeding frequency, to 24 months of age. Maternal pre-pregnancy BMI was found not to be related to feedings per day or oligosaccharide composition of human milk but predicted poorer infant cognitive development (β = -0.31, P = 0.03). Feedings per day (β = 0.34) and 2′-FL concentration (β = 0.59) at one month predicted better infant cognitive development (both P ≤ 0.01). The association of feedings per day with infant cognitive development was no longer significant after further adjustment for 2′-FL. There were no associations of feedings/day and 2′-FL at six months with infant cognitive development.

The study authors acknowledged several limitations of the study, namely that the study was not a randomised design, the results are specific to a small sample of Hispanic mother-infant pairs located in the South-western United States, and the cohort was somewhat homogenous in socioeconomic status, education level, and cultural practices regarding food choice and eating behaviours.

Leung et al. (2020) described a randomised, controlled, double blind, parallel-group trial, in which healthy Chinese children aged 1 – 2.5 years were assigned to consume two 200 mL/day servings of one of four different young child formulas for six months. Children received either a standard milk formula or one of three trial formulas containing varying quantities of milk fat, immunoglobulins, lactoferrin and transforming growth factor-β (TGF-β). Two of the formulas also contained 3 g/L 2′-FL. No significant differences in anthropometric measures were observed in children given the test formulas compared with those given control formula. Additional details of this study, including tolerance outcomes are provided in Section 4.2.2.

Ramirez-Farias et al. (2021) was a non-randomised, single-group, multicenter study, evaluating growth and tolerance in infants with persistent feeding intolerance, suspected protein allergy sensitivity or other conditions where an extensively hydrolyzed formula (eHF) was deemed appropriate. Infants (n = 36; <60 days of age; mean = 33 days old) were examined over a two-month feeding trial, using eHF supplemented with 2′-FL at 0.2 g/L. The primary outcome was weight for age z-score (WAZ) and secondary outcomes included stool

analyses, feeding volume, and anthropemetric factors including weight, length and head circumference. Analyses of WAZ showed a statistically significant improvement from study day 1 to study day 60 (mean change ± standard error: 0.32 ± 0.11, p = 0.008). Additional details of this study, including tolerance outcomes are provided in Section 4.2.2.

5.3 Key findings of the nutrition assessment

The previous assessment of application A1155 by FSANZ concluded that the addition of 2′-FL to infant formula at levels normally found in human milk is unlikely to affect growth. Assessment of additional studies for the present application does not alter that conclusion.

6 Dietary intake assessment

FSANZ completed a dietary intake assessment of 2′-FL for application A1155 (FSANZ 2019). The concentration of $2'\text{-FL}_{micro}$ in infant formula, follow-on formula and FSFYC considered in the dietary intake assessment was 2.4 g/L. This level is in the middle of the range of concentrations found in mature human milk for secretors of 2′-FL. Evidence suggests that 70-80% of women are secretors of 2′-FL, due to the functional expression of the enzyme α-1,2-fucosyltransferase (Sprenger et al. 2017; Totten et al. 2012). At the time of the A1155 assessment, the range of concentrations reported in mature human milk for secretor mothers was 1.0 – 7.8 g/L. The estimated dietary intake of $2'\text{-FL}_{micro}$, based on 2.4 g/L in infant formula and follow-on formula, was similar to 2′-FL intakes for 3 and 9 month old breastfed infants (FSANZ 2019). Estimated mean intakes of $2'\text{-FL}_{micro}$ from FSFYC, based on 2.4 g/L, for 12 month old infants and 2-3 year old children were similar to or less than those for younger formula-fed and breastfed infants (< 12 months). A summary of the results from the dietary intake assessment for A1155 are reproduced in Table 6.1.

The concentration of $2'\text{-FL}_{human}$ in human milk varies between mothers, over the stages of lactation and across regions of the world (Austin et al. 2016; Chaturvedi et al. 2001; Erney et al. 2000; Kunz et al. 2017; McGuire et al. 2017). The analytical methods used to quantify $2'\text{-FL}_{human}$ vary between studies, as do the regions of the world the samples were collected in and the breadth of time across which the samples were taken. Some studies report the concentration of $2'\text{-FL}_{human}$ in human milk for secretors only, while others report the concentration from all milk samples pooled. FSANZ undertook a literature search for concentration data for $2'\text{FL}_{human}$ in human milk published since the assessment of A1155. A number of relevant studies were identified (Lagström et al. 2020; McJarrow et al. 2019; Plows et al. 2021; Saben et al. 2020; Saben et al. 2021; Tonon et al. 2019b; Torres Roldan et al. 2020; Wu et al. 2020), with the range of concentrations reported for secretors, 0.6 – 4.0 g/L, falling within a similar range to concentrations reported in previous studies. It was concluded that an additional dietary intake assessment was not required for application A1190.

Table 6.1: *Summary of estimated dietary intakes of 2'-FL (human and microbial sources) for infants aged 3, 9 and 12 months and children aged 2-3 years (reproduced from A1155[14])*

Unit	Age group	Mean dietary intake		90th percentile dietary intake	
		From microbial fermentation*	From human milk♦	From microbial fermentation*	From human milk♦
g/day	3 months	2.1	1.8 – 2.2	4.2	3.5 – 4.4
	9 months	1.4	1.2	2.8	2.4
	12 months	$0.97 - 1.9^{\Omega}$	n/a	$1.9 - 3.9^{\Omega}$	n/a
	2-3 years	$1.1 - 2.2^{\Omega}$	n/a	$2.2 - 4.4^{\Omega}$	n/a
g/kg bw/day**	3 months	0.33	0.28 – 0.34	0.66	0.55 – 0.69
	9 months	0.16	0.13	0.32	0.27
	12 months	$0.10 - 0.20^{\Omega}$	n/a	$0.20 - 0.40^{\Omega}$	n/a
	2-3 years	$0.077 - 0.15^{\Omega}$	n/a	$0.15 - 0.31^{\Omega}$	n/a

*Estimated intake is from infant formula / follow on formula / FSFYC only. The minor contribution to intake from other food sources of 2′-FL is not included. Ω Lower bound of the range is for the FSFYC serve size of 230 ml; the upper bound of the range is for the FSFYC serve size of 115 ml. ♦ Lower bound of the range is for human milk 60+ days post-partum; upper bound of the range if for 10-60 days post-partum. ** Mean body weights used: 6.4 kg for 3 months, 8.9 kg for 9 months and 9.6 kg for 12 months. For 2-3 year olds, each individual's body weight was used for the calculation before summary statistics for the group were derived.

7 Benefit assessment

The [ministerial policy guideline on the regulation of infant formula products](#)[8] requires that 2′-FL for use in infant formula should have: a substantiated beneficial role in the normal growth and development of infants or children; or a technological role, taking into account, where relevant, the levels of comparable substances in human milk.

Substantiation of the role of 2′-FL in normal growth and development requires appropriate evidence to link its physiological, biochemical and/or functional effects to specific health outcomes for infants, in infancy or childhood.

In order to assess the evidence of a beneficial role of 2′-FL in the normal growth and development of infants or children, FSANZ has applied a hierarchy to the different types of studies, as follows, in decreasing order of weight of evidence:

- Data from studies in humans:
  - human intervention studies: with greatest weight given to randomised, controlled, double-blinded studies
  - human observational studies: such as cohort studies, case-control studies, cross-sectional studies

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[8] [foodregulation.gov.au/internet/fr/publishing.nsf/Content/publication-Policy-Guideline-on-Infant-Formula-Products](#)

- other studies in humans
- Non-human data:
  - animal studies;
  - *ex vivo* studies: eg using human or animal biological samples
  - *in vitro* data: typically analysing mechanistic aspects of an effect.

Data from well-controlled human studies are considered of greatest value in reaching conclusions about a possible beneficial role of 2′-FL. However, ethical constraints limit their availability when considering health outcomes from interventions in infants and children. Therefore, this assessment necessarily focusses mainly on less direct evidence for a link between physiological, biochemical or functional effects of 2′-FL and specific health outcomes. FSANZ has also considered any limitations or confounding factors in the studies which might affect the overall quality of the findings.

7.1 Previous FSANZ assessment (A1155)

FSANZ recently assessed the benefits of 2′-FL as part of application A1155[9], focusing on three proposed health benefits:

1. pathogen-binding inhibition effect;
2. intestinal microbiota modulating effect supporting a proliferation of bifidobacteria (bifidogenic effect);
3. immune modulation, improved barrier function and alleviation of allergic responses.

Pathogen-binding inhibition effect

FSANZ assessed the evidence for an inhibitory effect of 2′-FL on the binding of pathogenic strains of *Campylobacter jejuni* to the infant gastrointestinal epithelium and its effect on intestinal colonisation and the likelihood of illness (see Section 3 of A1155 Review Supporting Document 2[10]). Noting that the low number of relevant clinical trials constrains the assessment of beneficial health effects and any estimation of the magnitude of those effects, and acknowledging the limitations in the body of evidence, FSANZ concluded that:

- for infants, there is a consistent body of indirect evidence to demonstrate a credible mechanism for 2′-FL inhibition of the binding of pathogenic *C. jejuni* to intestinal epithelial cells, and limited, largely indirect, evidence for a reduction of intestinal colonisation by *C. jejuni* and the incidence of diarrhoea.

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[9] www.foodstandards.gov.au/code/applications/Pages/A1155.aspx

[10] www.foodstandards.gov.au/code/applications/Documents/A1155%20Review%20SD2%20Beneficial%20effects.pdf

- for young children, there is no direct evidence that inhibition of binding of *C. jejuni* occurs in children fed formulated supplementary foods for young children (FSFYC) supplemented with 2′-FL$_{chem.}$

The Independent Expert Advisory Group (IEAG) convened by FSANZ to provide guidance on the benefit assessment for A1155 (see A1155 Review Supporting Document 4[11]), concluded that:

- the approach to the assessment taken by FSANZ was appropriate; and

- there is a dose response effect in relation to the competitive inhibition by 2′-FL of binding of *C. jejuni* to its epithelial cell receptor; but this inhibitory effect at a cellular level cannot be linked causally to a reduction in infection rates in infants or children because, for obvious reasons, *C. jejuni* challenge studies in humans are unethical.

The evidence for a health effect of 2′-FL in protecting infants and young children against other pathogens and toxins is inconclusive and is primarily limited to *in vitro* inhibition studies, with no specific mechanism of action identified (FSANZ 2019).

Bifodogenic effect

FSANZ assessed the evidence for a bifidogenic effect of 2′-FL in infants and young children. Noting the limitations in the evidence base (see Section 2 of A1155 Review Supporting Document 2[15]), FSANZ concluded that:

- for infants, the addition of 2′-FL to infant formula leads to a *Bifidobacterium*-enriched microbiota that is more similar to that observed in breastfed infants than in those fed non-supplemented formula. However, the size of the effect of 2′-FL on bacterial populations is difficult to estimate. Evidence for a link between the presence of 2′-FL in human milk or formula and any specific health outcome is limited to secondary outcomes of one randomised control trial and observational studies of lower quality.

- evidence that a bifidogenic effect occurs in children fed formulated supplementary foods for young children (FSFYC) supplemented with 2′-FL$_{chem}$ is very limited. However, there is no reason to expect that the effect demonstrated in infants would not occur in young children.

The IEAG for A1155[15] concluded that:

- the approach to the assessment taken by FSANZ was appropriate; and

- there is a bifidogenic effect; but there is limited evidence in humans to estimate the size of the effect or to link the bifidogenic effect to a beneficial health outcome.

Immune modulation

In the studies assessed by FSANZ (FSANZ 2019), there is *in vitro* evidence that 2′-FL can mediate changes in immune signalling and trafficking. However, the clinical significance of

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[11] www.foodstandards.gov.au/code/applications/Documents/A1155%20Review%20SD4%20IEAG%20Summary%20of%20Conclusions.docx.pdf

this data is inconclusive, and an immune modulating effect for the proposed use of 2′-FL in IFP is, therefore, inconclusive.

Improved barrier function

In the studies previously assessed by FSANZ (FSANZ 2019), there is plausible *in vitro* evidence that 2′-FL may promote intestinal epithelial cell differentiation and maturation. 2′-FL had a limited protective role in experimental models of necrotising enterocolitis, but was beneficial post intestinal surgery. However, the observed effects have only been reported in animal models, and at considerably high doses of 2′-FL relative to body weight. None of the studies have demonstrated how 2′-FL mediates these effects, be it through by-products of microbiota processing; direct binding to intestinal epithelial cells; or cellular uptake. These studies have also not provided clinically relevant data to support the protective effect of 2′-FL in restoring barrier function in infants.

Alleviation of allergic responses

FSANZ previously assessed (FSANZ 2019) two studies examining the role of 2′-FL in allergic responses (Castillo-Courtade et al. 2015; Sprenger et al. 2017b). These studies did not provide strong evidence to support the protective effect of 2′-FL in preventing development of allergies in infants and children. Although 2′-FL was shown to have the potential to alleviate allergic responses post-sensitisation, the effect was only observed in an animal model, using a considerably high dose of 2′-FL relative to body weight. Thus, the relevance to infants is inconclusive. Of clinical relevance was the finding that 2′-FL did not prevent production of allergen-specific IgE-immunoglobulins in the animal study. Therefore, the risk of anaphylaxis remains.

7.2 Current assessment

In the current application, the addition of 2′-FL to IFP and FSFYC is stated by the applicant to confer functional benefits to infants and young children, consistent with the oligosaccharides fraction of human milk, with the following specified health effects:

1. pathogen-binding inhibition effect;

2. intestinal microbiota modulating effect supporting a proliferation of bifidobacteria (bifidogenic effect);

3. immune modulation, improved barrier function and alleviation of allergic responses;

4. beneficial impacts on learning and memory; and

5. improved gut motility.

FSANZ assessed data submitted by the applicant and information from published sources. Most of this data has previously been assessed, and is summarised in Section 7.1. Both direct and indirect evidence was assessed to establish the mechanism of action underpinning the stated health effect and the likelihood of the effect occurring in infants and young children fed formula supplemented with 2′-FL. This included *in vitro* and *ex vivo* studies, animal studies, and where available, human epidemiological studies and clinical trial data. In assessing the quality of individual studies, FSANZ assessed various elements of the study design and method. These included: the purpose of the study; appropriateness of the study design for the purpose; appropriateness of the outcome measures; the duration of the study; and the appropriateness of the statistical analyses undertaken.

Pathogen-binding inhibition effect

Human studies

The applicant provided evidence from two infant feeding studies. A study by Stepans et al. (2006) was excluded because the focus of the study was the impact on health outcomes of the lacto-*N*-fucopentaose-2; there was no examination of 2′-FL. A study by Kuhn et al. (2015) was also excluded, as the infants were breastfed and there was no characterisation of the individual oligosaccharide levels and/or secretor status of the mothers.

Animal studies

The applicant provided evidence from one animal study examining the impact of oligosaccharides supplementation on rotaviral infection (Azagra-Boronat et al. 2019a). This study was included in the A1155 Review (see Section 3 of A1155 Review Supporting Document 2[15]). The limited evidence provided from this rodent study implies 2′-FL$_{micro}$ may reduce the length of time of rotaviral infection in rats but not the severity. Furthermore, this study does not demonstrate a pathogen-binding inhibition effect of 2′-FL in infants.

In vitro studies

The applicant provided evidence from four studies, one of which—Weichert et al. (2013)— was included in the A1155 Review (see Section 3 of A1155 Review Supporting Document 2[15]).

Weichert et al. (2013) showed that the presence of 10 mg/ml (~20 mM) 2′-FL$_{micro}$ inhibited adhesion of *Salmonella enterica* serovar *fyris*, enteropathogenic *E. coli*, *Pseudomonas aeruginosa* and *C. jejuni* to Caco-2 cells by 12%, 18%, 17% and 26%, respectively. At the same concentration, 2′-FL inhibited adhesion of *P. aeruginosa* to human lung carcinoma cell line A549 by 24%. The study demonstrated limited inhibition by 2′-FL of pathogen binding to the cell lines used. The concentration of 2′-FL used was 3–4 times higher than the mean concentration of 2′-FL in human milk. In similar experiments, Coppa et al. (2006) showed that purified 2′-FL$_{human}$ had minimal impact on binding of *E. coli* and *S. enterica* serovar *fyris* to Caco-2 cells, while a mixture of low molecular weight neutral oligosaccharides found in human milk had significantly greater effect. The suitability of the Caco-2 cell line for these studies is open to question. It has been reported that only a subset of differentiated Caco-2 cells express the type H-2 histo-blood group antigen—the cellular receptor for which 2′-FL is a putative soluble mimetic (Murakami et al. 2013). It is possible that a significant proportion of the binding of pathogens to the Caco-2 cells in these studies was non-specific or mediated through other epitopes which are not susceptible to inhibition by 2′-FL.

Laucirica et al. (2017) showed that 2′-FL at 2.5 and 5 mg/ml inhibited binding of two clinical strains of rotavirus to African green monkey kidney epithelial cells by 15% to 62%, depending on incubation conditions. When 2′-FL was added at 5 mg/ml after virus absorption onto cells, infectivity was inhibited by 62% for one strain and 45% for the other.

Weichert et al. (2016) investigated the ability of 2′-FL$_{micro}$ to inhibit norovirus binding to histo-blood group antigens (HBGA)—an interaction thought to be important for infection. 2′-FL inhibited the binding of norovirus virus-like particles to HBGA in a dose dependent manner, with a half-maximal inhibitory concentration from 5.5 to 26.9 mM, depending on the source of HBGA used. Similar results were observed for 3-fucosyllactose and fucose. Binding of 2′-FL (and 3-FL) at the HBGA-binding site on the norovirus was demonstrated by X-ray crystallography.

These studies demonstrate binding inhibition *in vitro*. However, binding and inhibition of bacterial, rotavirus or norovirus infection in the infant gut has not been demonstrated. Epidemiological evidence links 2′-FL to a reduction in diarrhoea as a result of *Campylobacter* infection in infants and young children, but not to protection against calicivirus diarrhoea (Morrow et al. 2004).

Bifodogenic effect

Human observational study

The applicant provided evidence from one human observation study (Borewicz et al. 2019), which was included in the A1155 Review (see Section 2 of [A1155 Review Supporting Document 2](#)[15]). Results indicated that the breakdown of 2′-FL$_{human}$ and other oligosaccharides found in human milk, in transit through the infant gastrointestinal tract, was strongly associated with microbial composition, particularly with levels of *Bifidobacterium*, *Bacteroides* and *Lactobacillus*.

In vitro studies

Two studies examining bacterial growth and utilisation of 2′-FL were provided by the applicant. The study by Marcobal et al. (2010) was excluded because the oligosaccharide was a mixture. Thongaram et al. (2017) examined the ability of 12 *Lactobacillus* and 12 *Bifidobacterium* strains (*B. adolescentis*, *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* and *B. longum* ssp. *infantis*) to ferment a variety of oligosaccharides found in human milk, including 2′-FL$_{chem}$, on media containing simple sugars. Only the two *B. longum* spp. *infantis* strains were demonstrated to grow on 2′-FL as the sole carbon source.

Improved barrier function

No new studies were provided as supporting evidence, nor new studies identified from a literature search (performed April 2021).

Immune Modulation

Animal studies

The applicant submitted two studies investigating the impact of 2′-FL supplementation on vaccine responsiveness in mice. The study by van den Elsen et al. (2019) was excluded because the treatment arm had 2′-FL in the presence of FOS and GOS. In the second study (Xiao et al. 2018), adolescent mice were placed on a diet intervention, where the feed was supplemented with 0 to 5g 2′-FL$_{micro}$/total carbohydrate. On day 14, mice received a primary vaccination and, on day 35, a booster vaccination, using an inactivated influenza virus vaccine. Analyses were performed on days 44 and 45. Results showed that chronic supplementation of the vaccinated adolescent mice led to a significant dose-responsive effect on splenic B cell maturation and vaccine-specific serum antibody levels with 2′-FL from 0.25–1%. This was associated with an increased level of mature antigen presenting cells (dendritic cells (DC)) in the spleen from animals in the 1% 2′-FL group. Neither B cell or DC maturation was observed in mesenteric lymph node tissue from the supplemented animals. The authors suggest these results demonstrate that 2′-FL improves vaccine responsiveness. Although this study showed that antibody levels were higher in the treated animals, there was no demonstration that the antibodies were effective at preventing disease. Furthermore, the mice used are an inbred strain, maintained in a clean environment, and, therefore, do not

reflect the genetic diversity of humans nor the full environmental exposure of infants that can influence the development of the immune system (Boyd and Jackson 2015).

The applicant also provided a study examining 2′-FL$_{micro}$ supplementation on immune modulation in rat pups (Azagra-Boronat et al. 2019b). Chronic supplementation of rat pups led to significantly increased plasma levels of IgG compared to unsupplemented animals. The increase was 1.16–1.55 times at 7 and 15 days, respectively. This was associated with a 1.19–1.42 times increase in the Th1/Th2 immunoglobin ratio at 7 and 15 days, respectively, reflecting a shift to a more cell-based immune response driven by 2′-FL. On treatment day 15, supplemented animals showed a significant reduction in B cells in the mesenteric lymph nodes that changed the ratio of B to T cells from 0.49 to 0.24. Measurement of cytokines from a gut wash showed reduced levels of both pro- and anti-inflammatory cytokines in the supplemented animals. The authors suggest their results show that 2′-FL promotes a more mature immune response. However, no evidence was provided demonstrating that the observed changes actually resemble a more mature immune system. The result also demonstrates that, at the timepoints analysed, the immune system is actually in flux.

The evidence from both of these rodent studies provide further support that 2′-FL may mediate changes in immune signalling and trafficking. However, there is still no demonstration that 2′-FL is able to perform this function and thus provide a benefit in infants.

Alleviation of allergic responses

No new studies were provided as supporting evidence, nor new studies identified from a literature search (performed April 2021).

Learning and Memory

Animal studies

Three studies examining 2′-FL supplementation on the learning and memory of rodents (Vazquez et al. 2015; Vazquez et al. 2016; Oliveros et al. 2016). In the first study (Vazquez et al. 2015), long term (12-week) supplementation of adult mice with 2′-FL$_{chem}$ resulted in improved performance in a conditioning task using a Skinner box, and in a learning task in an IntelliCage system, compared to unsupplemented animals. This was associated with increased long-term potentiation in the hippocampus and significantly increased expression of protein markers for: synaptic signal transduction (PSD-95); rapid excitatory synaptic transmission (CaMKII); and synaptic plasticity (BDNF). All three markers were increased in the hippocampus, but BDNF was also increased in the striatum. The results from this study indicate that supplementation with 2′-FL can have an impact on development of memory and associative learning in adult mice. The authors concede that the mechanism of action has not been elucidated, especially as there is no evidence that ingested 2′-FL can cross the blood-brain barrier.

In the second study (Vazquez et al. 2016), long term (5-week) supplementation of adult rats with 2′-FL$_{chem}$ did not significantly improve performance in a conditioning task using the Skinner box, when compared to either unsupplemented animals or supplemented animals that had been vagotomised. Supplemented rats did, however, show increased long-term potentiation compared to both unsupplemented and supplemented + vagotomised animals. The authors suggest that data from this study shows a potential role of the vagus nerve in mediating the effects of 2′-FL on the central nervous system.

In the third study (Oliveros et al. 2016), performance in different behavioural tests used for studying learning and memory were compared based on age (4–6 week old vs 1 year old) and supplementation ± 2′-FL$_{chem}$ (from day 3 of life to weaning at 21 days). Supplementation did not significantly change response in a novel object recognition task in young rats. However, when the same rats were 1 year old, the supplemented animals spent significantly more time exploring the novel object. Using a Y maze test—where there was an initial blocked arm that was removed—adult rats from the supplemented group showed a significantly reduced latency; an increased number of animals visited the novel arm; and there was a nonsignificant increase in time spent in the novel arm compared to unsupplemented animals. Data was not collected on the young rats. Using the Morris water maze, neither supplementation nor age affected latency time or time spent in the same area as the platform. Although the author claims their data shows supplementation of pups was beneficial for cognitive skills in adult rats, this was only really demonstrated in one visual recall task, potentially showing effects on long-term memory.

The data from these studies indicate that 2′-FL may have the potential to influence learning and memory in rodents. However, the mechanism has not been identified, and there is still no demonstration that this occurs in infants.

Gut motility

Ex vivo study

The applicant submitted a study investigating if 2′-FL had an impact on gastrointestinal contractility in an *ex vivo* murine colon preparation (Bienenstock et al. 2013). The data showed that fucose and the fucosylated molecules 2′-FL$_{chem}$ and 3'-FL$_{chem}$ did reduce the frequency, amplitude and velocity of colonic motor contractions. The strongest effect was observed with 3'-FL. The authors noted that 2′-FL is not present in mouse milk, and suggested the reduced effect seen with 2′-FL compared to 3'-FL may be related to the murine intestinal cells not being adapted to respond to 2′-FL. The authors stated that further studies are required to determine if reduced gut motility could reduce functional gut disorders and colic in infants.

7.3 Key findings of the health benefit assessment

There is consistent evidence that 2′-FL is bifidogenic in infants and young children based on both *in vitro* and human studies. Specific strains can metabolise 2′-FL, providing a selective growth advantage over other species of bacteria. FSANZ notes limitations in the evidence base (see Section 2 of A1155 Review Supporting Document 2[15]). However, it has been concluded that:

- for infants, the addition of 2′-FL to infant formula leads to a *Bifidobacterium*-enriched microbiota that is more similar to that observed in breastfed infants than in those fed unsupplemented formula. However, the size of the effect of 2′-FL on bacterial populations is difficult to estimate. Evidence for a link between the presence of 2′-FL in human milk or formula and any specific health outcome is limited to secondary outcomes of one randomised control trial and observational studies of lower quality.

- evidence that a bifidogenic effect occurs in children fed formulated supplementary foods for young children (FSFYC) supplemented with 2′-FL is very limited. However, there is no reason to expect that the effect demonstrated in infants and adults would not occur in young children.

There is also plausible evidence that intestinal infection with invasive *C. jejuni* and subsequent pathogenesis is inhibited by 2′-FL. This has been demonstrated: by *in vitro* studies that have identified mechanism of action; animal studies where supplementation reduced infection, invasiveness and disease severity; and human studies where there is epidemiological evidence supporting 2′-FL having an anti-infective health effect in infants and young children.

Although there is a range of evidence that 2′-FL can mediate changes in the function of the immune response, the intestinal barrier, learning and memory and gut motility, there is limited information demonstrating this effect in humans. FSANZ concludes that for these stated health effects, the claims are not supported by the evidence.

8 Summary and conclusions

2′-fucosyllactose (2′-FL) is one of the predominent oligosaccharides identified in human milk, expressed by 70-80% of women, known as secretors. While the levels of 2′-FL varies throughout the lactation period and per individual, the overall range found in human milk from secretors is 0.6 – 7.8 g/L. The maximum level permitted in the Code is equivalent to 2.4 g/L. The concentration of 2′-FL$_{micro}$ requested by the applicant to be added to IFP and FSFYC is 2.0 g/L, which is well within the range found in human milk from secretors and below the maximum permitted level. As 2′-FL is already permitted in the Code, the purpose of this assessment is to determine the risk and safety of 2′-FL produced by a new production strain and with it's own specifications.

Estimates of dietary intakes of 2′-FL based on a concentration of 2.4 g/L for infants up to 12 months ranged between 0.1 – 0.33 g/kg bw/day at the mean and 0.2 – 0.66 g/kg bw/day at the 90th percentile, and for children 2-3 years from 0.077 – 0.15 g/kg bw/day at the mean and 0.15 – 0.31 at the 90th percentile. These ranges reflect typical intakes of infants and young children who receive human milk from mothers who are secretors.

Chemically and structurally, 2′-FL produced by fermentation was shown to be identical to the naturally occurring oligosaccharide found in human milk. The final product was shown to be free of fermentation-derived contaminants. The purity and other constituents of the final product have been identified and listed in the specification for the product. The shelf-life and specifications are appropriate for addition to IFP and FSFYC.

The examination of the source organisms concluded there were no safety concerns. The host organism is a well characterised bacterium with a recognised safe history of use for the production of industrial compounds and human therapeutics. It is neither pathogenic nor toxigenic. The production strains were confirmed to contain the introduced DNA, which was shown to be inherited across several generations.

FSANZ has previously determined that there are no safety concerns associated with the addition of 2′-FL to IFP and FSFYC at concentrations up to 2.4 g/L. A number of new studies evaluated as a part of this application did not indicate a reason to change this conclusion.

2′-FL was not genotoxic *in vitro* or *in vivo*. No adverse effects were observed in multiple subchronic oral toxicity studies in neonatal rats at doses up to 5000 mg/kg bw/day, or in older rats at doses > 7000 mg/kg bw/day. Three-week studies with neonatal piglets administered formula containing 2′-FL at concentrations up to 4 g/L also found no adverse effects. In human studies, infant formula supplemented with 2′-FL was well tolerated with no significant increases in adverse events. 2′-FL was also well tolerated in studies with children and adults.

Protein was not detected in the 2′-FL product, therefore 2′-FL is unlikely to pose an allergenicity concern.

Based on the available information from feeding studies, a nutritional assessment found no difference in growth for infants fed formula with 2′-FL compared to a control formula. Combined with the limited gastrointestinal absorption of 2′-FL, there is no evidence to indicate a nutritional concern at concentrations that are typically observed in human milk.

An assessment of the proposed beneficial role of 2′-FL in the normal growth and development of infants or children concluded that there was evidence to support a bifidogenic effect and inhibition of infection by pathogenic strains of *Campylobacter jejuni*.

In vitro studies clearly showed specific strains of *Bifidobacterium* can metabolise 2′-FL, providing these organisms a selective growth advantage. Studies examining the faecal microbiome of breastfed infants of mothers of known 2′-FL secretor status corroborate the *in vitro* studies, demonstrating the growth advantage afforded to bifidobacteria when fucosylated oligosaccharides, including 2′-FL, are present in the infant diet. Similarly, a feeding study performed in adults showed that 2′-FL supplementation resulted in increased relative abundance *of Bifodobacterium*. The data from these studies supports the likelihood that 2′-FL has a bifidogenic effect in infants and young children.

Available evidence supports the likelihood that 2′-FL can bind to invasive strains of *C. jejuni*, preventing attachment and subsequent infection and pathogenesis. The mechanism of action was established in an *in vitro* study which showed that α1,2-fucosylated oligosaccharides, including 2′-FL, specifically inhibit attachment of invasive *C. jejuni* to the intestinal H2 antigen binding receptor. The plausibility of this anti-infective health effect occurring in infants was demonstrated in an *in vivo* murine model. Animals supplemented with 2′-FL and challenged with invasive *C. jejuni* showed decreased intestinal infection, invasiveness and disease severity. A human study showing decreased incidence of *Campylobacter*-associated diarrhoea in infants and young children of mothers with a higher proportion of 2′-FL in their milk provides additional epidemiological evidence of 2′-FL having an anti-infective health effect.

FSANZ notes that the Independent Expert Advisory Group (IEAG) for A1155 concluded that there are many different factors in the microbiome which influence infant health, and that it is not possible to determine a linear effect from the presence of one substance in human milk and a specific health outcome. The IEAG advised that FSANZ should focus on specific mechanisms by which health outcomes could be modified by 2′-FL.

In summary, 2′-FL is naturally present in human milk in a range of concentrations, providing a history of safe human exposure for breastfed infants. The conclusion from the assessment is there are no public health and safety concerns associated with the addition of 2′-FL to IFP and FSFYC at the requested level of 2 g/L, or at higher estimated dietary intakes based on 2.4 g/L, which is the level already permitted in the Code.

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