

6 December 2021
182-21

Supporting document 1

Food technology and safety assessment – Application A1233 **2'-FL from new GM source for infant formula**

Executive summary

Friesland Campina Ingredients has applied to amend Schedules 3 and 26 of the Australia New Zealand Food Standards Code (the Code), to include a new source organism and specification for 2'-fucosyllactose (2'-FL).

The new source organism is a genetically modified (GM) *Escherichia coli* K-12 derived strain expressing the α -1,2-fucosyltransferase gene from *Bacteroides vulgatus*. The fucosyltransferase enzyme mediates the intracellular transfer of a fucosyl group to lactose, producing 2'-FL.

The Code already permits 2'-FL from a different source organism for addition to infant formula products. The maximum permitted level is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment is therefore to assess the safety of 2'-FL produced by the new production strain.

The Applicant's 2'-FL is chemically and structurally identical to the naturally occurring substance isolated from human milk. Stability studies conducted by the Applicant demonstrated that the final product is suited for the intended food uses. The Applicant has requested that the specification established in the EU in 2019 be applied to their 2'-FL_{micro} preparation. Multi-batch analyses showed that the final product is consistently within the proposed specifications, with impurities and/or contaminants resulting from the fermentation process either minor or absent.

E. coli K-12 has a long history of use for the production of recombinant proteins and does not pose a risk to humans. Analyses of the gene donors also confirmed there were no safety concerns. Characterisation of the production strain confirmed the expression plasmid carrying the α -1,2-fucosyltransferase gene was both genetically stable and fully functional.

FSANZ has previously determined that there are no safety concerns associated with the addition of 2'-FL to infant formula products at concentrations up to 2.4 g/L, which is within the range of naturally occurring levels in human milk from the majority of women (0.6 – 7.8 g/L). Newly available information relating to previously assessed studies of 2'-FL produced by the Applicant and another manufacturer 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

juvenile and adult 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.

The proposed specification does not raise any safety concerns. 2'-FL is unlikely to pose an allergenicity concern because the protein content of the 2'-FL product is below the limit of quantitation.

FSANZ's previous assessments of 2'-FL found no evidence of a nutritional concern at concentrations typically observed in human milk. No new information was provided that would indicate a need to change these conclusions. The evidence for a beneficial role of 2'-FL in the normal growth and development of infants will be reassessed in a review to be completed by March 2026.

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
GLP	good laboratory practice
g	gram
GM	genetically modified
GOS	galactooligosaccharide
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
IFP	infant formula products
kg	kilogram
L	litre
LNnT	lacto-N-neotetraose
LOD	limit of detection
mg	milligram
NMR	nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
scFOS	Short-chain fructooligosaccharide
µg	microgram

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1 Introduction

FSANZ has received an Application from Friesland Campina Ingredients to amend Schedules 3 and 26 of the Australia New Zealand Food Standards Code (the Code), to include a new source organism and specification for 2'-fucosyllactose (2'-FL).

The new source organism is a genetically modified (GM) *Escherichia coli* K-12 derived strain expressing the α -1,2-fucosyltransferase gene from *Bacteroides vulgatus*. The fucosyltransferase enzyme mediates the intracellular transfer of a fucosyl group to lactose, producing 2'-FL.

The Code already permits 2'-FL from a different source organism for addition to Infant Formula Products (IFP). The maximum permitted level is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment is therefore to assess the safety of 2'-FL produced by the new production strain.

2 Food Technology Assessment

The food technology assessment provides information on the chemical identification, physicochemical properties and specifications for 2'-FL. 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.

2.1 Chemical properties

2.1.1 Chemical names, properties, and structures

2'-FL is an oligosaccharide that is naturally found in human milk. The substance has been isolated from human milk, or produced using chemical synthesis or microbial fermentation for research and commercial purposes. This Application is in relation to the production of 2'-FL by microbial fermentation and subsequent purification of the substance for commercial purposes.

The chemical names, properties and structures for 2'-FL have been previously described in detail in A1155 (gazetted 26 March 2021, [FSANZ 2019a](#)) and A1190 ([FSANZ 2021](#)). A summary of the nomenclature and chemical properties of 2'-FL is presented in Table 2.1. The Applicant's 2'-FL is referred to as 2'-FL_{micro} in this assessment, as opposed to the 2'-FL_{human} isolated from human milk and 2'-FL_{chem} produced by chemical synthesis.

Table 2.1 *The 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-fucosyllactose 2'-O-L-fucosyl-D-lactose 2'-Fucosyl-D-lactose 2'-fucosyllactose
Alternative names ¹	fucosyl- α -1,2-galactosyl- β -1,4-glucose Fuc- α -(1→2)-Gal- β -(1→4)-Glc α -L-Fuc-(1→2)- β -D-Gal-(1→4)-D-Glc 2'-FL
CAS registry number	41263-94-9
Chemical formula	C ₁₈ H ₃₂ O ₁₅
Molecular weight	488.44 g/mol

¹ Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose

The Applicant's 2'FL (2'-FL_{micro}) is available as a white powder and will be marketed as a nutritive substance ingredient to be added to IFP. The general physical properties of the final powder product are listed in Table 2.2 below.

Table 2.2 *General physical properties of the Applicant's 2'-FL_{micro}*

Property	2'-FL _{micro}
Form	Homogenous powder
Colour	White
Purity	≥ 90%
Stability (shelf-storage conditions) (25°C/ 60% humidity)	36 months
Stability (accelerated storage conditions) (40°C/ 75% humidity)	6 months

2'-FL is a trisaccharide consisting of the monosaccharides L-fucose, D-galactose and D-glucose. It can also be described as the disaccharide D-lactose at the reducing end and the monosaccharide L-fucose at the terminal galactose in α (1→2) linkage to form the trisaccharide (Figure 2.1).

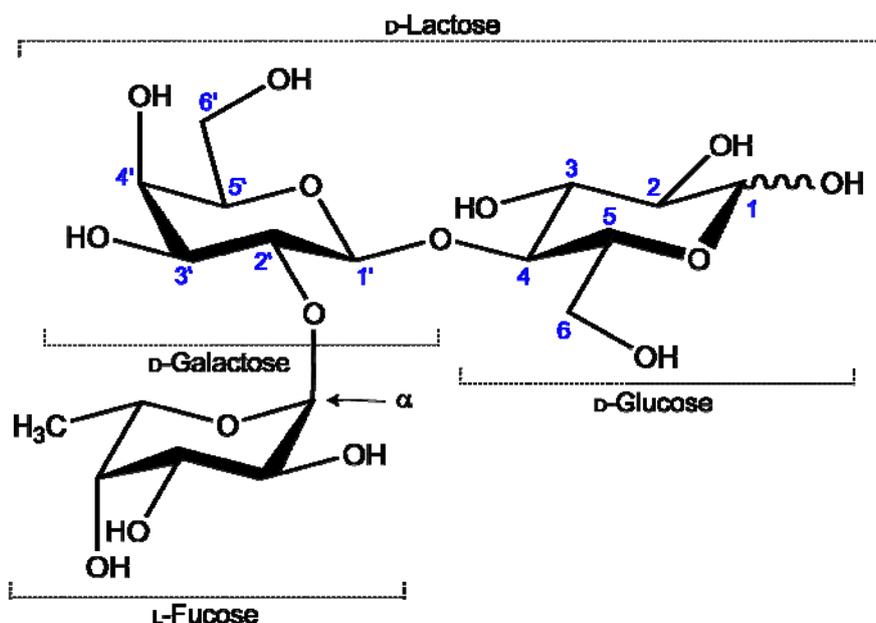


Figure 2.1 Molecular structure of 2'-FL

2.1.2 Structural identification

The identity of the Applicant's 2'-FL_{micro} was confirmed by nuclear magnetic resonance (NMR) spectroscopy performed by third-party analytical service providers. The Applicant developed a method using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to characterise the final product. The structural and chemical equivalence of Applicant's 2'-FL_{micro} was compared to a 2'-FL standard, of which the 2'-fucosyllactose was identified and quantified with quantitative NMR (qNMR). The analytical methods used (NMR and HPAEC-PAD) are accepted analytical techniques for the determination of oligosaccharides, as clearly articulated in Application A1190 ([FSANZ 2021](#)). The Applicant has provided sufficient information on how these methods were used.

The analyses performed on five non-consecutive batches of the Applicant's 2'-FL_{micro} confirmed its identification with a 2'-FL standard. The multi-batch analytical results indicated that 2'-FL was the primary constituent of the final product, with a purity of no less than 90%.

2.1.3 Structural equivalence of 2'-FL based on source

FSANZ has previously reviewed structural analyses for 2'-FL_{human}, 2'-FL_{chem} and 2'-FL_{micro} and confirmed that 2'-FL_{micro} is chemically and structurally identical to the naturally occurring 2'-FL isolated from human milk and the chemically synthesized 2'-FL ([FSANZ 2019a](#)).

2.2 Analytical methods for detection

Currently there are no internationally recognised methods for the analysis of 2'-FL. However there is a large body of scientific literature on the isolation, quantitation and characterisation of human milk oligosaccharides (Ruhaak et al. 2012, Grabarics et al. 2017). The Applicant has developed appropriate analytical methods to characterise 2'-FL_{micro} in addition to methods developed by third-party analytical service providers. Methods that were developed by the Applicant have been validated by a third party. The methodology including method validation data was submitted to FSANZ. The information provided included:

- detailed methods to confirm the identity of 2'-FL using NMR, a well-established analytical technique for structural determination. These method have sufficient sensitivity to detect other by-products and contaminants. Low levels (<2%) of two

other human milk sugars (3-fucosyllactose and lactodifucotetraose) were detected in the Applicant's 2'-FL_{micro} product

- details of the methodology used to characterise 2'-FL_{micro} using HPAEC-PAD, including a method validation report by a third party confirming that the method was fit for purpose
- detailed method used to measure the protein content in purified 2'-FL_{micro} product using a modified Bradford assay, including a method validation report by a third party confirming that method was fit for purpose
- a number of internationally recognised test methods (ISO¹) were employed to determine other impurities and contaminants such as metals, microbial presence and toxins in the final 2'-FL_{micro} product
- detailed method used to evaluate the absence of residual genetic materials from the *Escherichia coli* (*E.coli*) K12 production strain by quantitative polymerase chain reaction (qPCR)
- detailed information regarding reagents, reference materials and standards, solution preparation, run parameters and procedures, evaluation and calculations, chromatograms and method validation data.

The Applicant conducted analyses on five non-consecutive batches of 2'-FL_{micro} product. The multi-batch analyses results demonstrated that 2'-FL_{micro} can be produced according to the proposed manufacturing process, with the purity and impurities profile consistent with the specifications proposed by the Applicant (see Section 2.4).

2.3 Manufacturing processes

2.3.1 Fermentation and purification

The manufacturing process consists of two stages: fermentation and purification. 2'-FL_{micro} is produced via batch fermentation, using glucose as the primary energy and carbon source. Precursors for 2'-FL are glucose and disaccharide α -lactose, which is comprised of galactose and glucose. The production host is a GM strain of *E.coli* K12 (See Section 3). The fermentation is performed in a well-defined minimal medium and under sterile conditions. Microbial fermentation is a well-established production method for food and food ingredients.

After fermentation, *E.coli* cells are separated from the growth medium by microfiltration. Since the 2'-FL has been excreted into the medium, the product is purified from the medium with multiple filtration steps and chromatography separation (Figure 2.2). The purified 2'-FL is then concentrated using nanofiltration or reverse osmosis, then spray dried in a spray drier to obtain high purity 2'-FL_{micro} powder.

The Applicant has provided details of the production and purification processes, including identification and specifications including certificate of analysis of the raw materials and processing aids. Detailed information of the production host organism (*E.coli* K12), methodology for the purification and isolation steps, and quality controls are also submitted to FSANZ.

¹ International Organization for Standardization

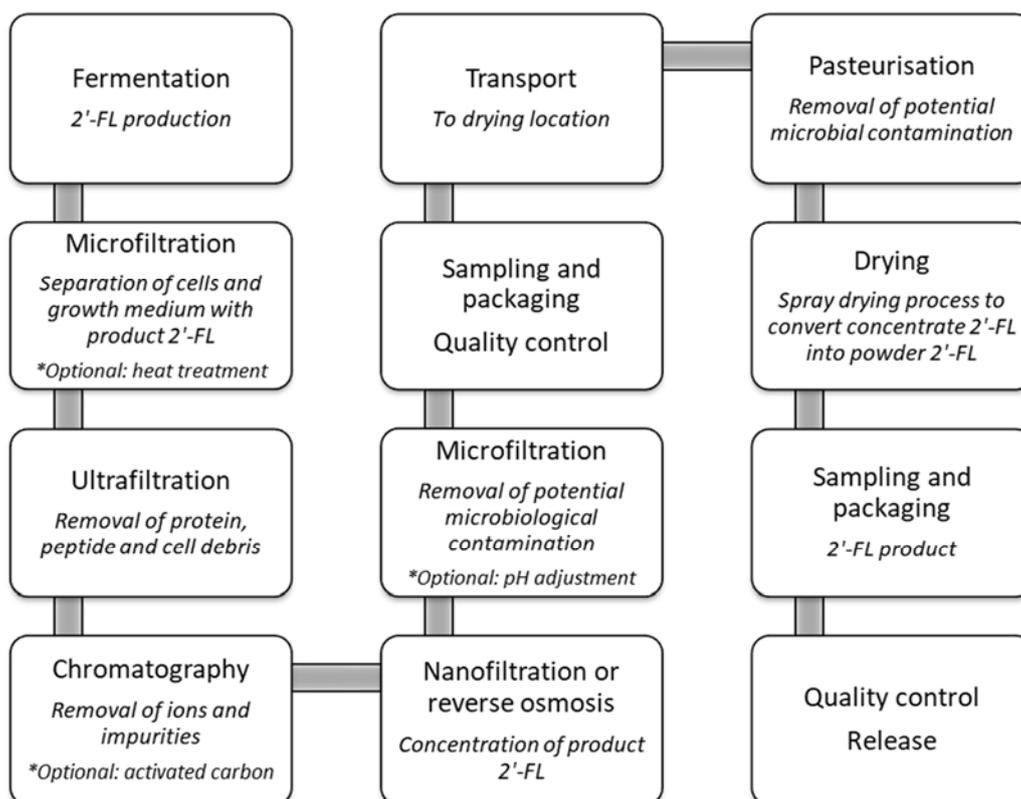


Figure 2.2 Flow chart of the manufacturing process

2.3.2 Fermentation by-products

The Applicant's 2'-FL_{micro} final product was analysed to determine if there were any carbohydrate impurities and contaminants as a result of the fermentation process. These contaminants could include the bacterial DNA (production strain *E.coli* K12), proteins, microbial contamination and microbial toxins such as *E.coli*-derived endotoxins and fungal-derived aflatoxins. The presence of these microbial toxins could indicate microbial contamination of the fermentation system.

During the fermentation process, it is expected that other carbohydrate impurities will be produced or will remain in the fermentation medium. The typical carbohydrate impurity profile showed the presence of monosaccharides glucose, galactose and fucose and disaccharides lactose and allo-lactose. These substances are naturally found in human milk and their presence was determined using HPAEC-PAD. Multiple batch analyses showed that amounts of these substances were consistently within the specifications proposed by the Applicant, with the either low levels detected ($\leq 3\%$) or none detected at the detection limits.

The presence of residual bacterial DNA in 2'-FL_{micro} product was determined using three qPCR assays. These qPCR assays have been developed to detect unique chromosomal target regions of production strain *E.coli* K12 (E997), with the LOD ranging from 4.5×10^{-7} ng/ μ L to 4.5×10^{-4} ng/ μ L cDNA. Concentration of total protein in 2'-FL_{micro} product was measured using a Bradford protein assay with the LOQ of 0.01% (m/m). Results from analyses of five non-consecutive batches showed that there was no detectable levels of residual *E.coli* DNA and proteins present in the final product. The absence of microbial contamination and microbial toxins (endotoxins and aflatoxin M₁) was confirmed using internationally recognised methods. Multi batch analyses of the final 2'-FL_{micro} product showed that microbial contamination and microbial toxins were consistently absent or within the specifications proposed by the Applicant.

The Application included a description of quality control measures and analytical methods for parameters such as pH and water content. Multi batch analyses demonstrated that the pH and water content for 2'-FL product were within the proposed specifications. The presence of other contaminants and/or impurities including ash, metals (aluminium, lead, arsenic, cadmium and mercury), nitrite, nitrate and scorched particles from the growth medium were determined using internationally recognised methods. Results from multi-batch analyses showed that these contaminants and/or impurities were either insignificant or absent in five non-consecutive batches of final 2'-FL_{micro} product.

2.3.3 Stability

Stability studies were conducted on 2'-FL_{micro} product under ambient shelf-storage conditions (25°C/60% relative humidity) and accelerated storage conditions (40°C/75% relative humidity). The data demonstrated that 2'-FL_{micro} did not show significant changes in the appearance, moisture, microbiological profiles, impurity profiles or degradation of the ingredient over the study duration. The Applicant's 2'-FL_{micro} is stable for at least 6 months under accelerated storage conditions and at least 36 months under standard shelf-storage conditions.

The stability of 2'-FL_{micro} has been previously assessed as part of A1155 ([FSANZ 2019a](#)) and A1190 ([FSANZ 2021](#)). It has been reported that 2'-FL_{chem} is stable when incorporated into infant formula products and the reconstitution process of dry infant formula products has no significant effect on 2'-FL levels ([FSANZ 2019a](#)). Stability studies conducted on 2'-FL_{micro} confirmed that 2'-FL_{micro} is stable when incorporated into IFP and the reconstitution process of dry IFP has no significant effect on 2'-FL levels ([FSANZ 2021](#)).

While no stability studies specifically for the Applicant's 2'-FL_{micro} in a food matrix such as IFP was provided, it is unlikely that the Applicant 2'-FL_{micro}'s stability profiles differ to 2'-FL_{micro} and 2'-FL_{chem} based on their chemical and structural equivalence (see Section 2.1.3). In addition, the impurities present in the preparation of the Applicant's 2'-FL_{micro} are not suspected to cause or accelerate the degradation of 2'-FL in the final product (see Section 2.3.2).

2.4 Product specifications

The Applicant has established product specifications for 2'-FL_{micro} however has requested that the specification established by the European Union (EU) in 2019 (European Union 2019) be applied to their 2'-FL preparation. A relevant specification for 2'-FL is listed in Schedule 3 under section S3—2(2) of the Code ([A1155](#) gazetted 25 March 2021).

The Applicant's specification prescribes maximum amounts for carbohydrate impurities and other contaminants resulting from the manufacturing process. These specifications are dissimilar to those listed in Schedule 3 under section S3—40.

The proposed specifications for the Applicant's 2'-FL_{micro}, and existing specifications for 2'-FL established in the EU and in the Code, are presented in the Table 2.3. Submitted certificates of analysis for five non-consecutive batches of 2'-FL_{micro} demonstrated that the product consistently meets the specifications proposed by the Applicant across all batches tested. Multi-batch analyses showed that 2'-FL_{micro} also meets requirements for lead, arsenic, cadmium and mercury prescribed in Schedule 3 under section S3—4 of the Code. The finished product also meets the specifications established in the EU for 2'-FL produced with *E.coli* K12. Additionally, either an internationally recognised method, or a method that was developed by the Applicant and/or a third-party analytical service provider was specified for the analysis of the Applicant's 2'-FL_{micro} for each parameter in the proposed specifications.

Table 2.3 Proposed specifications for 2'-FL

Parameters	Friesland Campina Specifications	EU Specifications ¹	Specifications in the Code (S3—40) ²
Physical and chemical			
Appearance	Homogenous powder	Powder	Powder or agglomerates
Appearance colour	White	White to off-white	White to off white
2'-FL content	≥ 90%	≥ 83%	≥ 94%
pH	3.0 to 7.5 (10% solution)	3.0 to 7.5 (20°C, 5% solution)	3.2 to 5.0 (20°C, 5% solution)
Water	≤ 5.0%	≤ 9.0%	≤ 5.0%
Ash (sulphated)	≤ 0.2%	≤ 2.0%	≤ 1.5%
Lactose	≤ 3%	≤ 10.0% (D-lactose)	≤ 3.0% (D-lactose)
Allo-lactose	≤ 2%	NS	NS
Glucose	≤ 2%	NS	NS
Galactose	≤ 2%	NS	NS
Fucose	≤ 2%	≤ 2.0% (L-fucose)	≤ 1.0% (L-fucose)
Difucosyl-D-lactose	NS	≤ 5.0%	≤ 1.0% (difucosyllactose)
2'-Fucosyl-D-lactulose	NS	≤ 1.5%	≤ 1.0%
Sum of saccharides	NS	≥ 90.0% (sum of 2'-Fucosyllactose, D-Lactose, L-Fucose, Difucosyl-D-lactose, 2'-Fucosyl-D-lactulose)	≥ 96.0% (sum of 2'-FL, lactose, difucosyllactose and fucose)
Residual proteins	≤ 0.01%	≤ 0.01%	≤ 0.01%
Acetic Acid	NS	≤ 1.0%	≤ 1.0% (as free acid and/or sodium acetate)
Nitrite	≤ 1 mg/kg	NS	NS
Nitrate	≤ 50 mg/kg	NS	NS
Scorched particles	Max. disc A	NS	NS
Metals			
Aluminium	≤ 4.8 mg/kg	NS	
Lead	≤ 0.05 mg/kg	NS	≤ 0.1 mg/kg (S3—40) ≤ 2 mg/kg (S3—4)
Arsenic	≤ 0.1 mg/kg	NS	≤ 1 mg/kg (S3—4)

Cadmium	≤ 0.01 mg/kg	NS	≤ 1 mg/kg (S3—4)
Mercury	≤ 0.05 mg/kg	NS	≤ 1 mg/kg (S3—4)
Microbiological			
Aerobic mesophilic bacteria total count	≤ 3,000 cfu/g	≤ 3000 cfu/g	≤ 500 cfu/g (total plate count)
Yeasts	≤ 10 cfu/g	≤ 100 cfu/g	≤ 10 cfu/g
Moulds	≤ 10 cfu/g	≤ 100 cfu/g	≤ 10 cfu/g
<i>Salmonella</i>	Absent in 25 g	NS	Absent in 25 g
Enterobacteriaceae	Absent in 10 g	NS	Absent in 10 g
<i>Cronobacter (Enterobacter) sakazakii</i>	Absent in 25 g	NS	Absent in 10 g
<i>Listeria monocytogenes</i>	NS	NS	Absent in 25 g
<i>Bacillus cereus</i>	≤ 100 cfu/g (presumptive)	NS	≤ 50 cfu/g
<i>E. coli</i>	Absent in 10 g	NS	NS
<i>Staphylococcus aureus</i>	Absent in 1 g	NS	NS
Sulphite reducing clostridia spores	≤ 30 cfu/g	NS	NS
<i>Clostridium perfringens</i>	Absent in 1 g	NS	NS
Residual Endotoxins	≤ 10 EU/mg	≤ 10 EU/mg	≤ 10 EU/mg
Aflatoxin M1	≤ 0.2 µg/kg	NS	NS
GMO detection	Negative	NS	NS

Abbreviations: NS: Not Specified; EU: endotoxin unit; cfu: colony forming unit

¹ European Union (2019) Specifications for 2'-FL produced with genetically modified *E. coli* K12. The applicant has requested that this specification be applied to their 2'-FL preparation by being referenced or included in Schedule 3 of the Code.

² Schedule 3 Identity and Purity (S3—40) Australia New Zealand Food Standards Code

2.5 Food technology conclusion

The Applicant's 2'-FL_{micro} is chemically and structurally identical to the naturally occurring substance isolated from human milk (2'-FL_{human}) and chemically synthesized substances (2'-FL_{chem}). The Code currently permits 2'-FL to be used as a nutritive substance in IFP, which generally has a shelf life of two years. The final product is stable for up to 36 months at shelf-storage conditions, supporting the position that the Applicant's 2'-FL_{micro} preparation is suited for intended food uses. The Applicant has requested that the specification established in the EU in 2019 be applied to their 2'-FL_{micro} preparation. Multi-batch analyses showed that the

final product is consistently within the proposed specifications, with impurities and/or contaminants resulting from the fermentation process either minor or absent.

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 K-12 is the most common bacterial laboratory strain in use globally. It was isolated from a human stool sample in 1922 (Bachmann 1996). Comparative genome sequencing and proteomic analysis of the K-12 strain and its derivatives, to well characterised pathogenic strains, have identified differences in the K-12 cell wall structure associated with reduced ability to colonise the human intestinal tract, and absence of adhesive proteins and virulence factors that meet requirements for pathogenicity (Bachmann 1996; EPA 1997; Sahl et al. 2013). These studies have also shown reduced toxin production in K-12 strains and absence of plasmids encoding antibiotic resistance. Under the U.S. National Institutes of Health (NIH) Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines², 2016), *E. coli* K-12 is classified as a Risk Group 1 agent which is reserved for organisms that are not human or animal pathogens.

E. coli K-12 has a long history of use in the human biopharmaceutical industry, with ~30% of currently approved recombinant therapeutic proteins in the United States (US) being produced in *E. coli* K-12, starting with the US FDA approval of biosynthetic human insulin in 1983 (Huang et al. 2012; Jozala et al. 2016). The use of this bacterium as a source for the production of food enzymes began in the 1980s (JECFA 1991). *E. coli* K-12 is permitted as a source microorganism for the production of chymosin in the Food Standards Code. Schedule 26 permits the production of 2'-O-fucosyllactose by *Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*. *E. coli* K-12 is considered a model organism and has been thoroughly characterised for use in research and industry, it is therefore considered a safe organism.

The host strain, ATCC 55151 (GI 724 strain), has been optimised for large-scale production, through standard manipulation of metabolic pathways, to ensure sufficient pools of GDP-fucose and lactose, the two primary reactants for production of 2'-FL. The intermediate host strain has been identified as *E. coli* E638 and the production strain as *E. coli* E997. The Applicant has undertaken complete genome sequencing that confirms the host strain lacks virulence genes and is very closely related to the *E. coli* K12 MG155 and W3110 lineages.

Two of the three DNA segments in strain E638 that are not formally derived from the ancestral wild-type *E. coli* K12 include a tetracycline resistance gene and the kanamycin resistance gene. E638 is transformed with plasmid pG217 to generate the production strain

² [NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules \(NIH Guidelines\) - April 2019](#)

E997, and the plasmid includes the TEM116 beta-lactamase gene that encodes ampicillin resistance. However, the risk from the presence of antimicrobial resistance in this organism is managed through the production process. Data provided by the Applicant demonstrates that the final product is highly purified protein (see section 4.2.3), with neither the production strain, nor its DNA, detectable in the final product. The remaining biomass from the fermentation is also inactivated. Data provided also indicate the manufacturing process involves appropriate controls to prevent microbial contamination, and the microbial quality of the final enzyme preparation meets the specifications required by the European Union³.

3.1.2 Gene donor organism(s)

The synthetic *futN* gene encoding α -1,2-fucosyltransferase was sourced from *Bacteroides vulgatus*. *B. vulgatus* ATCC 8482 is a gut commensal organism (Li et al. 2016) and a Biosafety Level 2 organism. As the gene is synthetically produced there are no safety concerns.

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. No risks were identified in the assessment of the gene donors.

3.2 Characterisation of the genetic modification(s)

3.2.1 Preparation of the 2'-FL production strain

The *E. coli* K-12 production strain E997 was generated by transformation of E638 with an expression plasmid containing the α -1,2-fucosyltransferase gene. The fucosyltransferase mediates the transfer of a fucosyl group from GDP-fucose to lactose thus making 2'-fucosyllactose.

The fucosyltransferase gene *futN* was sourced from *B. vulgatus*, and is flanked by the leftward promoter from lambda phage and a terminator from a well characterised *E. coli* K-12 gene. Also included on the expression plasmid, are two expression cassettes for selectable markers: an antibiotic resistance gene, used only during the plasmid developmental phase and not during the production of 2'-FL; and a metabolic marker gene for maintaining presence of the plasmid in the production strain, when grown in minimal media. A fourth expression cassette contains a regulator to increase the intracellular pool of the fucosyl donor, GDP-fucose.

3.2.2 Characterisation of the genetically modified organisms

Characterisation of introduced DNA

Data was provided showing the production of 2'-FL after five distinct fermentation runs and from growth studies performed in minimal media. These data confirmed the presence and functionality of the plasmid expression system in the production strain E997.

Data was also provided from whole genome sequence analysis. The sequence confirmed the production strain contained the expression plasmid, whereas the expression plasmid was absent in the *E. coli* reference genome.

³ [European Union \(2019\)](#)

Genetic stability and inheritance of the introduced DNA

The Applicant provided data from whole genome sequencing, comparing the production strain at generation 0 and generation 100. No changes in the DNA sequence were identified in the plasmid expression cassettes, indicating the plasmid was genetically stable and was inherited across generations.

3.3 Key findings of the microbiological and biotechnology assessment

E. coli K-12 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 strain confirmed the expression plasmid carrying the α -1,2-fucosyltransferase gene was both genetically stable and fully functional.

On the basis of the data provided, no potential safety concerns were identified in the assessment of the 2'-FL production strain E997.

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 Applications A1155 and A1190 (FSANZ 2019a; FSANZ 2020; FSANZ 2021).

Data derived using Glycom's 2'-FL_{micro}, Glycom's 2'-FL_{chem}, Chr. Hansen's 2'-FL_{micro} and Friesland Campina's 2'-FL_{micro} were evaluated, as well as studies of 2'-FL_{micro} in combination with other oligosaccharides found in human milk.

As noted in Section 2.1.2, 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 assessments of 2'-FL_{micro} produced by Glycom and Chr. Hansen confirmed their chemical and structural identity to 2'-FL_{human}. Studies with all these forms of 2'-FL are therefore considered to be relevant for assessing the safety of the Applicant's 2'-FL_{micro}.

Studies previously 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), lacto-N-fucopentaose I (LNFP-I) 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
- Short-term toxicity studies of 2'-FL in combination with DFL or LNFO-I in neonatal rats
- A short-term toxicity study in rats of 2'-FL in combination with four other oligosaccharides found in human milk
- Human clinical studies in infants, children and adults.

The assessments for A1155 and A1190 concluded that there are no safety concerns

associated with the addition of 2'-FL to IFP at concentrations up to 2.4 g/L.

4.2 Newly available data

The Applicant provided full reports of two *in vitro* genotoxicity studies with 2'-FL_{micro}, a 14-day dose-range finding study in rats and a 90-day toxicity study in rats. Results of the genotoxicity study and 90-day toxicity study are summarised in van Berlo et al. (2018), which FSANZ reviewed as part of the assessment of A1155.

An additional clinical study in children published since FSANZ's last evaluation was identified in a literature search conducted in PubMed on 12th August 2021 for papers published from 29th April 2021 (the date of FSANZ's last previous literature search) using the search terms '2'-fucosyllactose OR 2'-O-Fucosyllactose'.

4.2.1 Toxicological studies with 2'-FL

Short-term toxicity studies conducted with the Applicant's 2'-FL_{micro}

14-day dietary dose-range finding study with 2'-FL_{micro} in rats (Triskelion 2016) Regulatory status: Non-GLP; Non-guideline

Groups of four male Wistar (CrI:WI(Han)) rats aged 6-7 weeks were fed diets containing 0, 3, 6 or 10% 2'-FL (purity 94%) for 14 days, equal to 0, 2670, 5050 or 7990 mg/kg bw/day. Clinical signs were monitored daily and body weights were recorded at the start of the study and twice weekly thereafter, with the last measurement made at study termination. Food consumption was measured over successive three- or four-day periods during the treatment period. After 14 days of dosing the animals were killed and examined for gross pathological changes. The kidneys, liver and caecum (full and empty) were weighed and organ weights relative to body weight were calculated.

All rats survived to the end of the study and no clinical signs of toxicity were observed. There were no differences in body weight and food consumption between the groups. Relative liver weights were significantly lower than controls in the mid and high dose groups, with the absolute liver weight also significantly lower in the high dose group compared with controls. These changes were not considered to be of toxicological significance. Absolute and relative weights of the filled and empty caecum were increased in the mid and high dose groups compared with controls. These changes were statistically significant with the exception of the absolute empty caecum weight in the mid dose group. The study authors considered that the increased caecum weights were a physiological adaptation to the administration of large amounts of the test substance, known to occur upon consumption of poorly digestible, fermentable sugars, rather than a toxic effect.

The authors concluded that the concentrations tested in the dose-range finding study were suitable for a 13-week oral toxicity study of 2'-FL.

13-week oral toxicity study with 2'-FL_{micro} in juvenile rats (van Berlo et al. 2018) Regulatory status: GLP; conducted in compliance with OECD Test Guideline (TG) 408 (1998)

Juvenile Wistar Han IGS rats (CrI:WI(Han)) (10/sex/group; aged 25 days) were administered 2'-FL (purity 94%) for 90 days at concentrations of 0, 3, 6 and 10% (w/w). Mean intake of 2'-FL was calculated to be 2170, 4270 and 7250 mg/kg bw/day for males and 2450, 5220 and 7760 mg/kg bw/day for females in the low, mid and high dose groups, respectively. Animal condition and behaviour were monitored twice daily with more detailed clinical observations conducted weekly. A functional observation battery and motor activity assessment was performed in all animals 12 weeks after initiation of treatment. Ophthalmological

examinations were conducted on the control and high dose animals in the last week of the exposure period. Body weight was recorded weekly and food consumption was measured twice weekly. Blood samples were collected at necropsy for haematology and clinical chemistry evaluations, and urine was collected from all animals in the final week of the study. Macroscopic examination was performed at necropsy and organ weights were determined. Histopathological examination was performed on organs and tissues from animals in the control and high dose group, and from animals that died during the study.

No treatment related mortality or clinical signs were observed. One female animal in the mid dose group died in the fourth week of the study. No cause of death could be established following histopathological examination of organs and tissues from this animal. As no further deaths occurred in any dose group the mortality was not considered to be due to 2'-FL exposure. Detailed clinical observations, the functional observational battery and the motor activity assessment did not reveal any neurotoxic potential of 2'-FL. No exposure-related changes were found upon ophthalmological evaluation. There were no significant differences in body weights between the control and test groups throughout the course of the study. Food consumption was significantly decreased in females in the high dose group, but the reduction was slight (< 10%) and body weights were not affected. No treatment related adverse effects on haematology, clinical chemistry and urinalysis parameters were observed. A few slight but significant differences in haematology, clinical chemistry and urinalysis parameters were not considered to be treatment related because they were observed in one sex only, the difference compared with controls was small, and no changes were observed in any of the associated parameters investigated. Relative liver weights were significantly increased (+8%) in high dose males, but in the absence of accompanying histopathological changes or alterations in clinical chemistry this was not considered to be an adverse effect. Absolute and relative weights of the filled and empty caecum were significantly increased in the mid and high dose groups in both sexes, as was the absolute weight of the filled caecum in low dose group males. This finding was considered to be a physiological response to consumption of the test substance, which is a non-digestible carbohydrate. No exposure-related macroscopic or microscopic histopathological changes were reported.

The NOAEL of 2'-FL in this study was the highest concentration tested, corresponding to 7250 mg/kg bw/day in males and 7760 mg/kg bw/day in females.

Genotoxicity studies conducted with the Applicant's 2'-FL_{micro}

Bacterial reverse mutation test with 2'-FL (van Berlo et al. 2018) Regulatory status: GLP; conducted in compliance with OECD TG 471 (1997)

The test article was 2'-FL, 94% purity. The solvent and negative control article was phosphate buffered saline (PBS). Test systems for this assay were *Salmonella typhimurium* strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* strain WP2 *uvrA*. Positive controls in the absence of S9 mix for metabolic activation, were sodium azide for TA 1535 and TA 100, 9-aminoacridine for TA 1537, 2-nitrofluorene for TA 98 and N-ethyl-N-nitrosourea for WP2 *uvrA*. Positive controls in the presence of metabolic activation were benzo(a)pyrene for TA 1537 and 2-aminoanthracene for TA 1535, TA 98, TA 100 and *uvrA*. Five concentrations of the test article were tested ranging from 62 – 5000 µg/plate. All assays were conducted in triplicate using the plate incorporation method. The number of revertant colonies were counted following incubation of the plates at 37 °C for 48 – 72 hours.

The test article showed no evidence of toxicity, and there was no significant increase in the number of revertant colonies following treatment with the test item, with or without S9 mix. Mean numbers of revertant colonies in the negative controls were within the laboratory's historical control ranges. Positive controls produced significant increases in the number of revertant colonies as expected, confirming the validity of the assays.

It was concluded that 2'-FL was not mutagenic under the conditions of this study.

In vitro micronucleus assay in cultured human lymphocytes (van Berlo et al. 2018)
Regulatory status: GLP; conducted in compliance with OECD TG 487 (2016)

The test material for this assay was 2'-FL, purity 94%. The solvent and negative control was RPMI 1640 medium. The test system was cultured phytohaemagglutinin (PHA)-stimulated peripheral human lymphocytes obtained from two healthy, non-smoking volunteers (one volunteer per assay). Two assays were performed in duplicate. In the first, lymphocytes were treated with the test item (3.9 – 2000 µg/mL based on purity) for 4 hours in the presence or absence of metabolic activation (S9 mix), then cultured for a further 20 hours in fresh medium containing cytochalasin B to prevent cell division. In the second assay, lymphocytes were treated with the test substance (15.6 – 2000 µg/mL) without metabolic activation for 24 hours in the presence of cytochalasin B. Cyclophosphamide was used as a clastogenic positive control in the first assay in the presence of S9 to demonstrate both the activity of the S9-mix and the response of the test system. In the second assay vinblastine sulphate was used as an aneugenic positive control. At the end of the total incubation period cells were harvested and analysed for cytotoxicity (1000 per treatment) and the number of binucleated cells containing micronuclei (2000 per treatment).

No significant cytotoxicity was observed in any of the cultures treated with the test item. Based on this finding three dose levels (500, 1000 and 2000 µg/mL) were scored for micronucleus induction in binucleated cells in each of the assays, together with the solvent and positive controls. The test item did not induce a significant increase in the number of binucleated cells with micronuclei compared with negative controls in the absence or presence of metabolic activation under any treatment conditions. Negative control values and values following treatment with the test item were within the laboratory's historical negative control data ranges. The positive controls induced significant increases in the number of cells with micronuclei consistent with the laboratory's historical positive control data, demonstrating the validity of the test system.

It was concluded that 2'-FL was not clastogenic or aneugenic under the conditions of this study.

4.2.2 Human studies with 2'-FL

Child study with 2'-FL alone and in combination with Lacto-N-neotetraose (LNnT) (Forvig et al. 2021)

This paper provides details of a randomised, double-blind, placebo-controlled trial of Glycom's 2'-FL_{chem} and LNnT_{chem} involving 75 children admitted to a hospital childhood obesity program. Unpublished interim results of this study were reviewed by FSANZ as part of A1155 (FSANZ 2019a). Children that were overweight (including obesity) aged ≥ 5 – < 13 years were randomised to receive an oral bolus dose of 4.5 g placebo (glucose), 2'-FL or a 4:1 mixture of 2'-FL and LNnT once daily for 8 weeks. The primary endpoint was the change in faecal bifidobacteria in the intervention compared to placebo from baseline to the end of the intervention. In addition, safety was assessed by clinical evaluation of haematology and clinical chemistry parameters, exploratory blood biomarkers of gut barrier function and inflammation as well as faecal calprotectin. The incidence of adverse events was also considered, as well as gastrointestinal symptoms and bowel habits.

Data newly available in the present report consisted of measures of faecal calprotectin at week 8 and biomarkers of gut barrier function and inflammation at baseline and the end of the intervention. No significant differences in these parameters were observed between

groups at baseline and measures were generally unchanged at the end of the study.

The additional results presented in this paper do not indicate a need to revise FSANZ's previous conclusion that a daily dose of 4.5 g 2'-FL or a 4:1 mixture of 2'-FL and LNnT was well tolerated by overweight children aged 5 – 12 years.

4.2.3 Allergenicity and intolerance

The specification for the 2'-FL ingredient indicates that residual proteins must be below the limit of quantification of $\leq 0.01\%$ (Table 2.3), and analysis of 5 non-consecutive lots confirmed that this specification is met. On this basis, 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](#), 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 Applicant's 2'-FL ingredient indicates it may contain residual lactose, at a concentration of up to 3%, while the EU specification proposed for use specifies lactose at up to 10% (Table 2.3). Batch analysis found lactose was present at lower levels than this (0.5 – 1.1%). 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

As noted in the assessments of A1155 and A1190, 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 and 0.6 g/L LNnT at a ratio of 2:1 (EFSA 2015). Subsequent to EFSA's assessment, a substantial equivalence notification by Glycom for its 2'-FL_{micro} was supported by an assessment by the Food Safety Authority of Ireland (FSAI 2016), and a 2'-FL_{micro} preparation from Chr. Hansen was authorised in 2017 following an evaluation by the Netherlands Committee on Safety Assessment of Novel Foods (NFU 2016).

The Novel Foods Unit (NFU) of the Netherlands completed an evaluation of 2'-FL_{micro} produced by the Applicant in 2017. The NFU concluded that FrieslandCampina's 2'-FL_{micro} is substantially equivalent to the 2'-FL products that had already been authorised for use in the EU.

The US Food and Drug Administration (FDA) has responded with 'no questions' to the Applicant's self-assessment that its 2'-FL_{micro} is Generally Recognized as Safe (GRAS) for use in infant formula and toddler formula at a maximum level of 2.4 g/L as consumed⁴.

4.3 Key findings of the toxicology assessment

2'-FL has previously been assessed by FSANZ (FSANZ 2019a; FSANZ 2019b; FSANZ 2020; FSANZ 2021). Data on 2'-FL produced from a variety of sources, including the Applicant's 2'-FL_{micro}, were considered as part of these evaluations. These assessments concluded that there are no safety concerns associated with the addition of 2'-FL to IFP at concentrations up to 2.4 g/L.

Information newly available to FSANZ since its previous assessments does not indicate a need to amend this conclusion. The full study reports of the *in vitro* genotoxicity assays and 90-day oral toxicity study in juvenile rats confirm that the Applicant's 2'-FL_{micro} is not

⁴ GRAS Notice (GRN) 735; details available at the US FDA's [GRAS Notice Inventory](#)

genotoxic and did not result in adverse effects following dietary exposure at doses up to 7250 mg/kg bw/day in males and 7760 mg/kg bw/day in females. Additional information on a study in overweight children aged 5 – 12 years previously considered by FSANZ also confirmed that a daily oral dose of 2'-FL (4.5 g/day) was well tolerated.

2'-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'-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 at concentrations up to 2.4g/L 2'-FL.

5 Nutrition assessment

FSANZ's previous assessments of 2'-FL (FSANZ 2019a; 2019b; 2020; 2021) concluded that 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 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*. No new information was provided that would indicate a need to change these conclusions. However, FSANZ notes that permissions for 2'-FL are subject to the outcome of a five year review⁵ (to be completed by March 2026) which will reassess evidence of a substantiated beneficial role of 2'-FL in the normal growth and development of infants.

6 Summary and Conclusions

The Code already permits 2'-FL from a different source organism for addition to infant formula products. The maximum permitted level is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment is therefore to assess the safety of 2'-FL produced by the new production strain.

The Applicant's 2'-FL_{micro} is chemically and structurally identical to the naturally occurring substance isolated from human milk (2'-FL_{human}) and chemically synthesized substances (2'-FL_{chem}). The Code currently permits 2'-FL to be used as a nutritive substance in IFP, which generally has a shelf life of two years. The final product is stable for up to 36 months at shelf-storage conditions, supporting the position that the Applicant's 2'-FL_{micro} preparation is suited for intended food uses. The Applicant has requested that the specification established in the EU in 2019 be applied to their 2'-FL_{micro} preparation. Multi-batch analyses showed that the final product is consistently within the proposed specifications, with impurities and/or contaminants resulting from the fermentation process either minor or absent.

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and does not pose a risk to humans. Analyses of the gene donors also confirmed there were no safety concerns. Characterisation of the production strain confirmed the expression plasmid carrying the α -1,2-fucosyltransferase gene was both genetically stable and fully functional.

⁵ <https://foodregulation.gov.au/internet/fr/publishing.nsf/Content/forum-communique-2020-November27>

FSANZ has previously determined that there are no safety concerns associated with the addition of 2'-FL to IFP at concentrations up to 2.4 g/L, which is within the range of naturally occurring levels in human milk from the majority of women (0.6 – 7.8 g/L). Newly available information relating to previously assessed studies of 2'-FL produced by the Applicant and another manufacturer 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 juvenile and adult 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.

The proposed specification does not raise any safety concerns. 2'-FL is unlikely to pose an allergenicity concern because the protein content of the 2'-FL product is below the limit of quantitation.

FSANZ's previous assessments of 2'-FL found no evidence of a nutritional concern at concentrations typically observed in human milk. No new information was provided that would indicate a need to change these conclusions. The evidence for a beneficial role of 2'-FL in the normal growth and development of infants will be reassessed in a review to be completed by March 2026.

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