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Supporting document 1

Risk and technical assessment – Application A1308

A1308 - 2′-FL from GM *Escherichia coli* W in infant formula products

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Executive summary

Food Standards Australia New Zealand (FSANZ) has assessed an application from Kyowa Hakko Bio Co., Ltd to amend the Australia New Zealand Food Standards Code (the Code) to permit a new source organism for the production of 2′-fucosyllactose (2′-FL). The applicant's 2′-FL is produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* (*E. coli*) W.

Schedule 26 of the Code already permits 2′-FL from several source organisms to be used as a nutritive substance in infant formula products. The maximum permitted amount of 2′-FL in infant formula products 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, produced by a microbial fermentation method of production, is chemically and structurally identical to the naturally occurring substance present in human milk. It is also chemically and structurally identical to 2′-FL previously assessed and permitted by FSANZ.

The *E. coli* W host organism has a long history of use for the production of recombinant proteins and other products, and is unlikely to pose a risk to humans. No safety concerns arising from the gene donor were identified. Characterisation of the GM production strain confirmed that the introduced alpha-1,2-fucosyltransferase gene is both genetically stable and 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. Newly available information did not indicate a reason to change this conclusion.

No treatment-related adverse effects were found in a 90-day oral toxicity study of the applicant's 2′-FL in rats. The NOAEL in this study was 2000 mg/kg bw/day, the highest dose tested. The applicant's 2′-FL was not genotoxic *in vitro* or *in vivo*.

The dietary intake assessment compared the estimated dietary intake of 2′-FL from infant

and follow-on formula to that of mature human milk for 3- and 9-month-old infants. As there is no requested change to the current permitted amount of 2′-FL in infant formula products, no extension of use, and no data suggesting a higher concentration in human milk since the most recent FSANZ assessment, estimated dietary intakes of 2′-FL from previous FSANZ assessments were used in this current assessment. These data showed that estimated mean and 90th percentile dietary intakes of 2′-FL at the maximum permitted amount in the Code from infant formula products fall within the range of estimated dietary intakes from mature human milk.

FSANZ has previously concluded that based on the available evidence the addition of 2′-FL to infant formula products is unlikely to pose a risk to normal growth of infants at levels typically found in human milk. No new relevant studies were identified for this assessment and therefore FSANZ maintains this conclusion.

Based on the available data, there are no public health and safety concerns associated with the addition of 2′-FL from the new source organism to infant formula products at the maximum permitted amount in the Code.

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1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Kyowa Hakko Bio Co., Ltd to amend the Australia New Zealand Food Standards Code (the Code) to permit a new source organism for the production of 2′-fucosyllactose (2′-FL). The applicant's 2′-FL is produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* (*E. coli*) W.

Schedule 26 of the Code already permits 2′-FL from several source organisms to be used as a nutritive substance in infant formula products. The maximum permitted amount of 2′-FL in infant formula products 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 chemical identification, physicochemical properties and specifications for the oligosaccharide proposed to be added to infant formula products (IFP). The assessment primarily aimed to address whether the microbiologically-synthesised 2′-FL proposed to be added to IFP is identical to that present in human milk. The assessment also considered the manufacturing process and the validity of analytical methods used to quantify and characterise 2′-FL during production and as a component of infant formula products.

FSANZ has assessed a number of recent applications requesting permissions for Human identical Milk Oligosaccharides (HiMO) in food. The information in this section has built on the reports written for the assessment of those applications. 2′-FL has been assessed by FSANZ in previous applications[1]. Application A1155 assessed permitting both 2′-FL and lacto-N-neotetraose (LNnT, a constitutional isomer of LNT) in IFP and other products. Application A1265 assessed a blend of four HiMO products, being 2′-FL and difucosyllactose (DFL) referred as 2′-FL/DFL; lacto-*N*-tetraose (LNT); 6′-sialyllactose sodium salt (6′-SL) and 3′-siallyllactose sodium salt (3′-SL). FSANZ has assessed two other applications A1277 (FSANZ 2023b) and A1283 (FSANZ 2024) that sought to also add 2′-FL to IFP.

2.1 Chemical and physical properties

2′-FL is a component of the human milk oligosaccharide (HMO) fraction of human milk. The applicant produces its 2′-FL via microbial fermentation using a GM strain of *E. coli* W, which is detailed in section 3.1.

The chemical name and properties of applicant's 2′-FL that is requested to be permitted is provided in Table 1 with information provided in the application and other references. The applicant claims its 2′-FL is chemically and structurally identical to the 2′-FL previously approved and permitted by FSANZ (see section 2.1.1 for discussion).

2′-FL is an oligosaccharide that contains the sugar fucose (a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$) and so is called a 'fucosylated' HMO. 2′-FL is a trisaccharide consisting of the monosaccharides L-fucose, D-galactose and D-glucose. It can also be described as the monosaccharide L-fucose, and the disaccharide D-lactose, connected by an alpha (1→2) glycosidic linkage (Figure 1).

[1] A1155, A1190, A1233, A1251, A1265, A1277 and A1283 (FSANZ 2019; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023a; FSANZ 2023b, FSANZ 2024 respectively)

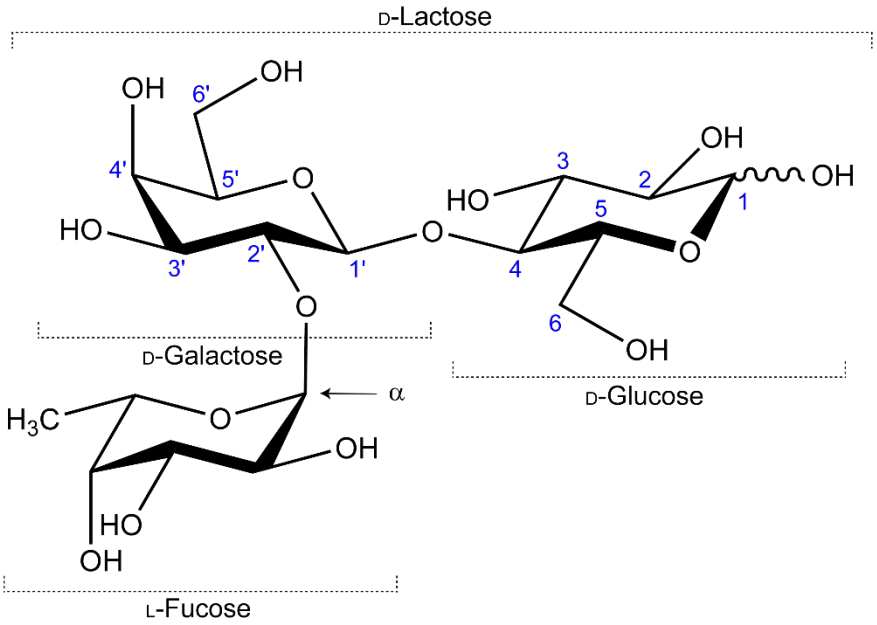

Figure 1 *Molecular structure of 2'-FL.*

2′-FL is a white to off-white homogeneous powder that is readily soluble in aqueous solutions. It is poorly soluble in organic solvents.

Table 1 *The nomenclature and chemical properties of 2'-FL.*

Property	2′-FL
Common name	2′-fucosyllactose
IUBMB[2] Chemical name	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
IUPAC[3] name	(2*S*,3*S*,4*R*,5*S*,6*S*)-2-[(2*S*,3*S*,4*R*,5*S*,6*S*)-4,5-dihydroxy-6-(hydroxymethyl)-2-[(2*R*,3*S*,4*R*,5*R*)-4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl]oxyoxan-3-yl]oxy-6-methyloxane-3,4,5-triol
Alternative common names	2′-O-fucosyllactose 2′-O-L-fucosyl-D-lactose 2′-fucosyl-D-lactose 2′-FL
Alternative names[a]	fucosyl-α-1,2-galactosyl-β-1,4-glucose α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc
IUPAC abbreviation[a]	Fuc-α-(1→2)-Gal-β-(1→4)-Glc
CAS[4] registry number	41263-94-9

[2] The International Union of Biochemistry and Molecular Biology
[3] The International Union of Pure and Applied Chemistry
[4] Chemical Abstract Service

Chemical formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol

[a] Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose

2.1.1 Chemical and structural equivalence of 2′-FL

The application included analytical data (including some Confidential Commercial Information (CCI)) to support the claim that 2′-FL produced using its microbial fermentation process is chemically and structurally identical to the substance naturally present in human milk as the reference standard. The analytical methods provided used one dimensional ^{1}H and ^{13}C as well as two dimensional ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) nuclear magnetic resonance (NMR) spectroscopy. Also liquid chromatograph – mass spectroscopy (LC-MS) was used. The applicant also had these NMR spectral studies interpreted using specific techniques such as COSY (homonuclear COrrelation SpectroscopY), TOCSY (TOtal Correlation SpectroscopY) and HETCOR (Heteronuclear Correlation Spectroscopy). FSANZ assessed the information provided and agreed with the applicant's conclusions that the NMR spectral analysis confirms that the microbially produced substance has the same stereochemical configuration and three-dimensional structure as those naturally occurring in human milk. The mass spectroscopy results further confirmed the chemical equivalence of the microbially produced substance to those naturally occurring in human milk.

In summary, FSANZ agrees with the applicant that its 2′-FL is chemically and structurally identical to 2′-FL naturally occurring in human milk and to those 2′-FL preparations already assessed and permitted by FSANZ from earlier applications.

2.1.2 Stability of 2′-FL under conditions of use

The applicant performed stability studies of its 2′-FL. These were studies where five lots of the product were stored under accelerated ageing conditions (40 ± 2°C and 75 ± 5% relative humidity (RH)) for 6 months. Storage studies were also conducted for one lot under ambient conditions (25 ± 2°C, 60 ± 5% RH) for 3 years (the proposed shelf life of the substance).

The results confirmed that the applicant's 2′-FL was stable for 3 years under ambient room temperature conditions and 6 months under accelerated ageing conditions.

The applicant did not perform stability trials of its 2′-FL contained within powdered infant formula to check for its stability in commercial products. It instead relied to the equivalence of its 2'-FL to other already permitted and assessed versions of the substance and comparable stability results conducted by other applicants and assessed as appropriate. FSANZ notes and agrees with this justification and the conclusions made.

2.2 Manufacturing processes

The method of production for the applicant's 2′-FL is similar to that of earlier applications so it is not reported in detail in this report. The production process for 2′-FL is summarised within SD1 of the 2nd CFS for A1155 (FSANZ 2019).

2′-FL is produced by a microbial fermentation process using a GM strain of *E. coli* W. The production process is conducted in two stages: upstream processing (USP) and downstream processing (DSP). The USP can be considered the fermentation steps while the DSP captures the isolation, purification and concentrations steps. In the main fermentation step glucose and lactose are used by the microorganism to synthesise the 2'-FL. For the

downstream processing filtration and cationic and anionic exchange steps are used, followed by concentration processes before spray drying to produce a powder form of the substance.

2.3 Specifications

As noted in section 2.1.1, the applicant's 2′-FL is chemically and structurally identical to 2′-FL already assessed and permitted by FSANZ from earlier applications.

The applicant provided its own proposed specification for its 2′-FL compared to the current specifications for 2′-FL in S3—40 (specification for 2′-fucosyllactose sourced from *Escherichia coli* K-12) and S3—45 (specification for 2′-fucosyllactose sourced from *Escherichia coli* BL21) in the Code. FSANZ notes that A1283 was assessed and approved before the current application was submitted. The outcome from the approval of A1283 is another slightly different 2′-FL specification, being S3—51 (specification for 2′-fucosyllactose sourced from *Corynebacterium glutamicum*).

As the applicant indicated there are slight differences to its 2′-FL compared to the other specifications, with a lower purity compared to some but not all products. Some parameters are slightly different however none raise safety concerns.

The applicant has proposed an in-house specification for the identity and purity of its nutritive substance to ensure its safety when added to food. Analytical results for three non-consecutive batches were included in the application, with additional details provided as CCI. These results demonstrate that the applicant can meet the specified requirements.

FSANZ assessed the analytical results of these batches of 2′-FL and agreed with the applicant's claim that their substance is chemically identical and of similar purity for most relevant parameters as currently permitted forms within S3—40, S3—45, and now S3—51 noting the very minor differences.

FSANZ used the applicant's specification to create a specification for its form of 2′-FL which is provided in Table 2 in a similar form to the current 2′-FL specifications (but as a table for simplicity). FSANZ notes that this specification, and S3—40, S3—45 and S3—51, are very similar to those provided in the updated European specification for 2′-FL (EU 2024) for the applicant's 2′-FL.

Table 2 *Proposed specification for the applicant's 2′-FL*

For 2′-fucosyllactose (2′-FL) sourced from *E. coli* W, the specifications are the following:

number	Parameter	Condition
a	chemical name	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
b	chemical formula	$C_{18}H_{32}O_{15}$
c	molecular weight	488.44 g/mol
d	CAS number	41263-94-9
e	description	White to off-white powder
f	2′-FL	Not less than 82% (water free)
g	D-lactose	Not more than 5.0 % (water free)
h	L-fucose	Not more than 1.0% (water free)
i	fucosylgalactose	Not more than 3.0% (water free)
j	difucosyllactose (difucosyl-d-lactose)	Not more than 3.0% (water free)
k	glucose + galactose	Not more than 1.0% (water free)
l	water	Not more than 9.0%
m	ash, sulphated	Not more than 0.5%
n	residual proteins	Not more than 0.01%
o	lead	Not more than 0.1 mg/kg
p	arsenic	Not more than 0.1 mg/kg
q	cadmium	Not more than 0. 1 mg/kg
r	mercury	Not more than 0.1 mg/kg
s	microbiological	
	i Aerobic mesophilic bacteria total count	Not more than 1000 CFU/g
	ii yeasts and moulds	Not more than 100 CFU/g
	iii Enterobacteriaceae	Absent in 10 g
	iv residual endotoxins	Not more than 10 E.U./mg

As was noted in the A1283 SD1 no microbiological limits have been listed for *Cronobacter* spp (absent in 100 g) or *Salmonella* (absent in 100 g) as they are provided in Schedule 27 as food safety microbiological limits in powdered IFP, even noting the nutritive substance is added at low levels into IFP.

2.3.1 Impurities

The levels of impurities of the applicant's 2′-FL comply with its specification, and are essentially consistent with those in S3—40, S3—45 and S3—51 specifications for comparable approved 2′-FL.

The application contained information relating to possible impurities in the final purified 2′-FL including the residual starting materials, D-lactose and glucose. Lactose, glucose and difucosyllactose are natural components of human milk (Sanchez et al. 2021) while other 2′-FL specifications also have limits for fucosylgalactose.

The production microorganism is removed during the processing and purifications steps during production of 2′-FL. Qualitative polymerase chain reaction methods were used to confirm that no residual DNA from the production microorganism remains in the final purified nutritive substance. Additionally, separate testing was conducted to confirm no viable production strain cells were present in the final nutritive substance preparation.

2.4 Analytical methods for detection

The applicant has in-house analytical methods for detecting and quantifying 2′-FL and other carbohydrates. The analytical method uses High Performance Liquid Chromatography coupled with Pulsed Amperometric Detector (HPLC-PAD). This analytical method can also be used to detect and quantify the presence of 2′-FL in foods to which it has been added (e.g. IFP). The applicant also used this analytical method for its stability trials.

2.5 Food technology conclusion

The applicant's 2′-FL produced by a microbial fermentation method of production is chemically and structurally identical to the naturally occurring substance present in human milk. It is also chemically and structurally identical to 2′-FL previously assessed and permitted by FSANZ.

The applicant's 2′-FL specification is similar to already permitted 2′-FL specifications, being S3—40, S3—45 and S3—51. FSANZ has written a comparable new specification specific for the applicant's 2′-FL.

Stability trials confirmed that the applicant's 2′-FL was stable for 3 years under ambient room temperature conditions (25 ± 2°C, 60 ± 5% RH) and 6 months under accelerated ageing conditions (40 ± 2°C and 75 ± 5% RH).

3 Safety assessment

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

3.1 GM production strain assessment

3.1.1 Host organism

Escherichia coli is a facultative anaerobic, Gram-negative, rod-shaped bacteria found in the gut of mammals (Guerra et al. 2019). *E. coli* strains can be pathogenic to humans causing a wide range of diseases, some of which can be fatal (Guerra et al. 2019; Mir and Kudva, 2018). However there are strains of *E. coli*, termed safe strains, that are used in research and industry specifically because of their inability to cause disease in humans (Bauer et al. 2008). *E. coli* W is one of these safe strains (Archer et al. 2011; Bauer et al. 2008; DeCanio et al. 2013).

E. coli W is a biosafety level 1 organism that was first isolated from the soil of a cemetery (Archer et al. 2011; Bauer et al. 2008). *E. coli* W is well characterised and its genome has been sequenced and annotated (Archer et al. 2011). Within *E. coli* W, the genes encoding pathogenicity are inactivated or missing key components (Archer et al. 2011). *E. coli* W is deemed a safe strain because it does not colonise the gut or cause disease in humans (Archer et al. 2011; Bauer et al. 2008). *E. coli* W has a long history of use in research laboratories and industry since the 1940's (Archer et al. 2011). *E. coli* W is considered a

safe, non-pathogenic and non-toxigenic microorganism (DeCanio et al. 2013).

The production strain used in this application was created by inserting a cloned α-1,2 fucosyltransferase gene from *Helicobacter mustelae* (ATCC 43772) into the *E. coli W* strain (ATCC 9637) (see section 3.1.3 for more information). The production strain in this application has been assessed by EFSA and the FDA, with no safety concerns raised (EFSA, 2023; FDA GRN 1051).

Whole genome sequencing results for the production strain were provided to FSANZ by the applicant. These results confirmed the identity of the production strain as *E. coli* W. The applicant also demonstrated the absence of the production organism in the final enzyme product with data from three representative product batches.

The microbiological risk assessment undertaken by FSANZ did not identify any public health and safety concerns associated with the use of *E. coli W* as a production organism for 2′-FL.

3.1.2 Gene donor organism

H. mustelae is a biosafety level 2 organism that primarily infects ferrets and other mustelids (O'Toole et al. 2010). *H. mustelae* shares many virulence factors with *Helicobacter pylori* (Croinin et al. 2007). The donor organism's identity was confirmed through CCI data. Endogenous vectors and other genetic material of *H. mustelae* are not relevant because only DNA encoding the α-1,2 fucosyltransferase gene was used in the construction of the modified bacterial strain. The expressed gene product is not associated with any potential toxicity or pathogenic traits of the donor organism.

3.1.3 Characterisation of the GM production organism

Development of the GM production strain

To develop the production strain, the α-1,2 fucosyltransferase gene was cloned from *H. mustelae* and mutated using site-directed mutagenesis (Kamada and Koizumi 2007). Using homologous recombination, the gene was then introduced into specific locations in the genome of the host *E. coli* W. The α-1,2 fucosyltransferase gene is under the control of a constitutive promoter from *E. coli* W.

Data provided by the applicant and analysed by FSANZ confirmed the identity of the α-1,2 fucosyltransferase enzyme.

Characterisation of introduced DNA

Data provided by the applicant confirmed the presence of the α-1,2 fucosyltransferase gene at the intended locations in the genome of the production strain. No antibiotic resistance markers are present in the final production strain.

Genetic stability and inheritance of the introduced DNA

The applicant provided data which confirmed the inserted gene remained stable over 5 successive generations.

Data was also provided showing that production of 2′-FL by the production strain was consistent over a minimum of 3 generations, providing further evidence of the stability and inheritance of the inserted DNA over this period.

3.1.4 Conclusion

E. coli W has a long history of use for the production of recombinant proteins and other products and is unlikely to pose a risk to humans. No safety concerns arising from the gene donor were identified.

Characterisation of the GM production strain confirmed that the introduced gene was both genetically stable and functional.

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

3.2 Toxicology assessment

3.2.1 Previous FSANZ safety assessments of 2′-FL

A range of toxicological and human clinical studies of 2′-FL have previously been reviewed by FSANZ as part of applications A1155, A1190, A1233, A1251, A1265, A1277 and A1283.

In summary, these assessments found 2′-FL to be structurally and chemically identical to the form present naturally in human milk. As such, no differences in pharmacokinetics or safety between the naturally occurring and manufactured forms of 2′-FL is expected. Data indicate that intestinal absorption is limited, and a significant proportion of 2′-FL reaches the large intestine where it is fermented by the microbiota or excreted unchanged in the faeces. Toxicity studies indicated 2′-FL is not genotoxic and does not produce adverse effects in short-term oral toxicity studies, including studies using neonatal animal models. In human clinical studies, consumption of infant formula containing 2′-FL was safe and well tolerated (FSANZ 2020, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024).

3.2.2 Newly available data

The applicant conducted a literature search to identify new toxicological and human clinical studies published from 2021. Two animal studies and 11 clinical studies were identified.

All but one of these studies were excluded from the present assessment for the following reasons:

- Study already evaluated in previous FSANZ safety assessments
- Study conducted in adult humans so not relevant for assessment of the target population for infant formula products

The applicant also submitted several proprietary toxicological studies conducted with their 2′-FL preparation which were reviewed in the present assessment:

- Bacterial reverse mutation assay
- In vitro mammalian cell micronucleus test
- In vivo micronucleus test in mice
- 90-day oral toxicity study in rats

Toxicological studies with the applicant's 2'-FL

90-day oral toxicity study in rats (CCI) Regulatory status: GLP; conducted in accordance with OECD Test Guideline (TG) 408 (1998)

In an OECD Test Guideline compliant 90-day oral toxicity study in rats, 2′-FL (purity 92%) was administered in water to male and female CrL:CD(SD) rats by oral gavage at doses of 0, 500, 1000 or 2000 mg/kg bw/day. All animals survived to the end of the treatment period. There were no treatment-related clinical signs or adverse effects on any of the parameters evaluated. The no observed adverse effect level (NOAEL) in this study was 2000 mg/kg bw/day, the highest dose tested.

Genotoxicity studies with the applicant's 2'-FL

Several genotoxicity studies with the applicant's 2′-FL were submitted. These studies were conducted in accordance with GLP and according to OECD Test Guidelines. The positive controls in these studies produced the expected responses. The results of these studies, as summarised in Table 3, showed no evidence of mutagenicity, clastogenicity or aneugenicity.

Table 3 *Genotoxicity studies of 2'-FL*

Test[1]	Test object	Concentration	Purity (%)	Results
Bacterial reverse mutation test (OECD TG 471 [1997])	*Salmonella typhimurium* strains TA100, TA1535, TA98 & TA1537; *Escherichia coli* strain WP2 *uvrA*	0, 313, 625, 1250, 2500 or 5000 µg/plate[2]	92%	Negative ± S9
Micronucleus test in vitro (OECD TG 487 [2016])	Chinese hamster lung cells (CHL/IU cells)	0, 500, 1000 or 2000 µg/L[3]	93.5%	Negative ± S9
Micronucleus test in vivo (OECD TG 474 [2016])	Slc:ICR mice bone marrow cells	0, 500, 1000 or 2000 mg/kg bw/day[4]	92%	Negative

[1] Study references are CCI.
[2] Main test conducted twice in triplicate; no precipitation observed.
[3] No cytotoxicity or precipitation observed. Cells exposed to 2′-FL with and without metabolic activation (S9 mix) for 6 hours and without S9 for 27 hours.
[4] Test item administered twice by oral gavage at 24 hour intervals.

Human studies of 2'-FL

One study not previously reviewed by FSANZ and considered relevant to the assessment was identified in the applicant's literature search.

In this study, healthy term infants were either exclusively fed formula containing 1.0 g/L 2′-FL and 0.5 g/L lacto-N-neotetraose (LNnT) (FF; n = 46), mixed fed (i.e. received both formula and human milk) (MF; n = 22) or exclusively breastfed (BF; n = 38) for 8 weeks. The formula was well-tolerated with Infant Gastrointestinal Symptom Questionnaire (IGSQ) scores in FF infants improving from baseline and showing no significant differences between groups when measured in weeks 4 and 8. No serious adverse events were recorded. In total 4/69 adverse events were considered potentially formula-related, including instances of lactose

intolerance, hard faeces, vomiting, and diarrhea. It was concluded that the formula showed good tolerance and safety (Jochum et al. 2023).

3.2.3 Safety assessments by other agencies

As noted in previous FSANZ assessments, the European Food Safety Authority (EFSA) has assessed 2′-FL from multiple sources as a novel food, including addition to infant formula and follow-on formula, and concluded that it is safe for its intended uses. In 2023, EFSA published a Scientific Opinion on the safety of the applicant's 2′-FL, which included a request to extend the use of 2′-FL to food supplements for infants. EFSA concluded that consumption of the applicant's 2′-FL at the proposed uses and use levels does not raise safety concerns (EFSA 2023).

The US Food and Drug Administration (US FDA) has responded that it has 'no questions' to the applicant's self-assessment that their 2′-FL is Generally Recognised as Safe (GRAS) under its intended conditions of use (US FDA 2023).

3.2.4 Summary of the toxicology assessment

Based on previous FSANZ assessments of 2′-FL and the toxicological assessment in the present application, it was concluded there are no public health and safety concerns associated with 2′-FL produced from the new GM source organism that is the subject of this application.

3.3 Microbiological assessment

FSANZ has undertaken microbiological risk and health benefit assessment on a number of previous applications in regards to production and addition of 2′-FL to infant formula products: A1155, A1190, A1233, A1251, A1265, A1277 and A1283 (FSANZ 2020; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023a; FSANZ 2023b; FSANZ 2024).

Based on these previous microbiological assessments, given the identical chemical structure and that the applicant has not requested any change in the maximum permitted amount of 2′-FL added to infant formula products, FSANZ has concluded that there are no microbiological public health and safety concerns. The associated health benefits from the use of 2′-FL as a nutritive substance in infant formula products for infants remain the same: (1) an anti-pathogenic effect; (2) immunomodulation and (3) development of the gut microbiome through supporting growth of *Bifidobacteria* spp.

3.4 Dietary intake assessment

3.4.1 Approach for the dietary intake assessment

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

FSANZ has previously conducted dietary intake assessments of 2'-FL under A1155, A1190, A1251, A1265 and A1283 (FSANZ 2019, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b). For A1155, A1251 and A1265 model diets for 3- and 9-month-old infants were included to represent consumption by exclusively formula-fed/breastfed infants, and infants who consumed food as well as follow-on formula or human milk respectively. A set of

model diets was not established for infants consuming infant formula products for special dietary uses as the energy/and or fluid requirements can vary depending on the medical conditions of the infant; in addition to the variability in energy content of the various infant formula products for special dietary uses.

In the Code, 2′-FL is permitted to be added as a nutritive substance to infant formula products at a maximum permitted amount of 96 mg/100 kJ (equivalent to 2.4 g/L), which was the concentration considered in the assessment of A1155. As the applicant proposed no changes to the currently permitted amount and no extension of use to foods other than infant formula products, a new dietary intake assessment of 2′-FL has not been conducted for this application. Due to composition specifications in A1251 and A1265 being different to those relevant to this application, estimated dietary intakes of 2′-FL from infant formula and follow on formula as assessed under A1155 are presented in Table 4**.**

Dietary intakes of 2′-FL from mature human milk were most recently estimated in the assessment under A1265, using mean 2′-FL concentrations from a systematic literature review (Soyyilmaz et al. 2021). The present dietary intake assessment therefore aimed to identify and evaluate any newly available concentration data for 2′-FL in mature human milk, that were not included in previous FSANZ dietary intake assessments for 2′-FL or the systematic literature review by Soyyilmaz et al. (2021).

From the studies identified, data were excluded if the studies involved only pre-term infants, if the data were not presented by secretor status, if the data were not for mature human milk (10 days post-partum and above), if lactation periods were not defined, or if the data were reported as relative abundance, as percentages or in nmol/mL.

The current application did include a literature search that aimed to identify publications relevant to the safety of 2′-FL. Data from 4 of these studies were included in the literature search for this assessment (Li et al. 2022, Liu et al. 2023, Nguyen et al. 2022, Sudarma et al. 2023).

3.4.2 Previous FSANZ dietary intake assessments of 2′-FL

In the dietary intake assessment for A1155, FSANZ estimated the dietary intakes of 2′-FL_{micro} and concluded that intakes for 3- and 9-month-old infants were similar to the estimated intakes of 2′-FL_{human}. This was due to the proposed maximum use level of 2′-FL_{micro} in infant and follow-on formula (2.4 g/L / 96 mg/100 kJ) considered in the application being similar to the mean concentration of 2′-FL_{human} for human milk (secretors) (FSANZ 2019).

In the dietary exposure assessment for A1265, FSANZ concluded that mean estimated dietary intakes of 2′-FL from infant formula products were comparable to mean estimated dietary intakes from mature human milk, and high (90th percentile) estimated dietary intakes from infant formula products did not exceed estimated dietary intakes from mature human milk at high consumption and high concentration levels (FSANZ 2023a).

For A1190 and A1283, FSANZ undertook a literature search for concentration data for 2′-FL in human milk published since the assessment of A1155, and A1265 respectively. Several relevant studies were identified in each independent literature search, with the range of concentrations reported for secretors aligning with concentrations reported in previously reviewed studies. New dietary intake assessments were therefore not required (FSANZ 2021, FSANZ 2023c).

3.4.3 Key findings of the dietary intake assessment

In the literature search for this assessment, FSANZ identified 8 primary studies, including 4

primary studies also identified by the applicant (Berger et al. 2020, Cheema et al. 2022, Li et al. 2022, Liu et al. 2023, Mao et al. 2024, Nguyen et al. 2022, Oliveros et al. 2021, Sudarma et al. 2023) that met the inclusion criteria. Across these studies, mean 2′-FL concentrations in mature human milk ranged from 1.56 g/L to 3.16 g/L, and median 2′-FL concentrations ranged from 0.95 g/L to 2.69 g/L. As these concentration levels are lower than the highest individual study mean for 2′-FL previously identified, at 4.28 g/L, 15-90 days lactation (Soyyilmaz et al. 2021), it was concluded that an additional dietary intake assessment of 2′-FL from mature human milk was not required for this application. Estimated dietary intakes of 2′-FL from mature human milk as calculated under A1265 are presented in Table 4 as a comparison with estimated dietary intake from infant formula products. For both 3- and 9-month-old infants, estimated mean and 90th percentile dietary intakes of 2′-FL at the maximum permitted amount in the Code from infant formula products fall within the range of estimated dietary intakes from mature human milk.

Table 4 *Summary of estimated dietary intakes of 2′-FL from infant formula, follow-on formula and mature human milk for infants aged 3 and 9 months (reproduced from A1155* and A1265#)*

	Mean dietary intake (g/kg bw/day)[1]		90th percentile dietary intake (g/kg bw/day)[1]	
Age group	From infant/ follow-on formula*β	From human milk#t	From infant/ follow-on formula*β	From human milk#t
3 months	0.33	0.26 – 0.49	0.66	0.52 – 0.98
9 months	0.16	0.092 – 0.24	0.32	0.18 – 0.48

β Assumes 2′-FL concentration of 2.4 g/L / 96 mg/100 kJ.

t Lower bound of the range assumes mean of means 2′-FL concentration (15-90 day lactation period) from Soyyilmaz et al. (2021) and mean human milk consumption; upper bound of the range assumes maximum mean 2′-FL concentration (15-90 day lactation period) from Soyyilmaz et al. (2021) and 90th percentile human milk consumption.

[1] Mean body weights used: 6.4 kg for 3 months of age and 8.9 kg for 9 months of age

3.5 Nutrition assessment

3.5.1 Objective of the nutrition assessment

The objective of the nutrition risk assessment is to determine the effect (if any) of the addition of 2′-FL to infant formula products on infant growth. Schedule 26 of the Code permits 2′-FL produced by several source organisms (as described in Section 1 above) to be added as a nutritive substance to infant formula products at a maximum permitted amount of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant has requested a new GM strain of *E. coli* W for the production of 2′-FL but has not requested a change to the maximum permitted amount.

3.5.2 Previous FSANZ assessments of 2′-FL

FSANZ has assessed the effect of addition of 2′-FL to infant formula products on growth in

seven previous applications[5].

In these assessments FSANZ considered twenty clinical trials and cohort studies that measured the effects of 2′-FL alone or in combination with other HMOs or oligosaccharides on infant growth endpoints including mean weight, length, head circumference, height and weight for age z-scores, mean weight gain per day, fat mass index and weight velocity (Marriage et al. 2015; Kajzer et al. 2016; Puccio et al. 2017; Sprenger et al. 2017; Reverri et al. 2018; Larsson et al. 2019; Storm et al. 2019; Berger et al. 2020; Lagström et al. 2020; Leung et al. 2020; Román et al. 2020; Vandenplas et al. 2020; Parschat et al. 2021; Ramirez-Farias et al. 2021; Alliet et al. 2022; Cohen 2022; Gold et al. 2022; Lasekan et al. 2022; Vandenplas et al. 2022 and Wallingford et al. 2022). It was concluded that based on the available evidence, the addition of 2′-FL to infant formula products at levels that are normally present in human milk is unlikely to affect the normal growth of infants.

3.5.3 Current assessment

The applicant provided eight studies that investigated the effect of 2′-FL in infant formula on infant growth. All of the studies were excluded from the assessment for the following reasons:

- Six studies were previously assessed by FSANZ in application A1265 (Parschat et al. 2021, Laseskan et al. 2022), A1277 (Alliet et al. 2022, Wallingford et al. 2022), and A1283 (Gold et al. 2022, Vandenplas et al. 2022).
- Bauer et al. (2021) was a conference abstract that presented data from a study previously assessed by FSANZ in application A1265 (Cohen 2022).
- An open-label cohort study by Jochum et al. (2023) studied the effect of infant formula containing 1.0 g/L 2′-FL and 0.5 g/L LNnT compared to human milk and mixed feeding on infant growth but did not include a control group without 2′-FL and was of insufficient duration (8 weeks) to assess changes in anthropometric parameters.

FSANZ conducted a literature search[6] in PubMed on 20 August 2024 to identify any additional studies published since the previous assessment. The search returned 20 studies. No studies were relevant to include in the nutrition assessment as they did not report the effect of adding 2′-FL to infant formula products on infant growth.

3.5.4 Summary of nutrition assessment

Schedule 26 of the Code currently permits the addition of 2′-FL produced by several source organisms as nutritive substances to infant formula products at a maximum permitted amount of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant did not request any change to the maximum permitted amount.

FSANZ has previously assessed the effect of the addition of 2′-FL to infant formula products on infant growth in seven applications. Twenty studies were assessed in the body of evidence using several growth endpoints. It was concluded that the addition of 2′-FL to infant formula products does not pose a risk to normal growth in infants at levels typically found in human milk.

No new relevant studies were identified in the present assessment to alter the conclusions from previous assessments. Therefore based on the available evidence FSANZ maintains

[5] A1155, A1190, A1233, A1251, A1265, A1277 and A1283 (FSANZ 2019, FSANZ 2020, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023a, FSANZ 2023b, FSANZ 2024).
[6] Search terms: “2'FL or 2'-FL or 2'-fucosyllactose or 2'fucosyllactose” and “milk or breast or formula” and “anthropometric or weight or growth or development” and “child or infant or baby or maternal”

the conclusion that the addition of 2′-FL to infant formula products is unlikely to affect infant growth at levels normally found in human milk.

4 Conclusions

Schedule 26 of the Code currently permits the use of 2′-FL from different source organisms as nutritive substances in infant formula products. The maximum permitted amount is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment was therefore to assess the safety of 2′-FL produced by the new production strain.

The applicant's 2′-FL, produced by a microbial fermentation method of production, is chemically and structurally identical to the naturally occurring substance present in human milk. It is also chemically and structurally identical to 2′-FL previously assessed and permitted by FSANZ.

The *E. coli* W host organism has a long history of use for the production of recombinant proteins and is unlikely to pose a risk to humans. Characterisation of the GM production strain confirmed that the introduced gene was genetically stable and 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. Newly available information did not indicate a reason to change this conclusion.

Intestinal absorption of HMOs is limited and a significant proportion reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces. Toxicity studies previously reviewed by FSANZ indicated 2′-FL is not genotoxic and does not produce adverse effects in short-term oral toxicity studies, including studies using neonatal animal models. In human clinical studies, consumption of infant formula containing 2′-FL was safe and well tolerated. In addition, no treatment-related adverse effects were found in a 90-day oral toxicity study of the applicant's 2′-FL in rats. The NOAEL in this study was 2000 mg/kg bw/day, the highest dose tested. The applicant's 2′-FL was not genotoxic *in vitro* or *in vivo*.

As the applicant's 2′-FL is identical to naturally occurring 2′-FL it is not anticipated that there will be any significant differences in pharmacokinetics or safety between naturally occurring and manufactured forms of these substances.

FSANZ maintains the conclusion that, based on the available evidence, the addition of 2′-FL to infant formula products at levels typically found in human milk does not pose a risk to normal infant growth.

The dietary intake assessment compared the estimated dietary intake of 2′-FL from infant and follow-on formula to that of mature human milk for 3- and 9-month-old infants. As there is no requested change to the current permitted amount of 2′-FL in infant formula products, no extension of use, and no data suggesting a higher concentration in human milk since the most recent FSANZ assessment, estimated dietary intakes of 2′-FL from previous FSANZ assessments were used in this current assessment. These data showed that estimated mean and 90th percentile dietary intakes of 2′-FL at the maximum permitted amount in the Code from infant formula products fall within the range of estimated dietary intakes from mature human milk.

Based on the available data, there are no public health and safety concerns associated with the addition of 2′-FL from the new source organism to infant formula products at the maximum permitted amount in the Code.

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