23 February 2024
282-24

Supporting document 1

Risk and technical assessment – Application A1283

A1283 - 2′-FL from GM *Corynebacterium glutamicum* in infant formula products

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Executive summary

Food Standards Australia New Zealand (FSANZ) has assessed an application from Advanced Protein Technologies Corp. 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), a human milk oligosaccharide. The applicant's 2′-FL is produced by microbial fermentation using a genetically modified (GM) strain of *Corynebacterium glutamicum.*

The Code already permits 2′-FL from different source organisms to be used as nutritive substances in infant formula products. The maximum permitted amount is 96 mg/100 kJ, equivalent to 2.4 g/L. 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. The applicant is not requesting a change to the maximum permitted amount. The primary 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.

C. glutamicum has a long history of documented use for the production of biomolecules, including food additives, and poses no risks to human health. No safety concerns arising from the gene donors were identified. Characterisation of the production strain confirmed the expression plasmid carrying the introduced genes 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 *C. glutamicum.* Based on previous FSANZ assessments of 2′-FL and the toxicological assessment in the present application, it was concluded that 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.

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 the addition of 2′-FL to infant formula products at levels typically found in human milk does not pose a risk to normal growth of infants. Two studies provided by the applicant reported no difference in growth in infants fed formula containing lacto-N-neotetraose and 2′-FL compared to control, however were not directly relevant to the assessment because any effect (or lack of effect) on growth cannot be attributed to 2′-FL. No new relevant studies were identified for this assessment, and therefore FSANZ maintains the previous conclusion that the addition of 2′-FL to infant formula products at levels normally found in human milk is unlikely to affect growth.

Based on previous microbiological assessments, given the identical chemical structure and noting the applicant has not requested any change in the maximum permitted amount of 2′-FL added to infant formula products, 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.

Overall the safety assessment concluded there are no public health and safety concerns associated with the addition of 2′-FL synthesised 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 Advanced Protein Technologies Corp. 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 *Corynebacterium glutamicum).*

Schedule 26 of the Code already permits 2′-FL from several source organisms to be used as nutritive substances in infant formula products (*E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*; *E. coli* BL21 containing the gene for alpha-1,2-fucosyltransferase from *E. coli* O126; *E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Bacteroides vulgatus*). FSANZ is also currently considering an application to permit 2′-FL from *E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter enhydrae* in Application A1277. 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. The assessment primarily aimed to address whether the microbiologically-synthesised 2′-FL proposed to be added to infant formula products 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 in applications A1155, A1190, A1233, A1251, A1265 and A1277 (FSANZ 2019a; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023a; FSANZ 2023b). Application A1155 assessed permitting both 2′-FL and lacto-N-neotetraose (LNnT, a constitutional isomer of LNT) in infant formulas 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 is currently assessing another application A1277 (FSANZ 2023b) seeking permission for 2′-FL to also be added to infant formula products.

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 *Corynebacterium glutamicum*, which is detailed in section 3.1.

The chemical name and properties of 2′-FL that is requested to be permitted is provided in Table 1 with information as provided in the application and other references and claimed by the applicant to be chemically and structurally identical to the 2′-FL of previously FSANZ approved and permitted applications (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).

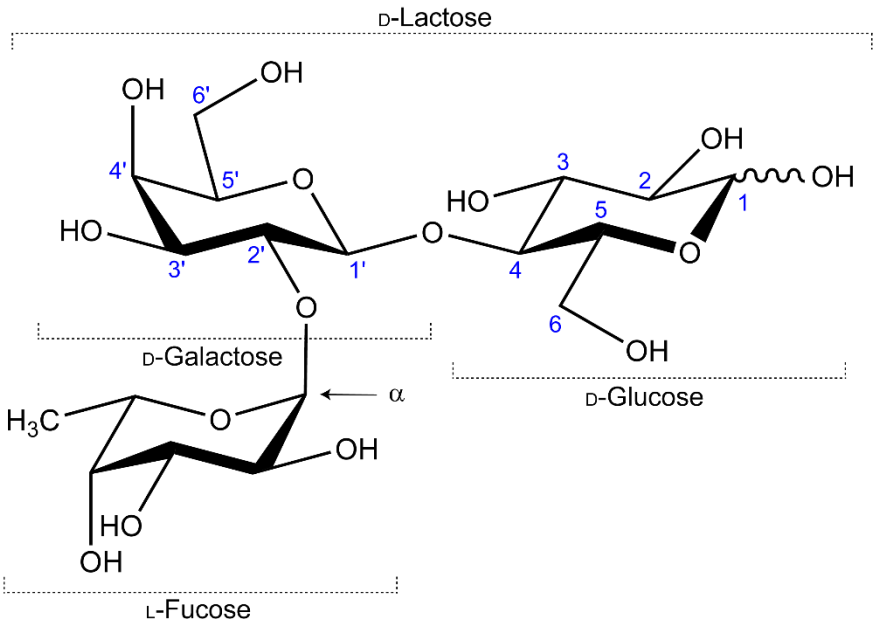

Figure 1 *Molecular structure of 2'-FL.*

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[1] Chemical name	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
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[2] abbreviation[a]	Fuc-α-(1→2)-Gal-β-(1→4)-Glc
CAS[3] registry number	41263-94-9
Chemical formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol

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

[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 (Confidential Commercial Information) 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 and two dimensional ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) nuclear magnetic resonance (NMR) spectroscopy. The applicant also had these NMR spectral studies interpreted using specific techniques such as COSY (homonuclear COrrelation SpectroscopY), TOCSY (TOtal Correlation SpectroscopY) and HSQC-TOCSY (Heteronuclear Single Quantum Coherence - TOtal 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 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) within the Code. As the applicant indicated there are slight differences to its 2′-FL compared to the other specifications, where some parameters are refined due to the enhanced purity of the product. Some parameters are slightly different however none raise safety concerns. The applicant also provided analytical results from five non sequential batches of product, from two different final processing steps, being crystallisation from solvent or spray drying. 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 at least as pure with relevant parameters as currently permitted forms within S3—40 and S3—45, noting the very minor differences.

In summary, FSANZ agrees with the applicant that its 2′-FL is chemically and structurally identical to 2′-FL 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 was stored under ambient conditions (25°C, 60% relative humidity (RH)), and accelerated storage (40°C and 75% RH), both for 2 years.

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

Separately the applicant performed stability trials of its 2′-FL contained within powdered infant formula to check for its stability in commercial products. These samples were stored at various storage temperatures, 4°C, 25°C and 37°C, and analysed at 3, 6, 12 and 18 months. These results confirmed that the applicant's 2′-FL incorporated within powdered infant formula is stable for 18 months stored at 4°C, 25°C and 37°C.

2.2 Manufacturing processes

The method of production for the applicant's 2′-FL is the same as 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 2019a).

2′-FL is produced by a microbial fermentation process using a modified strain of *C. glutamicum*. 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 purification, isolation and concentrations steps. The difference with the applicant's method of production to that detailed in the above earlier FSANZ reference relates to the final processing step; either crystallisation from solvent or spray drying to produce the final purified powder. Either method produced equivalent product that meets the applicant's purity specifications, as confirmed by analytical results provided in the application.

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 a specification for its 2′-FL as well as a comparison of its specification to S3—40 and S3—45. 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 and S3—45, are very similar to those provided in the European specification for 2′-FL (EU 2023).

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

For 2′-fucosyllactose (2′-FL) sourced from *Corynebacterium glutamicum*, 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/ivory powder
f	2′-FL	Not less than 94% (water free)
g	D-lactose	Not more than 3.0 % (water free)
h	L-fucose	Not more than 3.0% (water free)
i	3-fucosyllactose	Not more than 3.0% (water free)
j	difucosyl-D-lactose	Not more than 2.0% (water free)
k	glucose	Not more than 3.0% (water free)
l	galactose	Not more than 3.0% (water free)
m	water	Not more than 9.0%
n	ash, sulphated	Not more than 0.5%
o	ethanol	Not more than 1,000 mg/kg (for crystallised product from solvent only)
p	residual proteins	Not more than 0.005%
q	lead	Not more than 0.02 mg/kg
r	arsenic	Not more than 0.03 mg/kg
s	cadmium	Not more than 0.01 mg/kg
t	mercury	Not more than 0.05 mg/kg
u	microbiological	
	i total plate count	Not more than 500 CFU/g
	ii coliforms	Not more than 10 CFU/g
	iii yeasts and moulds	Not more than 100 CFU/g
	iv aflatoxin M1	Not more than 0.025 µg/kg
	v residual endotoxins	Not more than 10 EU/mg

Comments on some of these specification parameters and why they differ to those in S3—40 and S3—45 are that:

- a new parameter of ethanol, but only for 2′-FL that has been purified by crystallisation from solvent (ethanol)

- a number of the purity parameters are on the dried matter basis, (water free) so that condition has been added

no microbiological limits have been listed for *Cronobacter* (absent in 10 g) or *Salmonella* (absent in 25 g) as they are provided in Schedule 27 as food safety microbiological limits in powdered infant formula products.

2.3.1 Impurities

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

The applicant's own specification and analytical results provided in the application indicate that its 2′-FL also meets the requirements of S3—4 related to contamination limits for lead, arsenic, cadmium and mercury.

The application contained information relating to possible impurities in the final purified 2′-FL including the residual starting materials, D-lactose and L-fucose as well as manufacturing by-products such as difucosyllactose and 2′-fucosyl-D-lactulose. Again, these are consistent with the comparable specifications S3—40 and S3—45 for approved forms of 2′-FL. Lactose, fucose and difucosyllactose are natural components of human milk.

The production microorganism is removed during the processing and purifications steps during production of 2′-FL. Qualitative polymerase chain reaction (qPCR) methods were used to confirm that no residual DNA from the production microorganism remains in the final purified nutritive substance.

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 Anion Exchange Chromatography (HPAEC) coupled with Pulsed Amperometric Detector (HPAEC-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. 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 very similar to already permitted 2′-FL specifications, being S3—40 and S3—45. FSANZ has written a comparable new specification specific for the applicant's 2′-FL.

Stability studies of the applicant's 2′-FL concluded that the nutritive substance as a powder is stable for 2 years when both stored at 25°C and 60% relative humidity, and at accelerated conditions of 40°C at 75% relative humidity. The applicant's 2′-FL incorporated within powdered infant formula is stable for 18 months stored at 4°C, 25°C and 37°C.

3 Safety assessment

3.1 GM production strain assessment

3.1.1 Host organism

Corynebacterium glutamicum 534 [ATCC 13032; DSM 20300; NCIB 10025] type strain was isolated from wastewater as part of a screen for bacteria secreting L-glutamate in the late 1950s. It is internationally recognised as a transformational host and has been whole genome sequenced (GenBank: BA000036.3). No virulence genes or genes associated with antibiotic resistance genes were identified. Other closely related strains have been whole genome sequenced, some of which secrete other amino acids as by-products of fermentation.

C. glutamicum has a biotechnological history spanning over 60 years, with industrial scale production first recorded in the 1960s. Since then, it has been used extensively for production of amino acids, and a diverse range of other biomolecules (e.g. diamines, organic acids, carotenoids, proteins and biopolymers), some via metabolic pathway restrictions and others via genetic modification (Sgobba et al 2018; Wendisch et al 2016; Tsuge and Yamaguchi, 2021). Approximately 2 million tonnes of amino acids are produced annually by *C. glutamicum*, of which more than 1 million tonnes is sodium glutamate, used by the food industry as a flavour enhancer, and 0.5 million tonnes of L- lysine, a feed additive.

Because of its industrial importance in producing a wide range of biomolecules that can be used in many different applications, *C. glutamicum* is one of the most intensively studied and documented microorganisms. It is considered to be a safe organism for the production of food and feed additives and poses no risks to human health.

C. glutamicum APC199, the production strain, is a genetically modified version of the type species *C. glutamicum* 13032.

3.2 Characterisation of the GM production organism

3.2.1 Development of the GM production strain

The *C. glutamicum* production strain APC199 was generated by transforming the *C. glutamicum* host strain 13032 with an expression plasmid containing genes that direct the biosynthesis of 2′-FL. The genes were all synthesised *de novo*, ensuring that no genetic material, other than the desired genes and non-coding genetic elements, were introduced into the production strain.

The expression plasmid contains four genes within an expression cassette. The expression cassette consists of the genes, flanked by the *translational elongation factor* (*tuf)* promoter from *C. glutamicum* and the T7 terminator from a pET21a vector. The four genes encode:

- lactose permease from *E. coli*;
- GDP-L-fucose synthase from *E. coli*;
- GDP-D-mannose-4,6-dehydratase from *E.coli*; and
- α-1,2-fucosyltransferase from *Pseudopedobacter saltans*.

Together, these four genes facilitate the uptake of lactose and the production of GDP-fucose. GDP-fucose is an important intermediate in the synthesis of 2′-FL. Figure 2 provides an overview of the functions of the introduced genes in the biosynthesis of 2′-FL.

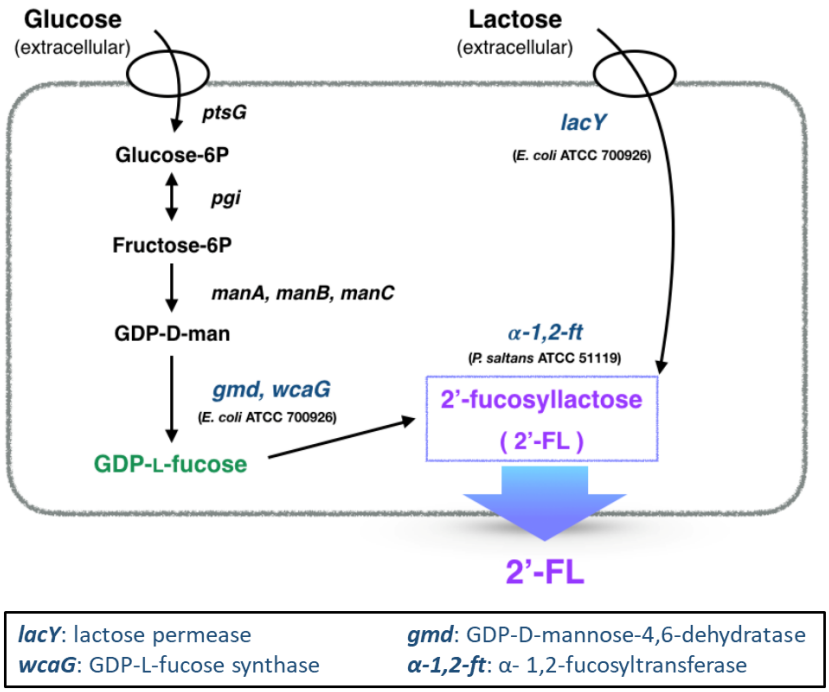

Figure 2: *The role of the four introduced genes in directing the biosynthesis of 2'-FL in the production strain. lacY allows the entry of lactose into the cell; gmd and wcaG facilitate the conversion of GDP-D-mannose into GDP-L-fucose; and α-1,2-ft catalyses the conversion of lactose and GDP-fucose into 2'-FL.*

The expression plasmid also contains the *nptII* gene from *E. coli* K-12, which encodes kanamycin resistance and is used to maintain the presence of the expression plasmid in the production strain. As described in section 2.3.1, the production strain containing the *nptII* gene, is removed during the final production and processing steps.

3.2.2 Characterisation of introduced DNA

The expression plasmid is maintained episomally and is not incorporated into the genome of the host organism. Southern blot analysis was performed using probes that covered the entire plasmid. The analysis confirmed the absence of the expression plasmid sequences in the genome of the production strain.

Data provided by the applicant showed that the production strain contains on average 5 expression plasmids per cell.

3.2.3 Genetic stability and inheritance of the introduced DNA

To provide evidence of the stability of the introduced genes, the applicant provided protein expression data for all introduced genes over multiple generations. The generations included 1, 2, 4, 8, 16 and 32 subcultures of the production strain. Protein expression was measured using enzyme-linked immunosorbent assay (ELISA) and showed that the expression of the introduced genes were consistent across the 32 subcultures.

Further data was provided showing the production of 2′-FL from 0, 8, 16 and 32 subcultures of the production strain. These data showed that production of 2′-FL was consistent across 32 subcultures and the expression plasmid is functional.

Together, these data confirm the stability and inheritance of the expression plasmid in the

production strain over multiple generations.

3.2.4 Conclusion

Corynebacterium glutamicum has a long history of documented use for the production of biomolecules, including food additives, and poses no risks to human health. No safety concerns arising from the gene donors were identified.

Characterisation of the production strain confirmed the expression plasmid carrying the introduced genes 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.

3.3 Toxicology assessment

3.3.1 Previous FSANZ safety assessments of 2′-FL

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

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 between the naturally occurring and manufactured form 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 that 2′-FL is not mutagenic or genotoxic *in vitro* and does not produce adverse effects in oral studies using neonatal animals. 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).

3.3.2 Newly available data

The applicant conducted a literature search to identify new toxicity and clinical studies. In total, 9 human clinical studies and 13 toxicity studies were identified. All of these studies were excluded from this assessment for the following reasons:

- Study already evaluated in previous FSANZ safety assessments; and/or
- Study experimental formulation contained other substances in addition to 2′-FL, and as such the safety outcomes related to the experimental formula could not be attributed directly to 2′-FL.

No additional relevant toxicity or human clinical studies were identified in a search of the published literature.

The applicant also submitted a number of commercial in confidence laboratory studies. The following studies were evaluated for the present application:

- Bacterial reverse mutation assay
- *In vitro* chromosomal aberration test
- *In vitro* micronucleus test
- *In vivo* micronucleus test
- Acute toxicity study in rats
- 90-Day oral toxicity study in rats

3.3.2.1 Toxicological studies with the applicant's 2'-FL

In vivo toxicity studies with the applicant's 2'-FL

Single oral dose toxicity study of 2'-Fucosyllactose in juvenile Sprague-Dawley rats (Biotoxtech, 2019a) Regulatory status: GLP; conducted in accordance with Republic of Korea's Ministry of Food and Drug Safety Notification No. 2017-71 (2017)

The potential acute toxicity of 2′-FL (97% purity) was assessed in juvenile (7-day old) male and female Sprague Dawley (Crl:CD(SD)) rats following a single oral exposure. 2′-FL was administered orally via gavage at doses of 0, 2500, 5000 or 7500 mg/kg body weight to rats (5/sex/group). The vehicle/negative control was water. The dose volume was 10 mL/kg body weight. Rats were housed under standard laboratory conditions with *ad libitum* access to rodent chow feed and drinking water.

Rats were observed immediately following dosing and 1, 2, 4 and 6 hours after dosing. A 2-week observation period followed, during which rats were observed daily for clinical signs and general condition. Body weights were recorded on days 0, 1, 3, 7 and the day of necropsy. All animals were euthanised on day 14 and subjected to a gross pathological examination (no details provided).

One female from the 7500 mg/kg body weight dose group was found dead on day 2 after dosing. This was not considered to be test material related as there were no macroscopic findings in the animal; nor were there any clinical signs or body weight changes in the other females administered this dose.

There were no treatment related effects on clinical signs or gross examination in any treatment group. Histopathological examination was not conducted. Males in the high dose group (7500 mg/kg bw) showed a significant decrease in body weight and body weight gain throughout the 14 day observation period. No other body weight changes were observed in the other groups. It was concluded that the lethal dose of 2′-FL was greater than 7500 mg/kg body weight in both males and females.

90-day repeated oral dose toxicity study with a four-week recovery period of 2'-Fucosyllactose in juvenile Sprague-Dawley rats (Biotoxtech, 2019b) Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

2′-FL (purity: 96.3-97.7%) was administered to juvenile Sprague-Dawley (Crl:CD(SD)) rats (7 days old; 10/sex/group) at doses of 0, 2500, 5000 or 7500 mg/kg body weight/day by oral gavage for 90 days (main group animals). An extra 5 animals of each sex were included in the control and high-dose groups, to assess reversibility of any toxicity during a 4-week recovery period at the end of dosing (recovery group animals). The vehicle/negative control was water and the dose volume was 10 mL/kg bw/day. The rats were group housed under standard laboratory conditions of environment and husbandry.

Animals were observed daily for clinical condition. Body weights were recorded twice weekly during the first four weeks of dosing and then weekly thereafter. Food consumption and detailed physical examinations for signs of toxicity were recorded weekly. Sensory reactivity, grip strength, and motor activity assessments were performed on the main group animals during weeks 12-13 and on the recovery group in weeks 3-4 post-dosing.

Ophthalmic examination was conducted on all animals of the control and high dose groups in week 13. Urine samples were collected for urinalysis over 24 hours from 5 males and 5 females per group a few days before necropsy. In addition urine samples were collected from all animals in the recovery group. At the end of the treatment period, blood samples were collected for haematology and clinical chemistry analysis. All animals underwent a

detailed necropsy at study termination (day 91 for the main group of animals and day 119 for the recovery group of animals). Full macroscopic and histopathological examinations were performed on an extensive range of organs and tissues.

One male of the mid dose group and one female of the high dose group were found dead on days 72 and 30, respectively. The mid dose group death showed distension of the caecum, enlargement of the liver and kidney, and two black foci on the kidney, with no corresponding histopathological lesions. This was deemed to be an unexplained sudden death (which has been documented in Sprague-Dawley rats) and therefore was not considered to be treatment related. The high dose group death showed a serous fluid-filled thoracic cavity and pulmonary congestion/oedema, however there were no morphological changes upon necropsy or on histopathological examination. The high dose group death was determined to be gavage-related error.

Diarrhoea and soft stools were observed in males and females of the high dose group from day 26 onwards, however these findings were not observed during the recovery period, and therefore this was considered to be treatment related. The diarrhoea and soft stools were considered to have little toxicological significance given no other findings were observed, including gross findings at necropsy and histopathological examination. Haematuria was observed in one male of the high dose group, however this was considered to have little toxicological significance given there was no haematuria-related morphological changes at necropsy or upon histopathological examination.

No treatment-related effects were observed on clinical signs, feed consumption, ophthalmology, or functional observations during the dosing period or recovery period. Body weights were significantly decreased in males in the mid dose and high dose groups on days 11 and 4, respectively. However, this was not considered to be treatment related as it was a temporary change with very little difference to the control group (5.8% decreases) and no dose dependency was observed. No body weight changes were observed during the recovery period.

Some minor changes in urinalysis, haematology and clinical chemistry parameters were observed following the treatment period in males and females from all dose groups. However these were not considered to be treatment related because the changes were within the range of historical reference data and were not dose dependent. Urinalysis, haematology and clinical chemistry changes were not observed after the four week recovery period. There were some minor changes in liver weights observed in males and females from all dose groups. However, these were not considered to be treatment related because the changes were within the range of historical reference data and were not dose dependent.

At necropsy, one female from the high dose group (of the main group) showed black focus on the mucosa of the glandular stomach, with erosion evident upon microscopy. One male from the high dose group (of the recovery group) showed a renal mesenchymal tumour in one kidney, at histopathological examination, which was reported as a common spontaneous tumour of the rat kidney. These two findings were not considered treatment related because they both only occurred in one animal and were not dose dependent.

No other treatment related macroscopic or histopathological findings were observed at necropsy, in either the main group or recovery group.

It was concluded that the no observed adverse effect level (NOAEL) of 2'-FL was 7500mg/kg bw/day, the highest dose tested, under the conditions of this study.

Genotoxicity studies with the applicant's 2'-FL

Bacterial reverse mutation test of 2'-Fucosyllactose (Biotoxtech, 2019d) Regulatory status:

GLP; conducted in accordance with OECD TG 471 (1997) and Republic of Korea's Ministry of Food and Drug Safety Notification No. 2017-71 (2017)

The potential mutagenicity of 2′-FL (purity 97%) was evaluated in *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535 and TA1537, and in the *Escherichia coli* strain WP2 *uvrA* (pKM101), with and without metabolic activation using rat liver homogenate (S9 mix). Appropriate positive control articles, as recommended by the OECD guideline, were used. Water was used as the vehicle/negative control. 2′-FL was tested in duplicate at concentrations of 313, 625, 1250, 2500 and 5000 µg/plate. Dose levels were determined from a dose-range finding study conducted using the same strains, methods and conditions.

No precipitation or toxicity was observed at any concentration of the test substance. No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed at any concentration level (up to and including 5000 µg/plate), with or without S9 mix. The expected increases in revertant colony numbers were observed with all the positive control articles used, therefore confirming the validity of the assay.

It was concluded that 2′-FL showed no evidence of mutagenic activity under the conditions of the assay.

In vitro mammalian chromosomal aberration test of 2′-Fucosyllactose (Biotoxtech, 2019e) Regulatory status: GLP; conducted in accordance with OECD TG 473 (2016) and Republic of Korea's Ministry of Food and Drug Safety Notification No. 2017-71 (2017)

The potential for 2′-FL (purity 97%) to induce chromosomal aberrations *in vitro* was evaluated in Chinese hamster lung (CHL/IU) cells. After pre-incubation of cells (24 hours), each plate was divided into three groups: (i) 6 hour treatment with metabolic activation (S9 mix); (ii) 6 hour treatment without metabolic activation; and (iii) continuous 24 hour treatment without metabolic activation. Experiments were performed in duplicate using 2′-FL concentrations up to 5000 µg/mL (based on a dose-range finding study). In the short term treatments, cells were cultured for an additional 18 hours after treatment.

The vehicle/negative control was water. Positive controls were benzo[a]pyrene and mitomycin C in the presence and absence of metabolic activation, respectively. At culture completion cells were prepared for chromosome observations. Three-hundred metaphases per concentration were evaluated for structural chromosome aberrations.

No cytotoxicity was observed. The frequency of cells with chromosome aberrations in both the short-term (with and without metabolic activation) and continuous (without metabolic activation) treatments was not statistically significantly different compared to the negative control group. The positive controls produced the expected increases in frequency of chromosome aberrations, confirming the validity of the test system.

It was concluded that 2′-FL was not clastogenic under the conditions of the study.

In vitro human lymphocyte micronucleus assay with 2′-Fucosyllactose (GenEvolutioN, 2021) Regulatory status: GLP; conducted in accordance with OECD TG 487 (2016)

The potential for 2′-FL to induce micronuclei formation in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected and pooled from four healthy, non-smoking adult volunteers (2 males and 2 females; aged 21-33 years).

The study consisted of a preliminary toxicity study (to determine concentrations for the main test) and a main test, with test procedures being the same. In the main test, lymphocyte cultures were treated with 2′-FL either in the presence or absence of rat liver homogenate (S9 mix) for 3 hours (short-term); or treated with 2′-FL for 24 hours (long-term) in the

absence of S9 mix.

Before treatment with 2′-FL, lymphocyte cultures were stimulated with phytohaemagglutinin A. Following treatment (3-hour exposure) or during treatment (24-hour exposure), cytokinesis was blocked with cytochalasin B. 2′-FL concentrations up to 5000 µg/mL were tested. The vehicle/negative control was water; the positive controls were mitomycin C and vinblastine in the absence of S9 mix; and cyclophosphamide in the presence of S9 mix. Cultures were performed in duplicate.

No precipitation of the test article was observed at any concentration, whatever the treatment duration, in both the presence and absence of S9 mix. In the short-term treatment, 2′-FL did not cause any statistically significant increase in the frequency of micronucleated cells, compared with the negative control, in either the presence or absence of S9 mix, up to the highest concentration tested. In the long-term treatment, a significant increase in micronucleated cells was observed at 2500 µg/mL; however, this effect was not deemed to be concentration related as similar findings were not observed at the higher concentrations of 4000 µg/mL and 5000 µg/mL. Furthermore, one culture out of the two cultures at 2500 µg/mL had a frequency of micronucleated cells within the laboratory's historical negative control data. Therefore, the isolated elevated frequency of micronucleated cells was not considered to be a cytogenetic effect, but rather due to variability of the cell culture in spontaneous micronucleated cell frequency. All positive controls induced significant increases in the proportion of cells with micronuclei, confirming the validity of the test system.

It was concluded that 2′-FL did not induce micronuclei under the conditions of the study.

In vivo micronucleus test of 2′-Fucosyllactose in ICR mice (Biotoxtech, 2019c) Regulatory status: GLP; conducted in accordance with OECD TG 474 (2016) and the Republic of Korea's Ministry of Food and Drug Safety Notification No. 2017-71 (2017)

The potential for 2′-FL (purity 97.56%) to induce micronuclei in polychromatic erythrocytes (PCE) in bone marrow of mice was investigated. Groups of 5 male ICR mice (age 8 weeks) were administered two gavage doses (with a 24-hour interval) of 0, 2500, 5000 or 7500 mg/kg bw 2′-FL. Dose levels were determined from a dose-range finding study conducted using the same strain, method and conditions. The vehicle/negative control was water; and the positive control was Mitomycin C (2 mg/kg bw). The dose volume was 10 mL/kg bw. Mice were housed under standard laboratory conditions with *ad libitum* access to rodent chow feed and drinking water.

Clinical signs were recorded on day 0 (immediately and 2 hours after administration of the first dose), day 1 (before, immediately and 2 hours after administration of the second dose), and day 2. Body weights were recorded prior to harvesting bone marrow cells. Animals were killed 24 hours following treatment with the second dose and bone marrow cells were collected for micronuclei analysis. 4000 PCEs per animal and 500 erythrocytes per animal were scored for the presence of micronuclei. The ratios between PCEs and micronucleated PCEs (MNPCE); and PCEs and total erythrocytes were assessed.

There were no treatment related effects on clinical signs or body weight in any treatment group. There were no increases in the frequency of micronucleated PCEs following treatment with 2′-FL compared with the negative control. No effects on the ratio of PCEs to total erythrocytes were observed following treatment with 2′-FL compared with the negative control, indicating a lack of cytotoxicity. The positive control induced a significant increase in micronucleus frequency.

It was concluded that 2′-FL did not induce micronuclei in mouse bone marrow cells under the conditions of this study.

3.3.3 Safety assessments by other agencies

As noted in previous assessments, the European Food Safety Authority (EFSA) has assessed 2′-FL as a novel food ingredient and issued scientific opinions on the safety of a mixture of 2′-FL/DFL. Uses evaluated included addition to infant formula, follow-on formula, and in food supplements for infants (EFSA 2015, EFSA 2019; EFSA 2022a and 2022b). EFSA concluded that 2′-FL is safe for use alone or in combination with DFL, under the proposed conditions of use. EFSA's assessments include the applicant's 2′-FL produced from fermentation with *C. glutamicum* strain APC199, which concluded that the 2′-FL that is the subject of this application is safe for its intended uses as a novel food ingredient (EFSA 2022b).

The US Food and Drug Administration (FDA) has responded that it has 'no questions' to self-assessments that 2′-FL and a 2′-FL/DFL mixture are Generally Recognized as Safe (GRAS) (FDA 2015a, FDA 2015b, FDA 2016, FDA 2018a, FDA 2018b, FDA 2019, FDA 2020a, FDA 2020b, FDA 2021a). This includes the applicant's 2′-FL produced from fermentation with *C. glutamicum* strain APC199 (FDA 2021b).

In 2018, Health Canada reported that it had 'no objection' to the use of 2′-FL from a genetically modified strain of *Escherichia coli* BL21, in infant formula products (Health Canada 2018).

3.3.4 Summary of the toxicology assessment

Based on previous FSANZ applications of 2′-FL and the toxicological assessment in the present application, it was concluded that 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.

3.4 Microbiology 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 and A12775 (FSANZ 2019b; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023).

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.

3.5 Dietary intake assessment

3.5.1 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 and A1265 (FSANZ 2019a, FSANZ 2021, FSANZ 2022b, FSANZ 2023a). 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 3.

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 published from 2021 onwards, 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 located, 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 table summarising the concentration data of 2′-FL in human milk from 31 studies, however data from these studies were excluded from the dietary intake assessment as they were all published prior to 2021.

3.5.2 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 2019a).

In the dietary intake assessment for A1190, FSANZ undertook a literature search for concentration data for 2′-FL in human milk published since the assessment of A1155. A number of relevant studies were identified, with the range of concentrations reported for secretors (0.6 g/L to 4.0 g/L) falling within a similar range to those reported in previously reviewed studies. It was concluded that an additional dietary intake assessment was therefore not required for A1190 (FSANZ 2021).

In the dietary intake assessment for A1251, estimated dietary intakes of 2′-FL combined with galacto-oligosaccharides (GOS) and/or inulin-type fructans (ITF) in infant formula products were compared with estimated dietary intakes of total oligosaccharides from human milk for 3- and 9-month old infants. In both age groups, intakes of 2′-FL combined with GOS and/or ITF in infant formula and follow-on formula were less than the estimated intakes of total oligosaccharides from human milk (FSANZ 2022b).

In the dietary intake assessment for A1265, FSANZ estimated the dietary intakes of 2′-FL (as a mixture with difucosyllactose (DFL)), in addition to 3 other oligosaccharides at proposed use levels in infant formula products. The results were compared with estimated dietary

intakes of all five oligosaccharides in human milk for 3- and 9-month old infants. FSANZ concluded that mean estimated dietary intakes of the five oligosaccharides 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, except for DFL (FSANZ 2023a).

3.5.3 Key findings of the dietary intake assessment

In the literature search for this assessment, FSANZ identified four primary studies (Biddulph et al. 2023; Menzel et al. 2021, Liu et. al. 2021, Siziba et al. 2021) that met the inclusion criteria. Across these studies, mean 2′-FL concentrations in mature human milk ranged from 1.43 g/L to 2.83 g/L, and median 2′-FL concentrations ranged from 1.15 g/L to 2.70 g/L. As these concentration levels are lower than the highest individual study mean for 2′-FL from Soyyilmaz et al. (2021) used in the assessment of A1265 (4.28 g/L, 15-90 days lactation) 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 1 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 3: *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#)*

Age group	Mean dietary intake (g/kg bw/day)[1]		90th percentile dietary intake (g/kg bw/day)[1]	
	From infant/follow-on formula *β	From human milk #♦	From infant/follow-on formula *β	From human milk #♦
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.
♦ Lower bound of the range assumes mean of means 2′-FL concentration from Soyyilmaz et al. (2021) and mean human milk consumption; upper bound of the range assumes maximum mean 2′-FL concentration 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.

3.6 Nutrition assessment

3.6.1 Objective of the nutrition assessment

Schedule 26 of the Code permits the use of 2′-FL as a nutritive substance produced by several source organisms (as described in Section 1 above) to infant formula products at a maximum permitted amount of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant has requested the use of *C. glutamicum* (strain APC199) for the production of 2′-FL but has not requested a change to the maximum permitted amount. The objective of the nutrition assessment is to determine the effect, if any, of the addition of 2′-FL to infant formula products on the growth of formula-fed infants.

3.6.2 Previous FSANZ assessments of 2′-FL

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

- A1155: 2′-FL and LNnT in infant formula and other products (FSANZ 2019a; FSANZ 2020);
- A1190: 2′-FL in infant formula and other products (FSANZ 2021);
- A1233: 2′-FL from new GM source for infant formula (FSANZ 2022a);
- A1251: 2′-FL combined with galacto-oligosaccharides and/or inulin-type fructans in infant formula products (FSANZ 2022b);
- A1265: 2′-FL/DFL, LNT, 6′-SL sodium salt and 3′-SL sodium salt for use as nutritive substances in infant formula products (FSANZ 2023a); and
- A1277: 2′-FL from GM *Escherichia coli* K-12 (gene donor: *Helicobacter enhydrae*) in infant formula products (FSANZ 2023b).

FSANZ considered eight infant studies in A1155 (FSANZ 2019a; FSANZ 2020). The studies evaluated the potential effect of 2′-FL on several growth endpoints, including height and weight Z-scores, mean weight gain per day, fat mass index, mean length, weight and head circumference, and weight velocity. Five studies were clinical trials (Marriage et al. 2015; Kajzer et al. 2016; Puccio et al. 2017; Storm et al. 2019; Román et al. 2020) and three were cohort studies (Sprenger et al. 2017; Larsson et al. 2019; Lagström et al. 2020). Based on the available evidence it was concluded that the addition of 2′-FL and LNnT to infant formula products at levels normally found in human milk is unlikely to affect growth.

In A1190 FSANZ assessed four studies on the effect of infant formula products containing 2′-FL on growth endpoints including body weight and weight-for-age Z-scores (FSANZ 2021). The studies included growth endpoints including body weight and weight-for-age Z-scores (Reverri et al. 2018; Berger et al. 2020; Leung et al. 2020; Ramirez-Farias et al. 2021). FSANZ maintained the conclusion that no difference in growth was observed in infants fed formula containing 2′-FL, compared to control formula.

No new relevant studies were identified for A1233 to alter the previous conclusions (FSANZ 2022a).

One clinical study was identified for A1251 (FSANZ 2022b). Vandenplas et al. (2020) investigated the effect of infant formula products containing 2′-FL, short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) on equivalence in mean weight gain per day, compared to control formula. It was concluded that, based on available evidence, no difference in growth is likely to occur in infants fed infant formula products that contain 2′-FL, scGOS and/or lcFOS at the permitted levels.

Three clinical studies were included in the body of evidence for A1265 (FSANZ 2023a). These studies evaluated the effect of infant formula products containing a mixture of HiMOs on growth using mean weight gain per day as the primary outcome (Parschat et al. 2021; Cohen 2022; Lasekan et al. 2022). In Parschat et al. (2021) and Lasekan et al. (2022), the HiMO mixture added to the experimental formula contained 2′-FL, LNT, 3′-SL sodium salt and 6′-SL sodium salt. The HiMO mixture added to the experimental formula in Cohen (2022) contained 2′-FL, DFL, LNT, 3′-SL sodium salt and 6′-SL sodium salt. FSANZ concluded that infants achieve normal growth when fed infant formula products containing HiMOs at levels that are normally present in human milk.

Two clinical trials were assessed in the body of evidence for A1277 (FSANZ 2023b). Alliet et al. (2022) and Wallingford et al. (2022) studied the effects of infant formula products supplemented with 1.0 g/L 2′-FL on mean weight gain per day. Both studies reported the difference in mean weight gain per day between experimental and control groups was less than the clinically relevant threshold of ± 3 g/day. It was concluded that the addition of 2′-FL to infant formula products is unlikely to pose a risk to the normal growth of infants.

3.6.3 Current assessment

The applicant provided nine human clinical studies to support their application. Seven of the studies were excluded:

- Six studies were previously assessed by FSANZ in A1190 (Ramirez-Farias et al. 2021), A1265 (Lasekan et al. 2022; Parschat et al. 2021), and A1277 (Alliet et al. 2022; Wallingford et al. 2022). Bauer et al. (2021) was a conference abstract that included data from a study already evaluated by FSANZ in A1265 (Cohen et al. 2022).
- A study by Hascoët et al. (2021) did not investigate the effect of 2′-FL in infant formula products on growth.

In two studies, the experimental formula contained 2′-FL and LNnT (Gold et al. 2022; Vandenplas et al. 2022), therefore any effects on growth could not be attributed to the presence of 2′-FL.

Gold et al. (2022) studied the effect of supplementing amino acid-based formula with 1.0 g/L 2′-FL and 0.5 g/L LNnT on the growth of infants with cow's milk protein allergy (CMPA), at six clinical sites in Australia, in a non-randomised, non-controlled study. Twenty-nine (20 male, 9 female) term infants with CMPA, and with no known underlying medical conditions impairing growth, aged 1-8 months were fed the formula for four months. Following the intervention, Z-scores were calculated for weight-for-age, length-for-age, head circumference-for-age, and body mass index (BMI)-for-age. Authors reported that growth within the four month intervention period generally progressed in line with the World Health Organization (WHO) Child Growth Standards (WHO 2008). The mean Z-scores for each anthropometric measure were within the normal range, defined as up to two standard deviations below and up to one standard deviation above the median (WHO 2008). The authors noted that the age range at enrolment may have affected the observed changes in growth over the four month intervention period. Another limitation was the lack of a control group.

Vandenplas et al. (2022) measured the effect of extensively hydrolysed formula containing 1.0 g/L 2′FL and 0.5 g/L LNnT on growth of infants with CMPA in a double-blind, randomised controlled trial. The study was conducted in 41 clinical sites in Europe, and 3 sites in Singapore. One hundred and ninety-four full-term infants aged 0-6 months at baseline with CMPA, and no significant congenital illness or malformation that could affect growth, were randomly allocated to receive experimental formula (n = 97) or control formula (n = 97), without 2′FL or LNnT. The primary endpoint was non-inferiority in weight gain per day compared to control formula.

Three infants in the experimental group and one in the control group did not commence treatment. Fifty-one percent of the experimental group and fifty percent of the control group were male. Fifteen infants (15.5%) in the experimental group and 24 (24.7%) in the control group withdrew before the four-month follow up period due to an adverse event, feeding difficulties, parental dislike of study formula characteristics, or other unknown reasons. A further 6 infants in the experimental formula group and 8 infants in the control formula group were excluded from the analysis due to major protocol deviations, such as an infant receiving non-study formula or treatment with oral corticosteroids. Therefore, the number of infants who completed the trial per-protocol was 73 (75.3%) and 64 (66.0%), for the experimental and control groups respectively.

The difference in mean weight gain per day between the experimental and control groups for the per-protocol analysis set at four months was -0.74 g/day (p = 0.0049). No significant differences were observed for weight, length, head circumference or BMI. Intention-to-treat analysis was also conducted for the 94 (96.9%) infants in the experimental group and the 96 (99.0%) infants in the control group who had commenced the treatment. The difference in

mean weight gain for the intention-to-treat population was 0.45 g/day ($p < 0.0001$), which study authors reported confirmed non-inferiority of the experimental formula. A limitation of the study was the intention-to-treat and per-protocol analyses were one-sided to detect non-inferiority, rather than the clinically relevant difference of ± 3 g/day (American Academy of Pediatrics 1988).

FSANZ undertook a literature search in PubMed on 27 October 2023 to identify any additional relevant studies published since the previous assessment in A1277 using the 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 for baby or maternal”. Twenty-five studies were returned through this search. The studies identified in this search were not relevant, as they did not assess the impact of the addition of 2′-FL to infant formula products, in comparison to control formula not containing 2′-FL, on the growth of infants. Therefore, no new relevant studies were identified for the nutrition assessment.

3.6.4 Summary of the nutrition assessment

Schedule 26 of the Code currently permits the use of 2′-FL produced by several source organisms as nutritive substances in infant formula products, at a maximum permitted amount of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant did not request changes 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 six applications: A1155, A1190, A1233, A1251, A1265 and A1277 (FSANZ 2019a; FSANZ 2020; FSANZ 2021, FSANZ 2022a; FSANZ 2022b; FSANZ 2023a; FSANZ 2023b). Eighteen studies were used in the body of evidence which investigated infant growth endpoints including body weight, mean weight gain per day, anthropometric Z-scores, fat mass index, and weight velocity. It was concluded that the addition of 2′-FL to infant formula products at levels typically found in human milk does not pose a risk to normal growth of infants.

Two new studies provided by the applicant reported no difference in growth in infants fed formula containing LNnT and 2′-FL compared to control. However the studies were not directly relevant to the assessment because any effect (or lack of effect) on growth cannot be attributed to 2′-FL.

No new relevant studies were identified in the present assessment. Therefore FSANZ maintains the conclusion that the addition of 2′-FL to infant formula at levels normally found in human milk is unlikely to affect growth.

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 is chemically and structurally identical to the naturally occurring substance in human milk.

C. glutamicum has a long history of documented use for the production of biomolecules, including food additives, and poses no risks to human health. No safety concerns arising from the gene donors were identified. Characterisation of the production strain confirmed the expression plasmid carrying all the introduced genes was 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 microbiological safety concerns were identified. 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.

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

FSANZ has previously assessed the effect of 2′-FL in infant formula products on growth in six applications. These assessments concluded that the addition of 2′-FL to infant formula products at levels typically found in human milk does not pose a risk to normal growth of infants. Two studies provided by the applicant reported no difference in growth in infants fed formula containing LNnT and 2′-FL compared to control, however were not directly relevant to the assessment because any effect (or lack of effect) on growth cannot be attributed to 2′-FL. No new relevant studies were identified for this assessment, and therefore FSANZ maintains the previous conclusions that the addition of 2′-FL to infant formula at levels normally found in human milk is unlikely to affect growth.

Based on the available toxicological and nutritional 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.

5 References

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