APPLICATION FOR THE APPROVAL OF 2'-FUCOSYLLACTOSE (2'-FL) FROM *CORYNEBACTERIUM GLUTAMICUM* AS A SUBSTANCE USED FOR A NUTRITIVE PURPOSE TO BE INCLUDED IN THE *AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE*

PREPARED BY:

[redacted]

Application for the Approval of 2'-Fucosyllactose (2'-FL) from *Corynebacterium glutamicum* as a Substance Used for a Nutritive Purpose to be Included in the *Australia and New Zealand Food Standards Code*

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INTRODUCTION

Advanced Protein Technologies Corp. ("APTech") is a Korean manufacturer of food ingredients that has developed the technology to produce 2'-fucosyllactose (2'-FL), a trisaccharide consisting of L-fucose, D-galactose, and D-glucose *via* a microbial fermentation process, which utilises a genetically modified strain of *Corynebacterium glutamicum* (strain APC199). Such advances in biotechnology have begun to enable infant formula manufacturers to produce marketable products that more closely resemble natural human breast milk. APTech's 2'-FL, produced from fermentation of *C. glutamicum* (strain APC199), is one component of this current market trend, which provides a safe and suitable manufactured 2'-FL ingredient for use in infant formula products designed to closely mimic natural human breast milk.

Generally, human milk oligosaccharides (HMOs) produced from microbial sources, such as APTech's 2'-FL, have been approved in a wide range of milk-based products, dairy analogues, cereal bars, table-top sweeteners, infant formulae, follow-on formulae, processed cereal–based food, baby food for infants and young children, foods for special medical purposes, meal replacement products, bread products, flavoured drinks, coffees, teas, and food supplements in the European Union. Similarly, in the United States (U.S.), a number of different HMOs obtained from various microbial sources, including APTech's 2'-FL, have been concluded to be Generally Recognized as Safe for the same food uses, which, upon notification to the U.S. Food and Drug Administration, has been responded to without questions on numerous occasions.

The first 2 HMOs to be included in the *Food Standards Australia New Zealand Food Code* ("the Code") are 2'-FL and lacto-*N*-neotetraose (LNnT). Both of these ingredients have gained approval for use in Australia and New Zealand in accordance with the specifications in *Schedule 3 – Identity and purity* and *Schedule 26 – Food produced using gene technology*, as currently defined in the Code. 2'-FL and LNnT are approved for use in infant formula products when obtained *via* fermentation of genetically modified *Escherichia coli* K-12, containing either the gene for *alpha*-1,2-fucosyltransferase from *Helicobacter pylori* (2'-FL) or the genes for *beta*-1,3-*N*-acetylglucosaminyltransferase from *Neisseria meningitides* and *beta*-1,4-galactosyltransferase from *H. pylori* (LNnT).

This application has been prepared to gain authorisation of 2'-FL produced from fermentation using an alternative microbial source, *C. glutamicum* APC199, as a nutritive substance in Australia and New Zealand. This ingredient is proposed for addition at levels of up to 2.4 g/L (*i.e.*, a maximum level of 96 mg/100 kJ) in infant formula products, which is in line with the currently approved food uses for 2'-FL in Australia and New Zealand. Moreover, APTech's 2'-FL currently meets all other established specifications for identity and purity for 2'-FL as defined in *Schedule 3 – Identity and purity*. The applicant, APTech, is therefore seeking to amend the Code to include the *C. glutamicum* APC199 production strain as an alternative permitted source in the production of 2'-FL.

This application dossier has therefore been prepared in accordance with the following guidelines, as presented in the *Food Standards Australia New Zealand Application Handbook*:

- *Guideline 3.1.1 – General requirements* (all sections)
- *Guideline 3.3.2 – Processing aids* (parts of Section C and all of Sections D and E)
- *Guideline 3.3.3 – Substances used for a nutritive purpose* (all sections)
- *Guideline 3.6.2 – Special purpose foods – Infant formula products* (all sections)

GENERAL REQUIREMENTS

This section has been completed in accordance with *Guideline 3.1.1 – General requirements* of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a).

1. Applicant Details [CONFIDENTIAL]

Contact Information

2. Purpose of the Application

This application is being submitted to Food Standards Australia New Zealand (FSANZ) to seek approval for the use of a purified human milk oligosaccharide (HMO) ingredient, produced *via* microbial fermentation, in infant formula products as a nutritive substance. APTech has developed the means to produce 2'-fucosyllactose (2'-FL) using a strain of *Corynebacterium glutamicum* (strain APC199) *via* a biosynthesis pathway that utilises the simple sugars glucose and lactose. APTech's final 2'-FL ingredient is a powder that contains not less than 94% 2'-FL as the primary constituent and lesser amounts of other carbohydrates, including difucosyllactose (DFL), glucose, and galactose. The final 2'-FL ingredient does not contain any chemical or microbial hazards that would pose a safety concern.

It is APTech's view that its 2'-FL ingredient meets the current definition of an ingredient that would be "*used as a nutritive substance*," as defined in Standard 1.1.2—12 of the *Australia New Zealand Food Standards Code* ("the Code"), on the basis that it is added to a food to "*achieve a nutritional purpose*" and is a "*substance that is identified in this Code as one that may be used as a nutritive substance*" (FSANZ, 2022a). At present, 2'-FL is "*permitted for use by Standard 1.5.2*" (*i.e.*, food produced using gene technology), as described in *Schedule 29 – Special purpose foods*, "Infant formula products—substances permitted as nutritive substances" (S29—5) (FSANZ, 2022b). The general purity parameter for 2'-FL, as listed in *Schedule 3 – Identity and purity*, "Specification for 2'-fucosyllactose sourced from *Escherichia coli* K-12" (S3—40) and "Specification for 2'-fucosyllactose sourced from *Escherichia coli* BL21" (S3—45), is specified as not less than 83% or 90% 2'-FL, respectively (FSANZ, 2022c). The specified 2'-FL content of APTech's 2'-FL produced from fermentation with *C. glutamicum* APC199 is consistent with the authorised 2'-FL.

However, as listed in *Schedule 26 – Food produced using gene technology* of the Code, "Permitted food produced using gene technology and conditions" (S26—3), 2'-FL of microbial origin must also comply with any corresponding conditions listed as outlined in Table S26—3(7) (FSANZ, 2022d). Given that APTech's 2'-FL is produced *via* fermentation using a microbial production organism that is not currently described in Table S26—3(7), the purpose of this application is to amend S26—3 to include this *C. glutamicum* strain as a source for 2'-FL: "*Corynebacterium glutamicum* strain APC199 containing the gene for α-1,2-fucosyltransferase from *Pseudopedobacter saltans*."

The applicant therefore recognises that the approval of this production strain as a source of 2'-FL, as a nutritive substance, would require the following changes:

- Amend Schedule 26—3 to permit 2'-FL derived from a new microbial source with distinct conditions of use (*i.e.*, exclusivity details).

- Amend Schedule 3 to include prescribed specifications for APTech's 2'-FL ingredient under "Specification for 2'-fucosyllactose sourced from *Corynebacterium glutamicum* APC199." New microbial sources inherently introduce slight variations in terms of the other minor carbohydrate components present in the final ingredient, which only slightly differ from those that were previously approved.

The information presented within this application support the safe and suitable use of 2'-FL produced *via* fermentation using the microbial production strain *C. glutamicum* APC199 for its proposed food use applications.

3. Justification for the Application

3.1 Need for the Proposed Change

2'-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. 2'-FL is currently authorised for use in infant formula products as a nutritive substance in Australia and New Zealand in accordance with S26—3. The currently authorised 2'-FL ingredient is manufactured using the same general microbial fermentation production techniques as APTech's 2'-FL ingredient; however, the only currently permitted sources of 2'-FL are "*Escherichia coli* K-12 containing the gene for *alpha*-1,2-fucosyltransferase from *Helicobacter pylori*" (Application A1155)[1] and "*Escherichia coli* BL21 containing the gene for *alpha*-1,2-fucosyltransferase from *Escherichia coli* O126" (Application A1190)[2]. 2'-FL produced by APTech is instead obtained from the microbial fermentation of a genetically modified strain of *C. glutamicum* (strain APC199), which has been demonstrated to be the chemically and compositionally similar to the 2'-FL ingredient currently on the Australian and New Zealand marketplace, in addition to the naturally occurring 2'-FL present in human breast milk. Addition of *C. glutamicum* (strain APC199) to the list of permissible sources of 2'-FL would provide infant formula manufacturers with an alternative source of 2'-FL aside from those that are currently permitted, which would promote healthy market competition that will ultimately benefit the Australian/New Zealand consumer.

Similar applications have been made by APTech in other countries to include *C. glutamicum* as a permissible source of 2'-FL for use in infant formula products. In the United States (U.S.), APTech's 2'-FL produced from *C. glutamicum* APC199 has Generally Recognized as Safe (GRAS) status for use in infant formula at a level of 2.4 g/L in non-exempt formula for term infants in addition to a variety of other food uses. Notification of this conclusion was filed under GRAS Notice (GRN) 000932 and has received a letter of "no questions" from the U.S. Food and Drug Administration (FDA) (Advanced Protein Technologies, Corp., 2020; U.S. FDA, 2021a). In the European Union (EU), APTech's 2'-FL from microbial fermentation with *C. glutamicum* APC199 received a positive scientific opinion from the European Food Safety Authority (EFSA), who concluded that this 2'-FL ingredient is safe for its intended use as a novel food (EFSA, 2022a). Formal approval for the use of APTech's 2'-FL was subsequently acquired under *Commission Implementing Regulation (EU) 2023/859 of 25 April 2023 amending Implementing Regulation (EU) 2017/2470 as regards the specifications of the novel food 2'-Fucosyllactose (microbial source) to authorise its production by a derivative strain of Corynebacterium glutamicum ATCC 13032* (EU, 2023). Similar approvals for the use of APTech's 2'-FL from *C. glutamicum* APC199 have also been issued in Korea, Thailand, Vietnam, and Malaysia (documentation included in Appendix A-5).

Therefore, APTech wishes to market its 2'-FL in infant formula products to facilitate the goal of harmonisation with international food standards as defined by the Australian and New Zealand governments.[3] The proposed change to include a wider range of permissible sources of 2'-FL in Australia and New Zealand will further the goal of alignment with current EU and U.S. permissions.

[1] https://www.foodstandards.gov.au/code/applications/Pages/A1155.aspx (FSANZ, 2021a).
[2] https://www.foodstandards.gov.au/code/applications/Pages/A1190.aspx (FSANZ, 2021b).
[3] https://foodregulation.gov.au/internet/fr/publishing.nsf/Content/system-aims-and-objectives.

3.2 Regulatory Impact Information

Based on the data presented within this application, inclusion of provisions in the Code to permit the use of APTech's 2'-FL in infant formula products:

- Is not expected to result in any adverse effects within target groups as a result of this change;
- Will not result in any additional cost to regulators, as 2'-FL is already approved for use in infant formula products;
- Remains consistent with existing permissions for 2'-FL ingredients;
- Promotes consistency between Australia and New Zealand food standards and those that have been established internationally; and
- Supports an internationally competitive food industry for infant formula products.

4. Information to Support the Application

Detailed supporting information and data contained within this application are provided to enable the objectives specified in Section 18 of the *Food Standards Australia New Zealand Act 1991*[4] ("the FSANZ Act") to be addressed. Briefly, these objectives, relevant to the consideration of the safety of a substance, are (i) the protection of public health and safety; (ii) the provision of adequate information relating to food to enable consumers to make informed choices; and (iii) the prevention of misleading or deceptive conduct.

To meet these defined objectives, technical information on the use and manufacture of APTech's 2'-FL has been provided in Section B of this application. Moreover, detailed safety data have been presented in Section C of this application and are based on the understanding that 2'-FL from fermentation is chemically and structurally equivalent to the 2'-FL naturally present in human breast milk. Accordingly, an extensive database of safety data already exists for 2'-FL preparations manufactured using fermentation techniques with a range of different production organisms. Numerous reviews by scientific bodies and regulatory authorities are therefore summarised in conjunction with any newly generated safety data since the previous FSANZ evaluations of 2'-FL obtained from fermentation of microbial sources.

This application is prepared in accordance with the relevant sections within the *Food Standards Australia New Zealand Application Handbook* (from 01 July 2019), including the following:

- *Guideline 3.1.1 – General requirements* (all sections)
- *Guideline 3.3.2 – Processing aids* (parts of Section C and all of Sections D and E)
- *Guideline 3.3.3 – Substances used for a nutritive purpose* (all sections)
- *Guideline 3.6.2 – Special purpose food – Infant formula products* (all sections)

[4] http://www8.austlii.edu.au/cgi-bin/viewdoc/au/legis/cth/consol_act/fsanza1991336/s18.html.

5. Assessment Procedure

APTech considers the General Procedure (Subdivision D), Cost Category Level 1 (up to 240 hours), to be the most appropriate assessment procedure for the application of 2'-FL as a substance used for a nutritive purpose. The safety of the proposed use of the 2'-FL ingredient is largely established and is further supported by the fact that the use levels will remain within the ranges that occur naturally within human breast milk or within ranges used in other product types internationally. Additionally, 2'-FL obtained from fermentation of microbial sources has been approved for use in infant formula products in Australia and New Zealand and is already contained within *Schedule 26 – Food produced using gene technology* of the Code. The nature of the anticipated amendments to the Code that would result from this current application are therefore not expected to be unduly complex.

6. Confidential Commercial Information (CCI)

Confidential commercial information (CCI), in relation to food, is defined in Section 4 of the FSANZ Act[5] as meaning either "*a trade secret relating to food*" or "*any other information relating to food that has a commercial value that would be, or could reasonably be expected to be, destroyed or diminished if the information were disclosed*." In accordance with this meaning, APTech requests that certain proprietary data and information forming part of the application dossier be treated as CCI. General summaries of the required proprietary data and information have been provided within the application. Data requested to be treated as CCI have been removed and are summarised in Table 6-1 below along with verifiable justification and are provided within Appendix A in full.

Table 6-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
1	Applicant and contact person contact details.	The contact details for the person responsible for the dossier are sensitive and should be treated as confidential. Public disclosure of this information is not required for the safety assessment of 2'-FL.
Appendix A	Raw materials and processing aids used in the production process. Detailed description of the production process. Description of the genetic modifications of the production microorganism.	The specific details of the manufacturing process are considered confidential and proprietary and are of significant commercial value to the applicant. A non-confidential summary has been provided in Section B.4.1. The raw materials and processing aids are a crucial part of the confidential and proprietary manufacturing process. The production microorganism is a key component of the manufacturing process (the fine details of which should also be considered as confidential). The publication of these data would provide a competitive advantage to other manufacturers.
Appendix A-1, A-2, and A-3	Nuclear magnetic resonance spectra of the applicant's ingredient. Comprehensive documentation pertaining to the production organism. Certificates of analysis for commercial lots.	These appendices contain sensitive information considered highly confidential and of significant commercial value to the applicant. Public disclosure of this information is not required for the safety assessment of 2'-FL and the *Corynebacterium glutamicum* source organism.

[5] http://www8.austlii.edu.au/cgi-bin/viewdoc/au/legis/cth/consol_act/fsanza1991336/s4.html.

Table 6-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
Appendix A-4	Unpublished and confidential toxicology studies.	The full unpublished toxicology study reports contain proprietary scientific data that support the safety of the applicant's 2'-FL ingredient. The studies were conducted with considerable financial investment by the applicant and include personal information related to involved laboratory personnel. This information has been redacted from the non-confidential full study reports included in Appendix E.

2'-FL = 2'-fucosyllactose.

7. Additional Confidential Information

No additional information from this application package is requested to remain confidential aside from what has been outlined in the section above.

8. Exclusive Capturable Commercial Benefit (ECCB)

As indicated in the sections above, APTech is seeking exclusive permission for the use of 2'-FL from *C. glutamicum* APC199 as a substance used for a nutritive purpose. Thus, it is anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act,[6] which states:

> *An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:*
>
> *(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and*
>
> *(b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application.*

Currently, APTech is not the only global manufacturer of 2'-FL; however, due to the nature of ingredient manufacturing *via* fermentation processes, it can be reasonably expected that only APTech will commercially benefit from the inclusion of *C. glutamicum* APC199 as a permitted source of 2'-FL in the Code upon successful approval of this application and, as such, an ECCB is expected to be conferred.

[6] http://www8.austlii.edu.au/cgi-bin/viewdoc/au/legis/cth/consol_act/fsanza1991336/s8.html.

9. International and Other National Standards

The current international and other national standards relevant to APTech's 2'-FL ingredient subject to this application are listed below. Data related to the safety of 2'-FL, as evaluated by the relevant authoritative bodies, are discussed in Section C.6, and the approved conditions of use are presented in Section D.5.

9.1 European Union

2'-FL is currently authorised for use as a novel food ingredient in the EU under *Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods* for use in a number of food and beverage categories, including infant formula and follow-on formula and food supplements (EU, 2017). Preparations of 2'-FL available in the EU must comply with the outlined specifications for 2'-FL as adopted by the European Commission. Presently, these specifications stipulate that 2'-FL must be produced from chemical synthesis or from microbial fermentation. Current permitted sources of 2'-FL from microbial fermentation in the EU include a genetically modified strain of *C. glutamicum* ATCC 13032, along with 2 genetically modified strains of *E. coli* (K-12 or BL21). This *C. glutamicum* production strain, permitted for use as a source of 2'-FL in the EU, is APTech's *C. glutamicum* APC199 as established in Commission Implementing Regulation (EU) 2023/859.

9.2 United States

In the U.S., 2'-FL produced from *C. glutamicum* APC199 has GRAS status for use as an ingredient at a level of 2.4 g/L of formula as consumed in milk- and soy-based, non-exempt infant formula for term infants; at a level of 2.4 g/L in drinks for toddlers and children ages 1 to 3 years, as consumed; at levels ranging from 0.24 to 1.2 g/serving in infant and toddler foods; and at levels ranging from 0.28 to 1.2 g/serving in beverage and beverages bases, breakfast cereals, dairy product analogues, frozen dairy desserts and mixes, gelatins, puddings, fillings, grain products and pastas, jams and jellies, milk and milk products, processed fruits and fruit juices, and sweet sauces, toppings and syrup. The GRAS status of 2'-FL produced from *C. glutamicum* APC199 has been notified to the U.S. FDA under filing number GRN 932, which has received a letter of "no questions" (Advanced Protein Technologies, Corp., 2020; U.S. FDA, 2021a).

Generally, 2'-FL preparations manufactured through either chemical synthesis or microbial fermentation have GRAS status in the U.S. in a wide range of products. Of the 14 GRAS Notices filed to-date, only 3 have been voluntarily withdrawn from review and several are currently undergoing review with the U.S. FDA. A summary of the GRAS Notices submitted to-date in the U.S. for 2'-FL preparations or those that contain 2'-FL is provided in Table 9.2-1, below.

Table 9.2-1 Summary of GRAS Notices Submitted to the United States Food and Drug Administration for 2'-FL (U.S. FDA, 2023)

Company	Substance	U.S. FDA Response	GRAS Notice No.
Glycom A/S	2'-O-fucosyllactose	No questions	GRN 000546
Jennewein Biotechnologie, GmgH	2'-Fucosyllactose	No questions	GRN 000571
Glycom A/S	2'-*O*-fucosyllactose	No questions	GRN 000650
Glycosyn, LLC and Friesland Campina Domo B.V.	2'-Fucosyllactose	No questions	GRN 000735
DuPont Nutrition & Health	2'-O-fucosyllactose	No questions	GRN 000749
Glycom A/S	2'-fucosyllactose and difucosyllactose	No questions	GRN 000815
BASF SE	2-fucosyllactose	No questions	GRN 000852

Table 9.2-1 Summary of GRAS Notices Submitted to the United States Food and Drug Administration for 2'-FL (U.S. FDA, 2023)

Company	Substance	U.S. FDA Response	GRAS Notice No.
Advanced Protein Technologies, Corp.	2'-fucosyllactose	Withdrawn	GRN 000859
DuPont Nutrition and Health	2-*O*-fucosyllactose	No questions	GRN 000897
Jennewein Biotechnologie GmbH	2'-fucosyllactose	Withdrawn	GRN 000924
Jennewein Biotechnologie GmbH	2'-fucosyllactose	No questions	GRN 000929
Advanced Protein Technologies Corp.	2'-fucosyllactose	No questions	GRN 000932[a]
Amyris, Inc.	2'-fucosyllactose	Withdrawn	GRN 000987
Chr. Hansen, Inc.	2'-fucosyllactose	No questions	GRN 001014
Glycom A/S	2'-fucosyllactose	No questions	GRN 001034
Chr. Hansen A/S	2'-fucosyllactose	Pending	GRN 001040
Kyowa Hakko Bio Co., Ltd.	2'-fucosyllactose	Pending	GRN 001051
Glycom A/S	2'-fucosyllactose	No questions	GRN 001060
Inbiose N.V.	2'-fucosyllactose	Pending	GRN 001091

2'-FL = 2'-fucosyllactose; FDA = Food and Drug Administration; GRAS = Generally Recognized as Safe; GRN = Generally Recognized as Safe (GRAS) Notice; U.S. = United States.
[a] The ingredient assessed under GRN 932 is the subject of this current submission to Australia and New Zealand.

9.3 Canada

2'-FL derived from a genetically modified strain of *E. coli* BL21 (DE3) (strain #1540) is approved for use as a novel food in Canadian infant formula products (Health Canada, 2018a,b). In its public notification to the petitioner, Health Canada stated that it has no objection to the use of the bacterially synthesised 2'-FL ingredient (≥90% purity) when used in infant formula for term infants to a maximum use level of 1.2 g 2'-FL/L of formula. The 2'-FL ingredient, from fermentation, was "*confirmed to be chemically equivalent to 2'-FL isolated from human milk.*"

Based on the described use level of 2'-FL in infant formula, the applicant estimated that an exposure of up to 0.31 g of 2'-FL/kg body weight/day could be reasonably expected. The margin of exposure (MOE) was calculated to be approximately 25 times lower than the referenced no-observed-adverse-effect level (NOAEL) from a 90-day subchronic oral toxicity study conducted in rats evaluated by Health Canada. 2'-FL produced from fermentation, as described in the assessed novel food submission [*i.e.*, fermentation with *E. coli* BL21 (DE3) #1540] was therefore concluded to not pose a safety concern at the defined use levels.

10. Statutory Declaration

A signed Statutory Declaration is provided in Appendix B.

11. Checklists

The completed checklists relating to the information required for submission with this application is provided in Appendix C.

A. INFORMATION ON THE USE OF THE NUTRITIVE SUBSTANCE

This section has been completed in accordance with Section A of *Guideline 3.3.3 – Substances used for a nutritive purpose* of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), which states the following information are to be provided:

1. A statement of the purpose(s) of the use of the nutritive substance in food. If such a substance has multiple purposes or functions, then these must all be briefly described. When the purpose for using a nutritive substance in food (including special purpose foods) relates to a nutritional purpose to deliver a potential beneficial physiological or health-related outcome, the application must:

    a. Include a brief description of all of the physiological or health-related function(s) of the substance at the proposed level; and

    b. Be stated in a way that can be measured – *i.e.*, as an outcome in clinical studies.

2. A statement as to whether the assessed nutritive substance is representative of the commercial product on which approval is sought. Scientific evidence must contain sufficient detail to enable an independent assessment of the methods and results to confirm the study conclusions, and any scientific evidence for a potential beneficial physiological or health-related outcome must:

    a. Be based on studies conducted on human subjects;

    b. Be based on foods or food groups which contain the nutritive substance rather than the use of the substance alone; and

    c. Relate to normal use by the target population group and the foods must contribute to the demonstrated nutritional role relevant to that target population.

APTech is seeking permission for the use of 2'-FL from *C. glutamicum* APC199 in Australia and New Zealand as a nutritive substance within infant formula products on the basis that it is manufactured *via* fermentation using a proprietary production strain. 2'-FL has been identified as the most abundant HMO in breast milk across different women with varying milk types (see Section C.1), and the use of APTech's ingredient in infant formula products is intended to bring the composition of infant formula products closer to that of human breast milk. The presence of HMOs in human breast milk indicates that these components likely play a beneficial role in the development of infants. Studies conducted with infants and various HMO preparations are discussed within Section C. Studies summarised in Sections C.3.1.1 and C.3.2.1 were conducted with APTech's 2'-FL from *C. glutamicum* APC199 ingredient—*i.e.*, a nutritive substance that is representative of the commercial product on which approval is sought.

B. TECHNICAL INFORMATION ON THE USE OF THE NUTRITIVE SUBSTANCE

Technical information on APTech's 2'-FL ingredient is described in this section. Specifically, this section has been completed in accordance with the requirements outlined in the relevant sections of *Guideline 3.3.3 – Substances used for a nutritive purpose* and *Guideline 3.3.2 – Processing aids* of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a). The pertinent requirements of each guideline have been addressed in the subsections below.

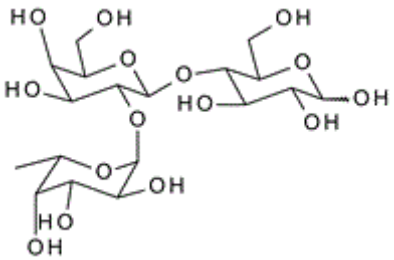
B.1 Information to Enable Identification

B.1.1 Identification of the Nutritive Substance

2'-FL produced by microbial fermentation of a genetically modified *C. glutamicum* (strain APC199) contains 2'-FL as the primary constituent (*ca.* 97%) and trace levels of other related carbohydrates, such as lactose, DFL, glucose, and galactose. The information to establish the identity of APTech's 2'-FL is included in Table B.1.1-1.

The 2'-FL produced by APTech has been analytically demonstrated by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy to be chemically and structurally identical to 2'-FL naturally present in human breast milk. The full analytical report is provided in Appendix A-1.

Table B.1.1-1 Identity of 2'-FL Produced by Microbial Fermentation of *Corynebacterium glutamicum* APC199

Chemical Name (CAS)	D-Glucose, *O*-6-deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)
Chemical Name (IUPAC)	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Structural Formula	$C_{18}H_{32}O_{15}$
Chemical Structure	
Common Name, Synonyms, and Abbreviations	2'-Fucosyllactose; 2'-*O*-Fucosyllactose; 2'-FL
Marketing Name	**Momstamin 2'-FL**
CAS Number	41263-94-9
Molecular Weight	488.44

2'-FL = 2'-fucosyllactose; CAS = Chemical Abstracts Service; IUPAC = International Union for Pure and Applied Chemistry.

B.1.2 Identification of the Production Strain

As APTech's 2'-FL is a biologically derived nutritive substance, the identifying information related to the production strain is also provided in Table B.1.2-1, below. Comparative genome analysis of the production organism, *C. glutamicum* (strain APC199), and the parent organism, *C. glutamicum* ATCC 13032, was conducted using DNA–DNA hybridisation. The production organism and parent organism were demonstrated to be 99.99% similar based on the comparative average nucleotide identity (ANI) value (see Appendix A-1).

Table B.1.2-1 Information on the Production Strain

Scientific (Latin) Name (Family, Genus, Species, Strain)	Phylum: Actinobacteria Order: Corynebacteriales Family: Corynebacteriaceae Genus: Corynebacterium Species: *Corynebacterium glutamicum* Strain: *Corynebacterium glutamicum* APC199
Synonyms That May be Used Interchangeably with the Preferred Scientific Name	*Brevibacterium lactofermentum*; *Brevibacterium flavum; Micrococcus glutamicus; Corynebacterium lactofermentum*
Verification of the Species and Strain Identity According to Internationally Accepted Methods (for Bacteria and Yeasts [Unicellular Organisms])	99.99% similar to *Corynebacterium glutamicum* ATCC 13032 based on DNA–DNA hybridisation (see Appendix A-2)
Origin of the Organism	*Corynebacterium glutamicum* ATCC 13032
Deposition in an Officially Recognised Culture Collection with Access Number	Deposited in Korean Collection of Type Cultures under KCTC 13735BP

ATCC = American Type Culture Collection.

B.2 Information on the Chemical and Physical Properties of the Nutritive Substance

B.2.1 Incorporation into Food Matrices

Size distribution of particles in a food can sometimes influence a range of food properties, including the processability and functionality of the final food product. APTech has therefore analysed the particle size of 3 commercial lots of its 2'-FL ingredient using an LA-950 laser scattering particle size distribution analyser. The median volume distribution (DV_{50}) was calculated to have a particle diameter size of 38~39 µm (see Table B.2.1-1). Moreover, the stability of APTech's 2'-FL has also been tested under a variety of conditions within the intended conditions of use, and the results are summarised in Section B.2.2.3, below.

Table B.2.1-1 Summary of Particle Size Distribution Analysis Results

Lot No.	DV_{50} (µm)
2'-FL-CG-011	38.5770
2'-FL-CG-012	38.4959
2'-FL-CG-013	38.4727

DV_{50} = median volume distribution.

B.2.2 Product Stability

The storage stability of 2'-FL produced by microbial fermentation of a genetically modified strain of *C. glutamicum* (strain APC199) was investigated when stored under normal (25°C, 60% relative humidity) and accelerated (40°C, 75% relative humidity) conditions for up to 56 weeks. Five lots of 2'-FL produced by microbial fermentation of *C. glutamicum* (strain APC199) (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, and 2'-FL-CG-015) were stored in sterile amber glass bottles with high-density polyethylene stopper. 2'-FL, moisture, and microbial contaminants were measured throughout the storage period. 2'-FL content was measured using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), while moisture content was measured using Karl-Fischer titration. Microbial contaminants were measured using methods of analysis by the Korean Ministry of Food and Drug Safety (MFDS). The results of the stability studies are discussed in the following sections.

B.2.2.1 Shelf-life Stability

The shelf-life stability of 5 lots of 2'-FL produced by microbial fermentation of a genetically modified strain of *C. glutamicum* (strain APC199) (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, and 2'-FL-CG-015) was investigated under normal storage conditions (25°C, 60% relative humidity) for 104 weeks. As shown in Table B.2.2.1-1 below, no appreciable changes in 2'-FL content, moisture content, or microbial contaminants were observed up to 104 weeks when stored at room temperature and ambient conditions.

Table B.2.2.1-1 Results of the Shelf-life Stability of 2'-FL Produced by Microbial Fermentation of *Corynebacterium glutamicum* APC199 Under Ambient Storage Conditions (25°C, 60% Relative Humidity)

Specification Parameter	Specification Limit	Timepoint (Week)									
		0	1	4	8	13	26	39	56	81	104
2'-FL (%)											
2'-FL-CG-011		96.67	96.84	96.97	96.71	96.73	97.27	97.10	96.88	97.23	97.05
2'-FL-CG-012		95.93	96.75	96.71	96.83	96.67	97.24	97.10	96.82	97.18	97.16
2'-FL-CG-013	≥94	96.24	96.88	96.71	96.87	96.59	97.27	97.09	96.76	97.18	97.12
2'-FL-CG-014		96.84	96.74	97.08	96.99	96.95	96.96	96.86	96.89	97.06	96.58
2'-FL-CG-015		97.99	97.92	98.20	97.79	97.99	98.09	97.84	98.11	98.04	97.69
Moisture (%)											
2'-FL-CG-011		1.67	1.67	1.69	1.82	1.69	1.84	2.00	2.00	1.94	1.87
2'-FL-CG-012		1.74	1.68	1.73	1.85	1.66	1.92	2.13	2.25	1.95	1.85
2'-FL-CG-013	≤9.0	1.64	1.71	1.66	1.88	1.64	1.86	1.95	2.03	1.87	1.83
2'-FL-CG-014		2.46	2.50	2.62	2.67	2.68	2.60	2.76	2.94	2.83	3.00
2'-FL-CG-015		2.70	2.75	2.79	2.88	2.96	2.82	3.00	3.15	2.98	3.19
Standard Plate Count (CFU/g)											
2'-FL-CG-011		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-012		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-013	≤500	ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-014		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND
2'-FL-CG-015		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND
Yeast and Mould (CFU/g)											
2'-FL-CG-011		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-012		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-013	≤100	ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-014		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND
2'-FL-CG-015		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND
***Salmonella* (in 25 g)**											
2'-FL-CG-011		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-012		ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-013	Negative	ND	N/A	N/A	N/A	N/A	N/A	NT	ND	NT	ND
2'-FL-CG-014		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND
2'-FL-CG-015		ND	N/A	N/A	N/A	N/A	N/A	ND	ND	NT	ND

2'-FL = 2'-fucosyllactose; CFU = colony-forming units; N/A = not applicable; ND = not detected; NT = not tested.

B.2.2.2 Accelerated Stability

The stability of 5 lots of 2'-FL produced by microbial fermentation of a genetically modified strain of *C. glutamicum* (strain APC199) (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, and 2'-FL-CG-015) was investigated under accelerated storage conditions (40°C, 75% relative humidity). Data for 3 lots (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, and 2'-FL-CG-013) were available up to 104 weeks, while data for 2 lots (Lot Nos. 2'-FL-CG-014 and 2'-FL-CG-015) were available up to 81 weeks. As shown in Table B.2.2.2-1 below, no appreciable changes in 2'-FL content or moisture content were observed up to 104 weeks when stored under accelerated conditions.

Table B.2.2.2-1 Stability Results of 2'-FL Produced by Microbial Fermentation of *Corynebacterium glutamicum* (Strain APC199) Under Accelerated Storage Conditions (40°C, 75% Relative Humidity)

Specification Parameter	Specification Limit	Timepoint (Week)									
		0	1	4	8	13	26	39	56	81	104
2'-FL (%)											
2'-FL-CG-011		96.67	96.87	96.89	96.54	96.59	97.09	96.84	96.65	96.91	96.65
2'-FL-CG-012		95.93	96.76	96.89	96.83	96.67	97.03	96.79	96.56	96.90	96.66
2'-FL-CG-013	≥94	96.24	96.83	96.85	96.75	96.58	96.97	96.72	96.49	96.79	96.64
2'-FL-CG-014		96.84	96.75	97.15	96.88	97.04	96.70	96.47	96.24	96.61	96.03
2'-FL-CG-015		97.99	97.72	98.02	97.84	98.10	97.88	97.70	97.69	97.74	97.29
Moisture (%)											
2'-FL-CG-011		1.67	1.72	1.66	1.87	1.67	1.99	2.16	2.14	2.36	2.48
2'-FL-CG-012		1.74	1.79	1.71	2.08	1.64	2.03	2.08	2.17	2.54	2.48
2'-FL-CG-013	≤9.0	1.64	1.69	1.72	1.96	1.71	2.03	2.18	2.17	2.41	2.56
2'-FL-CG-014		2.46	2.47	2.63	2.69	2.77	2.70	2.89	3.20	3.35	3.47
2'-FL-CG-015		2.70	2.77	2.78	2.85	3.01	2.92	3.11	3.36	3.46	3.64

2'-FL = 2'-fucosyllactose.

B.2.2.3 Stability Under the Intended Conditions of Use

The stability of 2'-FL produced by microbial fermentation of a genetically modified strain of *C. glutamicum* (strain APC199) (Lot No. 2'-FL-CG-015) was investigated in infant formula stored at 4, 25, and 37°C for 18 months. Three different samples were collected from the food product at 0, 3, 6, 12, and 18 months, and 2'-FL was extracted and purified using liquid and solid phase extraction. 2'-FL content was measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS). As shown in Table B.2.2.3-1, no significant changes in 2'-FL levels occurred after storage at 4, 25, and 37°C for up to 18 months.

Table B.2.2.3-1 Stability Results of 2'-FL from Microbial Fermentation of *Corynebacterium glutamicum* APC199 in Infant Formula After Up to 18 Months of Storage

Parameter and Storage Temperature	Timepoint (Months)				
	0	3	6	12	18
2'-FL (mg/100 g)					
4°C	NT	146.8 ± 20.7	138.2 ± 18.5	148.8 ± 13.3	145.1 ± 3.9
25°C	139.0 ± 20.2	149.5 ± 23.2	139.4 ± 10.6	146.6 ± 34.8	146.8 ± 14.2
37°C	NT	142.5 ± 24.7	144.8 ± 22.8	149.6 ± 17.4	142.5 ± 13.5

2'-FL = 2'-fucosyllactose; NT = not tested.

As discussed in Section B.1, 2'-FL produced by microbial fermentation of a genetically modified strain of *C. glutamicum* APC199 is chemically and structurally similar to other 2'-FL ingredients that are currently available globally. It is therefore expected for there to be no changes with respect to the stability profile of 2'-FL produced by APTech when used in similar food products. In 2015, the EFSA Nutrition, Novel Foods and Food Allergens (NDA Panel) reviewed and discussed the stability of 2'-FL produced by chemical synthesis in infant formula stored at 4, 20, 30, and 37°C over a period of 3 years (among other food matrices) (EFSA, 2015). No significant changes in the 2'-FL content were reported under the storage conditions for any of the above listed food matrices and it was concluded that 2'-FL is stable under different conditions of intended use (EFSA, 2015). Considering the chemical and structural similarities of APTech's 2'-FL to other 2'-FL preparations, the results of the stability studies conducted with other 2'-FL ingredients can also be extended to APTech's 2'-FL.

B.3 Information on the Impurity Profile

APTech has established product specifications for other significant carbohydrate components for the 2'-FL ingredient, such as lactose, fucose, 3-fucosyllactose, DFL, glucose, galactose, as described in Section B.5. Analysis of 10 production lots of 2'-FL from microbial fermentation of *C. glutamicum* (strain APC199) (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, 2'-FL-CG-015, SP2F22029, SP2F22031, SP2F22033, SP2F22035, and SP2F22041) demonstrate the absence of these other carbohydrate compounds (see Section B.5.2).

Five of these lots (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, and 2'-FL-CG-015) have also been thoroughly analysed for the confirmation of absence of viable cells or residual DNA from the *C. glutamicum* production strain. Results from these analyses indicate that there the final ingredient is absent of viable cells and that there is no detectable level of residual DNA (see Appendix A-2). Moreover, the same 5 production lots of 2'-FL from microbial fermentation of *C. glutamicum* (strain APC199) were analysed for trace elements and minerals potentially carried over from the fermentation medium. The results of the analysis are presented in Table B.3-1. The production process includes the use of nanofiltration, ion exchange chromatography, and treatment with activated carbon that sufficiently reduces the levels of elements and minerals that may be carried over from the fermentation medium. The elements and minerals were analysed using inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES) based on an Association of Official Analytical Collaboration (AOAC) method. Orthophosphate and ammonium were measured using ion chromatography.

Table B.3-1 Results of Analysis for Trace Elements and Minerals of 5 Lots of 2'-FL from Microbial Fermentation of *Corynebacterium glutamicum* (Strain APC199)

Element/Mineral	Manufacturing Lot No.				
	2'-FL-CG-011	2'-FL-CG-012	2'-FL-CG-013	2'-FL-CG-014	2'-FL-CG-015
Orthophosphate (mg/kg)	<0.1	<0.1	<0.1	<0.1	<0.1
Ammonium (mg/kg)	<0.1	<0.1	<0.1	<0.1	<0.1
Sulphate (mg/kg)	<30	<30	<30	<30	<30
Chloride (mg/kg)	<40	<40	<40	<40	<40
Sodium (mg/kg)	30.2	<24.8	<24.6	<25	<24.5
Potassium (mg/kg)	<123	<124	<123	<125	<123
Magnesium (mg/kg)	<24.6	<24.8	<24.6	<25	<24.5
Calcium (mg/kg)	<24.6	<24.8	<24.6	<25	<24.5
Iron (mg/kg)	<2.46	<2.48	<2.46	<2.5	<2.45
Zinc (mg/kg)	<0.49	<0.50	<0.49	<0.5	<0.49
Copper (mg/kg)	<0.246	<0.248	<0.246	<0.25	<0.245
Manganese (mg/kg)	<0.123	<0.124	<0.123	<0.125	<0.123

2'-FL = 2'-fucosyllactose.

B.4 Manufacturing Process

B.4.1 Manufacturing Process for 2'-FL

B.4.1.1 Identity of Raw Materials

The raw materials and processing aids used in the production of 2'-FL food grade and are detailed in Appendix A-3, along with their respective chemical formulae and Chemical Abstracts Service numbers, where applicable. Glucose is used as the carbon source and lactose as the substrate in the production of 2'-FL.

B.4.1.2 Production Process

The production of 2'-FL by microbial fermentation of genetically modified *C. glutamicum* (strain APC199) is conducted in compliance with current Good Manufacturing Practice (cGMP) and Hazard Analysis and Critical Control Points (HACCP) principles. A detailed overview of the manufacturing process for 2'-FL is provided in Appendix A, while the pertinent steps of the manufacturing process are provided in brief below.

The manufacturing process of 2'-FL consists of 2 stages: fermentation of the production strain to produce 2'-FL followed by purification of the resultant 2'-FL. In the first stage, glucose and lactose are converted to 2'-FL (released into the fermentation medium) by *C. glutamicum* (strain APC199) *via* the 2'-FL biosynthesis pathway. In the second stage, the 2'-FL is isolated and purified from the fermentation medium. This process includes a series of filtration and purification steps, followed by use of either crystallisation or spray drying as a final downstream process before packaging of the finished 2'-FL ingredient. Experimental data provided by APTech demonstrate the similarity of the finished 2'-FL ingredient produced through either of these downstream processing methods (see Section B.5.2). Following these steps, the production organism has been completely removed from the finished 2'-FL ingredient.

Notably, APTech's 2'-FL ingredient is currently manufactured in the Republic of Korea; it will not itself be manufactured in Australia or New Zealand. Thus, the raw materials, production organism, and processing aids used for its manufacture will not enter the territory.

B.4.1.3 Process Controls and Quality Assurance

The production of 2'-FL by microbial fermentation of genetically modified *C. glutamicum* (strain APC199) is conducted in accordance with cGMP and HACCP principles. The production process includes 3 critical control points to minimise the potential of impurities introduced into the final product. The food safety management system in place encompasses all stages of manufacturing from receipt and storage of the raw and packaging materials to the final ingredient and complies with Food Safety Systems Certification 22000 (FSSC 22000). The list of critical control points, including the critical limit and monitoring procedures, are provided in Appendix A.

B.4.2 Description of the Source Microorganism

B.4.2.1 Scientific Name, Taxonomy, and Other Names

Corynebacterium glutamicum are Gram-positive microorganisms that were first isolated in the 1950s from Japanese soil during a quest for natural L-glutamate producers (Vertès *et al*., 2012). They were initially characterised by their unique ability to produce large amounts of L-glutamate from sugar and ammonia. Since 1961, these species have been historically used in the production of amino acids (Kinoshita *et al.*, 1961a,b). The taxonomic information for the recipient or parental microorganism (*Corynebacterium glutamicum*) is provided in Table B.4.2.1-1 below.

Table B.4.2.1-1 Taxonomic Information for the Recipient or Parental Microorganism

Phylum	Actinobacteria
Order	Corynebacteriales
Family	Corynebacteriaceae
Genus	Corynebacterium
Species	*Corynebacterium glutamicum*
Deposit Number	ATCC 13032 or NCIMB 10025 or DSM 20330 or IMET 10482

ATCC = American Type Culture Collection; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures GmbH); IMET = formerly National Kurturensammlung für Mikroorganismen, Zentralinstitut für Mikrobiologie und Experimentelle Therapie; NCIMB = National Collection of Industrial, Food and Marine Bacteria.

There is no known history of previous genetic modifications of the *C. glutamicum* strain ATCC 13032. This organism was originally isolated from sewage for its ability to produce high quantities of glutamate. This strain has since been genetically modified for a number of different applications, primarily to optimise production performance (Wieschalka *et al*., 2013). The American Type Culture Collection (ATCC) lists *C. glutamicum* strain ATCC 13032 as a glutamate transformation host (ATCC, 2022). The parental strain, *C. glutamicum* ATCC 13032, is considered a wild-type strain that APTech utilises as the host organism for its production strain, *C. glutamicum* APC199.

B.4.2.2 Phenotypic and Genetic Markers

Corynebacterium species are Gram-positive, non-motile rods, with sizes between 2 to 6 µm in length and 0.5 µm in diameter (Public Health England, 2014). These microorganisms are aerobic or facultatively anaerobic and exhibit a fermentative metabolism. The optimal growth temperature for these microorganisms is 37°C. *C. glutamicum* ATCC 13032 was first isolated from the sewage as a producer of glutamate in the early 1950s (Kinoshita *et al*., 1958). The strain was originally named *Micrococcus glutamicus* and was deposited in the ATCC under 13032 and the National Collection of Industrial, Food and Marine Bacteria (NCIMB) under 10025. The genome of *C. glutamicum* ATCC 13032 consists of a single circular chromosome and is approximately 3.3 megabase pairs (Mbp) in size (Meyer *et al.*, 2003). The guanine and cytosine (G+C) content of the genome is approximately 53.8%.

The whole genome of *C. glutamicum* ATCC 13032 has been sequenced and is available under GenBank No. BA000036.3.

In addition, whole genome sequencing of the production organism has revealed an absence of virulence and antibiotic resistance genes. The whole genome sequence of the production organism, *C. glutamicum* APC199, was interrogated for the presence of genes encoding for or contributing to antimicrobial resistance (AMR). For this purpose, searches were conducted against 3 curated AMR databases: Comprehensive Antibiotic Resistance Database (CARD), Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT), and ResFinder. The findings are summarised below.

The search conducted using the resistance gene identifier (RGI) tool of the CARD database using default values (≥95% identity) did not identify any resistant phenotype. The AMR genes from the ARG-ANNOT database were downloaded and the *C. glutamicum* APC199 genome was searched using the National Center for Biotechnology Information (NCBI) BLASTx program, and no AMR genes were identified using the criteria recommended by EFSA[7] of >80% and >70% query coverage. Finally, a search was conducted using the ResFinder (v.4.1) tool maintained by the Centre for Genomic Epidemiology. This search revealed the possible presence of a *cmr* gene encoding for multi-drug resistance to erythromycin, tetracycline, puromycin, and bleomycin. The *cmr* gene was originally isolated from *C. glutamicum* ATCC 13032 and was demonstrated to be inactive in this species (Jäger *et al*., 1997).[8] Jäger *et al.* (1997) investigated the resistant phenotype of *C. glutamicum* ATCC 13032 containing multiple copies of the *cmr* gene (harbouring 0 to 2 plasmids containing the gene) and reported the strain to be susceptible to 2 µg/mL of erythromycin, tetracycline, puromycin, and bleomycin. The authors concluded that the cloned gene, in multiple copies, was not sufficient to cause resistance in *C. glutamicum*, possibly due to low expression rate of the gene. In comparison, when cloned into *E. coli*, resistance to erythromycin, tetracycline, puromycin, and bleomycin was observed. The findings of this study indicate the presence of the *cmr* gene in *C. glutamicum* ATCC 13032 does not pose a risk for AMR. The nucleotide sequence of the *cmr* gene was searched against the nucleotide database from GenBank (default parameters: E-value <0.05; 1,-2 match/mismatch scores; linear gap costs), and a number of hits >80% identity and >80% query coverage to *cmr* genes from other strains of *C. glutamicum*, as well as *Corynebacterium crudilactis* and *Brevibacterium flavum* were identified, suggesting that this gene is intrinsic to this species and other species of the *Corynebacterium* genus. It is noted that the *C. glutamicum* APC199 production strain is obtained from *C. glutamicum* ATCC 13032. The host organism (ATCC 13032) is genetically modified to introduce 2'-FL biosynthesis genes for the expression of 2'-FL. The genetic modification is mediated *via* the kanamycin resistance gene and does not introduce any other AMR gene into the host organism. Therefore, the production strain does not harbour any additional AMR gene that would not be native to the host organism. The genome of the production strain was also searched using ResFinderFG (v.1.0), and the results predicted no resistance phenotype of the production strain, providing further evidence that the *cmr* gene present in *C. glutamicum* ATCC 13032 is not expressed such that the production strain would have an undesirable phenotype.

B.4.2.3 Information on the Pathogenicity and Toxicity of the Source Microorganism

The parental strain used to produce APTech's 2'-FL, *C. glutamicum* ATCC 13032, is classified as a Biosafety Class 1 microorganism (ATCC, 2022). *C. glutamicum* is known as a non-pathogenic bacterium commonly used for industrial amino acid production (Yang and Yang, 2017). The *C. glutamicum* species is also regarded as having qualified presumption of safety (QPS) when used as a production organism for amino acids and other food or feed ingredients (EFSA, 2019a). This is because *C. glutamicum* does not produce toxic compounds and there are no associated hazards to the presence of toxic metabolites in the fermentation broth of this species (EFSA, 2019a). It is therefore considered safe for the derivation of genetically modified strain lineages for use in the production of food additives and enzymes. In 2018,

[7] https://www.efsa.europa.eu/sites/default/files/2021-03/EFSA-statement-EFSA-Q-2019-00434.pdf (EFSA, 2021a).
[8] https://jb.asm.org/content/jb/179/7/2449.full.pdf.

the U.S. FDA received a GRAS Notice for cultured corn starch fermented by *C. glutamicum* (GRN 792) and responded with a "no questions" letter regarding the GRAS status of the ingredient (Nestec, S.A., 2018; U.S. FDA, 2019a). Furthermore, APTech's 2'-FL produced from *C. glutamicum* has GRAS status for a range of food and beverage products as notified to the FDA under GRN 932.

B.4.2.4 Information on the Genetic Stability of the Source Microorganism

Expression Site, Amount, and Metabolite of the Gene Product

To evaluate the genetic stability of *C. glutamicum* strain APC199, derived from *C. glutamicum* strain ATCC 13032 that is used in the production of APTech's 2'-FL, a series of subculturing techniques were conducted. Briefly, *C. glutamicum* strain APC199 was cultured at 30°C with shaking (250 rpm) in Luria-Bertani (LB) liquid culture medium, which contained 25 µg/mL kanamycin, an aminoglycoside bactericidal antibiotic. Fermentation samples were collected in triplicate from 3 separate cultures of APC119 and inoculated with cells measured to have an optical density at 600 nm (OD_{600}) of 0.8. The fermentation samples were collected every 12 hours over the course of 32 subcultures. For reference, 3 cultures of *C. glutamicum* ATCC 13032 were incubated under the same culture conditions, without kanamycin, as controls. Fermentation samples (n=3) from subculture 0, 8, 16, and 32, which had been inoculated with cells at the OD_{600} of 0.1 for 3 days, were analysed to determine expression location of the introduced genes, and concentrations of 2'-FL and other metabolites.

Expression Location of the Introduced Genes

The expression plasmid utilised in the manufacture of 2'-FL is not incorporated into the genome and the biosynthesis conducted *via* the expression plasmid, which is ultimately transferred out of the cell. The expressed proteins remain within the cells as demonstrated by the results of the following test.

1 mL of fermentation samples from APC199 and control cultures were taken into a test tube and centrifuged for 3 minutes at 15,000 Xg (relative centrifugal force). The upper liquid was filtered with a 0.2-µm filter and centrifuged again for 3 minutes at 15,000 Xg. Washed cell pellets were resuspended with phosphate-buffered saline (PBS) to produce an OD_{600} of 0.8. The resuspended cells were disrupted with an ultrasonic grinder and centrifuged at 15,000 Xg for 10 minutes and filtered with a 0.2-µm filter. To prepare the membrane protein LacY, 100 mL of a control culture sample was placed into a tube and centrifuged at 9,000 rpm for 20 minutes to separate the upper liquid from the cell pellet. To extract membrane proteins from the pellet, proteins were resuspended through the addition of 4 mL disintegration buffer per gram of wet cell, and cells were disrupted using glass beads (BioSpec, 0.5 mm diameter). To separate supernatant and cell debris, centrifugation was conducted for 5 minutes twice at 5,000 Xg. To retrieve membrane proteins, the supernatant was centrifuged at 20,000 Xg for 90 minutes. The membrane protein was then extracted by reacting the pellet with pre-chilled PBS. After the extraction reaction, the sample was centrifuged at 20,000 Xg for 90 minutes and the upper liquid was ready as the final sample. The final enzyme-linked immunosorbent assay (ELISA) measurement value was adjusted using the basal level measurement of PBS as the negative control.

First, ELISA plate wells were coated with 300 µL blocking buffer, which remained in the wells at room temperature for 2 hours. Next, 100 µL of the prepared samples and standard proteins were added to the wells to undergo immobilisation for 1 hour. 100 µL of diluted polyclonal antibodies of Gmd, WcaG, LacY, FT, and NPTII at 1/2,000, 1/5,000, 1/1,000, 1/1,000, and 1/500, respectively, were added and allowed to react for 1 hour at room temperature. Afterwards, 100 µL of diluted immunoglobulin G (IgG) with linked horseradish peroxidase (HRP) at ratios of 1/10,000, 1/5,000, 1/2,500, 1/2,000, and 1/1,000, respectively, were added and allowed to react for 1 hour at room temperature. 3,3',5,5'-tetramethylbenzidine (TMB) and HRP were then incubated for 5 minutes by adding 50 µL of a stop buffer. A plate reader was then used to measure the absorbance value at 450 nm.

No transformed proteins were detected in culture media and *C. glutamicum* ATCC 13032 cells. Transformed proteins were only detected inside of the APC199 cells, and no transformed proteins were detected in the external culture medium (see Table B.4.2.4-1).

Table B.4.2.4-1 Gmd, WcaG, LacY, FT, and NPTII Expression Sites

Sample		Positive/Negative (+/-)[a]	
		Cell	Media
Gmd	*Corynebacterium glutamicum* ATCC 13032	-	-
	Corynebacterium glutamicum APC199	+	-
WcaG	*Corynebacterium glutamicum* ATCC 13032	-	-
	Corynebacterium glutamicum APC199	+	-
LacY	*Corynebacterium glutamicum* ATCC 13032	-	-
	Corynebacterium glutamicum APC199	+	-
FT	*Corynebacterium glutamicum* ATCC 13032	-	-
	Corynebacterium glutamicum APC199	+	-
NPTII	*Corynebacterium glutamicum* ATCC 13032	-	-
	Corynebacterium glutamicum APC199	+	-

[a] Cut-off (ng/mL): Gmd: 0.0580; WcaG: 0.0631; LacY: 0.0503; FT: 0.0819; NPTII: 0.0536.

Protein Expression Quantity by Subculture

During the 32 subcultures of APC199, APTech sampled 1 mL of culture from 1st, 2nd, 4th, 8th, 16th, and 32nd subcultures for protein analysis. 1 mL of each culture sample was centrifuged at 15,000 Xg for 10 minutes to obtain cell pellets, and the supernatant was filtered with a 0.2-µm filter to obtain samples for testing. Membrane protein LacY was prepared with the same method as described above. APTech used appropriate dilutions for ELISA analysis of transformed proteins.

ELISA plate wells were then coated by addition of 300 µL blocking buffer into each well, allowing them to react at room temperature for 2 hours. Next, 100 µL of the prepared samples and standard proteins were added for 1 hour to be immobilised. 100 µL of diluted polyclonal antibodies of Gmd, WcaG, LacY, FT, and NPTII at 1/2,000, 1/5,000, 1/1,000, 1/1,000, and 1/500, respectively, were added and allowed to react for 1 hour at room temperature. Afterwards, 100 µL of diluted IgG with linked HRP at ratios of 1/10,000, 1/5,000, 1/2,500, 1/2,000, and 1/1,000, respectively, were added and allowed to react for 1 hour at room temperature. TMB and HRP were incubated for 5 minutes by adding 50 µL of a stop buffer, and a plate reader was used to measure the absorbance value at 450 nm.

Results from the gene expression analysis indicate that the concentrations of the transformed proteins did not change significantly across the 32 subcultures, indicating strong genetic stability of the production organism (see Table B.4.2.4-2). The mean concentration of the Gmd, WcaG, LacY, FT, and NPTII proteins were measured as 79.55 ± 6.86 µg/g, 165.85 ± 37.69 µg/g, 3.96 ± 0.53 µg/g, 0.73 ± 0.20 µg/g, and 2.50 ± 0.92 µg/g APC199, respectively, on a dry weight basis.

Table B.4.2.4-2 APC199 Gene Expression as Measured by Gmd, WcaG, LacY, FT, and NPTII Protein Concentration Over 32 Subcultures

Sample		Number of Subcultures						
		1	2	4	8	16	32	Mean
Gmd	Gmd/DCW[a]	76.29	80.59	76.64	72.61	87.57	83.59	79.55
	Error value	±7.84	±9.16	±8.52	±5.58	±3.22	±7.38	±6.86
WcaG	WcaG/DCW[b]	131.24	233.03	144.38	185.02	157.98	143.46	165.85
	Error value	±9.01	±5.92	±5.91	±15.69	±4.87	±4.59	±37.69
LacY	LacY/DCW[c]	3.64	3.31	4.07	3.60	4.62	4.51	3.96

Table B.4.2.4-2 APC199 Gene Expression as Measured by Gmd, WcaG, LacY, FT, and NPTII Protein Concentration Over 32 Subcultures

Sample		Number of Subcultures						
		1	2	4	8	16	32	Mean
	Error value	±0.15	±0.38	±0.30	±0.39	±0.17	±0.17	±0.53
FT	FT/DCW[d]	0.61	0.96	0.75	0.72	0.91	0.40	0.73
	Error value	±0.02	±0.14	±0.10	±0.08	±0.10	±0.05	±0.20
NPTII	NPTII/DCW[e]	2.37	3.91	2.04	3.15	1.26	2.26	2.50
	Error value	±0.34	±0.61	±0.60	±0.22	±0.37	±0.50	±0.92

DCW = dry cell weight.
[a] Gmd (µg)/DCW (g).
[b] WcaG (µg)/DCW (g).
[c] LacY (µg)/DCW (g).
[d] FT (µg)/DCW (g).
[e] NPTII (µg)/DCW (g).

Stability of the 2'-FL Production Strain Following Subculturing

Samples were collected at the initiation of subculturing and from the 8th, 16th, and 32nd subculture and moved to a new flask, where they were cultured for 3 days to measure 2'-FL production. 1 mL samples of each cell culture were centrifuged at 15,000 Xg for 3 minutes to separate the upper liquids from the pellet, and the supernatant was filtered using a 0.2-µm filter before being centrifuged again at 15,000 Xg for 3 minutes. Pure 2'-FL powder was diluted to 0.3%, 3%, and 30% (w/v) with sterilised distilled water to obtain a standard curve. Using a refractive index (RI) detector, connected with a high-performance liquid chromatography system (Agilent HPLC 1260), APTech analysed the concentration of 2'-FL in each sample. High-purity 2'-FL was purchased from Carbosynth (Cat No. OF06739) to be used as a standard for the quantification of APTech's 2'-FL.

Results from the analysis of APTech's 2'-FL, produced by cultures of APC199 across various subculture stages (0, 8th, 16th, and 32nd), indicate that a consistent level of 2'-FL was generated in the cells across the 32 subcultures (see Table B.4.2.4-3). Across the 32 subcultures, an average of 0.30 ± 0.09 g 2'-FL/g was produced by the APC199 production strain, which indicates genetic stability of the production strain.

Table B.4.2.4-3 APC199 Production of 2'-FL Across 32 Subcultures

Measured Parameter	Subculture Number				
	0 (initial)	8	16	32	Mean (all subcultures)
2'-FL (DCW)[a] (g)	0.30 ± 0.02*	0.28 ± 0.12*	0.32 ± 0.13*	0.29 ± 0.02*	0.30 ± 0.09*

2'-FL = 2'-fucosyllactose; DCW = dry cell weight.
[a] 2'-FL (g)/DCW (g).
* Mean ± standard deviation.

B.4.2.5 Information on the Genetic Modifications of the Source Microorganism

The production microorganism is a genetically modified strain of *C. glutamicum*; a species of Gram-positive microorganisms that was initially characterised by its unique ability to produce large amounts of L-glutamate from sugar and ammonia. These species have been historically used in the production of amino acids since 1961 (Kinoshita *et al.*, 1961a,b). *C. glutamicum* has been granted QPS status by EFSA for the production of amino acids and other food ingredients, such as flavouring compounds (EFSA, 2019a). According to the EFSA, *C. glutamicum* does not produce toxic compounds and there are no associated hazards to the presence of toxic metabolites in the fermentation broth of this species

(EFSA, 2019a), demonstrating that it would be a safe and suitable source organism for the production of food ingredients.

To clone the genes required for 2'-FL biosynthesis, a shuttle vector of *E. coli* and *C. glutamicum* was created. The vector, pCN01, contains the origin of replication pBL1 from *Corynebacterium* pBL1 plasmid and pUC from pUC18 vector *NPTII* encoding for kanamycin resistance. The multicloning site containing the genes *gmd*, *wcaG*, *lacY*, and *α-1,2-ft* required for 2'-FL biosynthesis and *tuf* promoter from *C. glutamicum* ATCC 13032 and T7 terminator from pET21a vector are inserted by *Eco*RI/*Xho*I digestion, thus transforming pCN01 to pFP110. The characteristics of pFP110 are outlined in Table B.4.2.5-1, including the inserted components and their respective origin and function.

Table B.4.2.5-1 Characteristics of pFP110 Vector

Component	Origin	Function
pBL1 origin (regulator)	*Corynebacterium glutamicum* pBL1 plasmid	*Corynebacterium* replication origin. Replication of *Corynebacterium* plasmids.
tuf promoter (transcription initiation factor)	*Corynebacterium glutamicum* ATCC 13032	RNA polymerase binding site for transcription.
α-1,2-ft	*Pseudopedobacter saltans* ATCC 51119	Encodes for α-1,2-fucosyltransferase. Transforms GDP-L-fucose and lactose to 2'-FL.
gmd	*Escherichia coli* ATCC 700926	Encodes for GDP-D-mannose-4,6-dehydratase. Transforms GDP-D-mannose to GDP-4-keto-6-deoxymannose.
wcaG	*Escherichia coli* ATCC 700926	Encodes for GDP-L-fucose synthase. Transforms GDP-4-keto-6-deoxymannose to GDP-L-fucose.
lacY	*Escherichia coli* ATCC 700926	Encodes for lactose permease. Transports lactose into cells.
T7 terminator (transcription terminator)	pET21a vector	Transcription termination.
KanR (*NPTII*) (selection marker)	*Escherichia coli* K-12 Tn903	Kanamycin resistance gene.
pUC origin (regulator)	pUC18 vector	*Escherichia coli* replication origin.

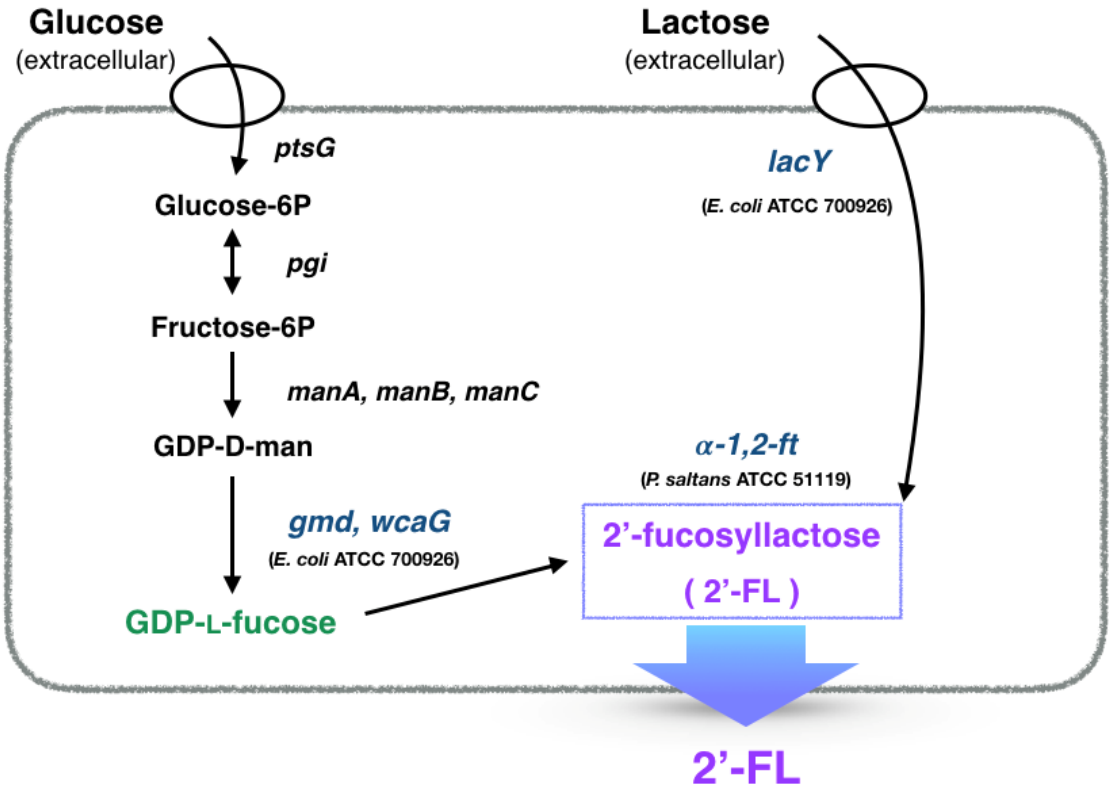
2'-FL = 2'-fucosyllactose; ATCC = American Type Culture Collection; GDP = guanosine diphosphate; RNA = ribonucleic acid.

The gene encoding for α-1,2-fucosyltransferase (*α-1,2-ft*) from *Pseudopedobacter saltans* ATCC 51119 (a non-pathogenic, non-toxigenic, Biosafety Level 1 organism) and the genes encoding for guanosine diphosphate (GDP)-D-mannose-4,6-dehydratase, GDP-L-fucose synthase, and lactose permease from *E. coli* ATCC 700926 (a non-pathogenic, non-toxigenic, Biosafety Level 1 substrain) were identified from a gene database and synthesised *de novo* and were therefore not isolated from their respective source organisms.

The pFP110 plasmid is transformed into *C. glutamicum* ATCC 13032 by electroporation. The plasmid is not incorporated into the genome of the host organism as confirmed by Southern blot analysis using probes that covered the entire plasmid, *tuf* promoter, and the introduced genes (*gmd*, *wcaG*, *lacY*, and *α-1,2-ft*). The mean number of plasmid copies in *C. glutamicum* APC199 was approximately 5 plasmids per cell. The full study reports are provided in Appendix A-2. The production organism, *C. glutamicum* (strain APC199) has been deposited in the Korean Collection for Type Cultures (Deposit No. KCTC 13735BP). The certificate of deposit is provided in Appendix A-2.

The production organism heterologously overexpresses the genes required for 2'-FL biosynthesis. The 2'-FL biosynthesis pathway in *C. glutamicum* (strain APC199) is shown in Figure B.4.2.5-1. The production strain uses glucose and lactose as substrates for 2'-FL biosynthesis. GDP-D-mannose is produced by *C. glutamicum* for cell wall biosynthesis (Jackson and Brennan, 2009; Mishra *et al*., 2011). The inserted genes, *gmd,* and *wcaG*, encoding for GDP-D-mannose-4,6-dehydratase and GDP-L-fucose synthase, respectively, converts GDP-D-mannose to GDP-L-fucose. As *C. glutamicum* is unable to utilise lactose, *lacY* encoding for lactose permease permits the production strain to transport lactose into the cell where α-1,2-fucosyltransferase (encoded by *α-1,2-ft*) converts lactose and GDP-L-fucose into 2'-FL. The 2'-FL is then transported outside of the cell where it is isolated and purified through a series of purification steps (see Section B.4.1).

Figure B.4.2.5-1 Biosynthesis Pathway for 2'-FL in *Corynebacterium glutamicum* (Strain APC199)



2'-FL = 2'-fucosyllactose; Frutose-6P = fructose-6-phosphate; GDP = guanosine diphosphate; GDP-D-man = GDP-D-mannose Glucose-6P = glucose-6-phosphate; *manA* = Mannose-6-phosphate isomerase; *manB* = phosphomannomutase; *manC* = mannose-1-phosphate guanylyltransferase; *pgi* = glucose-6-phosphate isomerase; *ptsG* = PTS system glycose-specific EIICB component.

B.5 Specification for Identity and Purity

B.5.1 Product Specifications for 2'-FL

2'-FL from *C. glutamicum* (strain APC199) is a purified carbohydrate ingredient that is primarily composed of 2'-FL and lesser amounts of related carbohydrates such as DFL, glucose, and galactose. It is a purified white to off-white/ivory powder that is produced by a microbial fermentation process, similar to the production method employed for the currently approved source of 2'-FL outlined in the Code (*i.e*., microbial fermentation of a genetically modified strain of *E. coli* K-12). The specified carbohydrates are all fully characterised and occur naturally in human breast milk.

As mentioned, APTech's 2'-FL from *C. glutamicum* APC199 has been concluded as GRAS in the U.S. for use in a wide range of food and beverage products and APTech's 2'-FL ingredient that is proposed to be marketed in Australia/New Zealand as subject to this application will meet the established product specification defined for the U.S. market. Additionally, the proposed specifications for APTech's 2'-FL from *C. glutamicum* APC199 are similar to the established specifications for 2'-FL produced by other microbial sources as defined in the European Union list. Despite this, the proposed product specifications for 2'-FL from fermentation with *C. glutamicum* APC199 do not comply with the current standard for 2'-FL identity and purity. Current specifications for the identity and purity of 2'-FL preparations in Australia and New Zealand are defined within S3—40 and S3—45 of *Schedule 3 – Identity and purity* of the Code. The proposed product specifications for APTech's 2'-FL ingredient are presented in Table B.5.1-1, below, alongside the established specifications for powdered 2'-FL preparations as currently permitted by the Code.

Minor differences in the composition of APTech's 2'-FL and the composition of already approved 2'-FL preparations are not considered to be a safety concern, as they relate to innocuous and structurally related carbohydrate components. The 2'-FL content is specified to be no less than 94%. Specification limits have been established for residual proteins (*i.e.*, ≤0.005 µg/g), as well as appropriate limits for heavy metals and microbiological parameters. Established specifications for APTech's 2'-FL produced *via* the 2 alternative downstream processes (*i.e.*, crystallisation or spray drying) remain largely similar, with the exception of a specification parameter for the solvent used during crystallisation. Specification limits for other oligosaccharides originating from the fermentation media, such as DFL and 3-fucosyllactose, have been lowered for APTech's spray-dried 2'-FL in order to be representative of the results of the batch analysis and to ensure a high purity in the finished product.

All analyses are conducted using internationally recognised methods or equivalent (*e.g.*, AOAC) at accredited testing laboratories (*e.g.*, Eurofins, Intertek, SGS), or are conducted using validated internal analytical methods.

Table B.5.1-1 Product Specification for 2'-FL Obtained by Microbial Fermentation of *Corynebacterium glutamicum* APC199

Parameter	FSANZ Specification for 2'-FL (S3—40) (FSANZ, 2022c)	FSANZ Specification for 2'-FL powder (S3—45) (FSANZ, 2022c)	Proposed Specification for APTech's 2'-FL from Fermentation with *Corynebacterium glutamicum* APC199	
			Specification	Analytical Method
Description				
Appearance (form)	Powder	Powder	Powder	USP 34 Rev. <994> or equivalent
Appearance (colour)	White to off-white	White to ivory	White to off-white/ivory	
pH (20°C, 5% solution)	3.0–7.5	NS	NS	N/A
Purity				
Sum of saccharides[a]	≥90%	NS	NS	N/A
2'-FL*	≥83%	≥90%	≥94%	Validated Internal Method (HPAEC-PAD)
D-Lactose*	≤10.0%	≤5.0%	≤3.0%	
L-Fucose*	≤2.0%	≤3.0%	≤3.0%	
3-Fucosyllactose*	NS	≤5.0%	≤3.0%	
Difucosyl-D-lactose*	≤5.0%	≤5.0%	≤2.0%	
Glucose*	NS	≤3.0%	≤3.0%	
Galactose*	NS	≤3.0%	≤3.0%	
Fucosyl-galactose	NS	≤3.0%	NS	N/A
2'-fucosyl-D-lactulose	≤1.5%	NS	NS	N/A
Water	≤9.0%	≤9.0%	≤9.0%	Karl Fischer titration; ASTM E203 or equivalent
Ash	≤2.0%	≤0.5%	≤0.5%	AOAC 923.03 or equivalent
Ethanol	NS	NS	1,000 mg/kg**	USP 467
Acetic acid	≤1.0%	NS	NS	N/A
Residual protein	≤0.01%	≤0.01%	≤0.005%	Bradford Assay; Bio-rad protein assay #5000006
Heavy Metals				
Lead	NS	≤0.02 mg/kg	≤0.02 mg/kg	AOAC 2011.19, 993.14, and 2015.01 or equivalent
Arsenic	NS	≤0.2 mg/kg	≤0.03 mg/kg	
Cadmium	NS	≤0.1 mg/kg	≤0.01 mg/kg	
Mercury	NS	≤0.5 mg/kg	≤0.05 mg/kg	

Table B.5.1-1 Product Specification for 2'-FL Obtained by Microbial Fermentation of *Corynebacterium glutamicum* APC199

Parameter	FSANZ Specification for 2'-FL (S3—40) (FSANZ, 2022c)	FSANZ Specification for 2'-FL powder (S3—45) (FSANZ, 2022c)	Proposed Specification for APTech's 2'-FL from Fermentation with *Corynebacterium glutamicum* APC199	
			Specification	Analytical Method
Microbiological Parameters				
Total plate count	≤3,000 CFU/g	≤10,000 CFU/g	≤500 CFU/g	AOAC 990.12 or equivalent
Coliforms	NS	Absent in 11 g	≤10 CFU/g	AOAC 991.14 or equivalent
Yeasts	≤100 CFU/g	≤100 CFU/g (combined)	≤100 CFU/g (combined)	ISO 21527-2 or equivalent
Moulds	≤100 CFU/g			
Salmonella	NS	Absent in 100 g	Absent in 25 g	ISO 6579-1 or equivalent
Cronobacter sakazakii	NS	Absent in 100 g	Absent in 10 g***	ISO/TS 22964 or equivalent
Staphylococcus aureus	NS	NS	<10 CFU/g	ISO 6888-1 or equivalent
Aflatoxin M1	NS	≤0.025 µg/g	≤0.025 µg/g	MFDS 9.2.3 (HPLC-FLD)
Residual endotoxins	≤10 E.U./g	≤10 E.U./g	≤100 E.U./g	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version 7.12B, Device 4486) cartridge type kit

2'-FL = 2'-fucosyllactose; AOAC = Association of Official Analytical Collaboration; ASTM = American Society for Testing and Materials; CFU = colony-forming units; E.U. = endotoxin units; HPAEC-PAD = high-performance anion exchange chromatography with pulsed amperometric detection; HPLC-FD = high-performance liquid chromatography with fluorometric detection; ISO = International Organization for Standardization; MFDS = Korean Ministry of Food and Drug Safety; N/A = not applicable; NS = not specified; Ph. Eur. = *European Pharmacopeia*; Rev. = Revised; TS = Technical Specification; USP = *United States Pharmacopeia*.

* % w/w on a dry matter basis.

** The residual ethanol product specification pertains only to APTech's 2'-FL that has undergone the crystallisation downstream process.

*** The current version of ISO/TS 22964 is described as a horizontal method for the detection of *Cronobacter* spp.

[a] Sum of saccharides for 2'-FL is defined in S3—40 as the sum of 2'-FL, D-Lactose, L-Fucose, difucosyl-D-lactose, and 2'-fucosyl-D-lactulose (FSANZ, 2022c).

B.5.2 Batch Analysis

The analytical results for 10 production lots (5 of each produced using alternative downstream processes; spray drying or crystallisation) of 2'-FL from microbial fermentation of *C. glutamicum* strain APC199 (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, 2'-FL-CG-015, SP2F22029, SP2F22031, SP2F22033, SP2F22035, and SP2F22041) are summarised in Table B.5.2-1 below. The certificates of analysis for these lots are provided in Appendix A-3. The methods of analysis employed in the measurement of all non-carbohydrate components of APTech's 2'-FL ingredient are internationally recognised methods (*e.g.*, MFDS, *European Pharmacopeia*, International Organization for Standardization). 2'-FL and other carbohydrates, as well as water content and protein content, were analysed using internal methods that have been validated and are addressed further in Section B.6. Results from these analyses demonstrate that the 2'-FL produced through the spray drying process is compositionally similar to the 2'-FL produced by solvent crystallisation, as described in APTech's U.S. and EU regulatory submissions. In addition, the results of the batch data demonstrate that 2'-FL is manufactured in a consistent manner that meets the defined product specifications, irrespective of the downstream manufacturing process employed (crystallisation *vs.* spray drying).

Table B.5.2-1 Results of Analysis for 10 Lots of 2'-FL from Microbial Fermentation of *Corynebacterium glutamicum* APC199

Specification Parameter	Specification Limit	Manufacturing Lot No.									
		Crystallisation					Spray Dried				
		2'-FL-CG-011	2'-FL-CG-012	2'-FL-CG-013	2'-FL-CG-014	2'-FL-CG-015	SP2F22029	SP2F22031	SP2F22033	SP2F22035	SP2F22041
Description											
Appearance (form)	Powder	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Appearance (colour)	White to off-white/ivory	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water content (%)	≤9.0	1.67	1.74	1.64	2.46	2.70	5.23	4.98	5.05	4.72	5.19
Purity											
2'-FL (w/w%)[a]	≥94%	97.47	98.26	98.04	98.67	98.85	95.83	95.36	95.35	95.25	95.73
Lactose (w/w%)[b]	≤3%	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.16	<LOQ	<LOQ	0.17	0.19
L-Fucose (w/w%)[c]	≤3%	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3-Fucosyllactose (w/w%)[d]	≤3%	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Difucosyl-D-lactose (w/w%)[e]	≤2%	<LOQ	0.35	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Glucose (w/w%)[f]	≤3%	0.27	0.34	0.31	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Galactose (w/w%)[g]	≤3%	0.21	0.22	0.22	0.17	0.08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Ash (%)	≤0.5	0.17	0.15	0.14	0.03	0.09	0.11	<0.1	<0.1	<0.1	<0.1
Residual ethanol (mg/kg)	1,000*	221	220	221	229	232	NT	NT	NT	NT	NT
Residual protein (%)[h]	≤0.005	<LOQ	<LOQ	0.000473	<LOQ	<LOQ	0.000529	0.000392	0.000316	0.000393	0.000326
Heavy Metals											
Arsenic (mg/kg)	≤0.03	<0.001	0.003	0.005	ND	ND	ND	ND	ND	ND	ND
Cadmium (mg/kg)	≤0.01	<0.001	<0.001	<0.001	ND	ND	ND	ND	ND	ND	ND
Lead (mg/kg)	≤0.02	<0.001	0.001	0.005	ND	ND	ND	ND	ND	ND	ND
Mercury (mg/kg)	≤0.05	<0.001	<0.001	<0.001	ND	ND	ND	ND	ND	ND	ND
Microbiological Parameters											
Total plate count (CFU/g)	<500	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Yeast and mould (CFU/g)	<100	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Coliform (CFU/g)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Cronobacter spp. (CFU/10 g)	Absent in 10 g	Negative[i]	Negative[i]	Negative[i]	Negative[i]	Negative[i]	Negative	Negative	Negative	Negative	Negative
Staphylococcus aureus (CFU/g)	Absent in 1 g	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Table B.5.2-1 Results of Analysis for 10 Lots of 2'-FL from Microbial Fermentation of *Corynebacterium glutamicum* APC199

Specification Parameter	Specification Limit	Manufacturing Lot No.									
		Crystallisation					Spray Dried				
		2'-FL-CG-011	2'-FL-CG-012	2'-FL-CG-013	2'-FL-CG-014	2'-FL-CG-015	SP2F22029	SP2F22031	SP2F22033	SP2F22035	SP2F22041
Salmonella (CFU/25 g)	Absent in 25 g	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin M1 (µg/kg)	≤0.025	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Residual endotoxins (E.U./g)	<100	<7.2	<5.7	<5	<5	35.5	13	<5.7	8	32	<6

2'-FL = 2'-fucosyllactose; CFU = colony-forming units; DM = dry matter; E.U. = endotoxin units; ISO = International Organization for Standardization; LOQ = limit of quantitation; ND = not detected; NT = not tested; TS = Technical Specification.

LOQs: [a] 2'-FL = 1.19% w/w DM; [b] D-Lactose = 0.14% w/w DM; [c] L-Fucose = 0.07% w/w DM; [d] 3-fucosyllactose = 0.32% w/w DM; [e] DFL = 0.26% w/w DM; [f] D-Glucose = 0.24% w/w DM; [g] D-Galactose = 0.07% w/w DM; [h] Residual protein = 0.00026%.

[i] Lots of 2'-FL produced *via* the crystallisation process were analysed using the 2006 version of ISO/TS 22964, which is used to detect *Cronobacter sakazakii* instead of *Cronobacter* spp.

* The residual ethanol product specification pertains only to APTech's 2'-FL that has undergone the crystallisation downstream process.

B.6 Analytical Method for Detection

APTech's 2'-FL can be detected using high-performance anion exchange chromatography (HPAEC), which selectively separates carbohydrates at high pH using a strong anion-exchange stationary phase based on the weakly acidic nature of carbohydrates. By coupling HPAEC with a pulsed amperometric detection (PAD), carbohydrates are directly quantified without derivatisation. HPAEC-PAD can therefore be applied to the analysis of 2'-FL, a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. This analytical method and its validation report are provided in Appendix D.

The described HPAEC-PAD analytical method can also be used to measure the presence of the 2'-FL nutritive substance in the foods in which it is proposed to be used and was employed in the stability tests discussed in Section B.2.2.

B.7 Information on the Proposed Food Label

APTech's 2'-FL is a highly purified ingredient and its use in food products would therefore follow any applicable food labelling standards established for ingredients used in the food products proposed for use in Australia and New Zealand, as defined in Section D. No additional labelling statements or claims would be used for food products containing 2'-FL.

C. INFORMATION RELATED TO THE SAFETY OF THE NUTRITIVE SUBSTANCE

The data to support the safety of APTech's 2'-FL ingredient under its proposed conditions of use are described in this section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of *Guideline 3.3.3 – Substances used for a nutritive purpose* and *Guideline 3.6.2 – Special purpose food – Infant formula products)* of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a). The pertinent requirements of each guideline have been addressed in the subsections below.

C.1 History of Safe Consumption from Human Breast Milk

2'-FL is a significant component of the HMO fraction in human breast milk and is one of the naturally occurring fucosylated oligosaccharides in human breast milk (EFSA, 2015). 2'-FL is not detected in the milk of approximately 20% of women who do not express α-1,2-fucosyltransferase in their mammary glands (termed “non-secretors”); some women also do not express α-1,4-fucosyltransferase required to synthesise “Lewis” antigens a and b. Thus, there are 4 different phenotypes for the categorisation of human milk (Thurl *et al*., 1997, 2010; Coppa *et al*., 2011). The mean level of 2'-FL in human milk is dependent on secretor and Lewis blood group status, ethnicity, lactation period, preterm birth, and maturity of the milk (EFSA, 2015). The levels also depend on the lactation period; higher levels of 2'-FL are reported in the colostrum, and the levels decrease thereafter. The pooled mean levels of 2'-FL across different phenotypes range between 1,100 and 4,260 mg/L in mature breast milk and 1,120 to 4,900 mg/L in colostrum. According to EFSA, the average intake of 2'-FL is approximately 170 to 660 mg/kg body weight/day, based on a 6.5-kg infant drinking approximately 1 L of breast milk per day (EFSA, 2015). For newborns, the average intake of 2'-FL from colostrum is approximately 80 to 360 mg/kg body weight/day, based on a 3.4-kg newborn infant drinking approximately 250 mL of breast milk per day**.**

C.2 Metabolic Fate

Analytical data demonstrate that APTech’s 2'-FL ingredient is chemically identical to the 2'-FL naturally present in human breast milk (see Appendix A-1). Therefore, it can be reasonably expected that APTech’s 2'-FL ingredient will be subjected to the same metabolic pathway as its natural counterpart present within human breast milk.

2'-FL is a non-digestible oligosaccharide that naturally occurs in human breast milk (EFSA, 2015). The absorption, distribution, metabolism, and excretion (ADME) profile of 2'-FL was discussed and evaluated in FSANZ Application A1155 (FSANZ, 2021a), as well as in an EFSA NDA Panel evaluation (EFSA, 2015). HMOs such as 2'-FL are resistant to hydrolysis by digestive enzymes (FSANZ, 2021a). These compounds pass through the upper gastrointestinal tract intact and are digested by anaerobic bacteria of the colon or excreted in the faeces. In infants, some levels of HMOs, including 2'-FL, may be absorbed intact and excreted in the urine (approximately 1 to 2% of the ingested amount) (EFSA, 2015). The data suggest that absorption of manufactured forms of 2'-FL would be limited and would not be any different from those natural occurring in breast milk (FSANZ, 2021a). Therefore, the majority of ingested 2'-FL would be digested in the large intestine or excreted in the faeces. The 2'-FL from *C. glutamicum* (strain APC199) has been analytically demonstrated to be chemically and structurally identical to 2'-FL that naturally occurs in human breast milk. Therefore, 2'-FL from *C. glutamicum* (strain APC199) will share the same ADME profile as the naturally occurring compound. As shown in Section B.5, APTech’s 2'-FL may also contain small quantities of other related carbohydrates, such as lactose, DFL, glucose, and galactose. Consumption of these related naturally occurring carbohydrates would be minimal and would be handled by the body in a similar manner as consumed through current dietary sources (*e.g*., dairy, breast milk, infant formula).

C.3 Toxicological Data for 2'-FL

A growing body of research is available to indicate that 2'-FL produced from chemical synthesis or microbial fermentation of genetically modified microbial strains are substantially equivalent. As discussed in Section B.1, the 2'-FL produced by APTech has been demonstrated analytically to be chemically and structurally identical to 2'-FL that is produced synthetically and naturally present in human breast milk by ^{1}H- and ^{13}C-NMR spectroscopy. In Australia and New Zealand, FSANZ has performed numerous evaluations of 2'-FL and concluded the same general conclusion of substantial equivalence with regard to 2'-FL produced from fermentation using different source microorganisms, as summarised in Table C.3-1 below.

Four previous submissions to FSANZ have been evaluated or recently submitted for 2'-FL produced by microbial fermentation with genetically modified organisms for use in infant formula (A1155, A1190, A1233, and A1251 – FSANZ, 2021a,b, 2022e,f).

Table C.3-1 Evaluations of 2'-FL Ingredients Produced by Genetically Modified Strains of *Escherichia coli* Conducted by Food Standards Australia New Zealand

Applicant	Ingredient	2'-FL Source	Evaluation	Status	Reference
Glycom A/S	2'-FL and LNnT in infant formula and other products	*Escherichia coli* K-12 (DH1) SCR6	FSANZ concluded that 2'-FL from this source is structurally and chemically identical to its counterpart found in human milk and that there are no concerns with its use in infant formula at levels up to 2.4 g/L.	Current	A1155 (FSANZ, 2021a)
Jennewein Biotechnologie (Acquired by Chr. Hansen A/S)	2'-FL in infant formula and other products	*Escherichia coli* strain BL21 (DE3)	FSANZ concluded that 2'-FL is naturally present in human milk in a range of concentrations, providing a history of safe human exposure. Thus, FSANZ concluded there are no safety concerns associated with the addition of 2'-FL from this source at the requested (*i.e.*, 2.0 g/L) and existing permissible use levels (*i.e.*, 2.4 g/L).	Forum	A1190 (FSANZ, 2021b)
FrieslandCampina Ingredients	2'-FL from new GM source for infant formula	*Escherichia coli* K-12 strain E997	FSANZ concluded that there is no safety concern with 2'-FL ingredients that adhere to EU specifications published in 2019, and that the new production organism does not pose a risk to humans. Newly available information did not alter previous FSANZ conclusions.	Gazetted	A1233 (FSANZ, 2022e)
Nutricia Australia Pty Ltd (Nutricia) & Chr. Hansen A/S	2'-FL combined with galacto-oligosaccharides and/or inulin-type fructans in infant formula products	*Escherichia coli* strain BL21 (DE3)[a]	FSANZ has not yet evaluated this application.	Forum	A1251 (FSANZ, 2022f)

2'-FL = 2'-fucosyllactose; EU = European Union; FSANZ = Food Standards Australia New Zealand; GM = genetically modified; LNnT = lacto-*N*-neotetraose.
[a] The 2'-FL ingredient in this submission was initially evaluated as part of A1190.

APTech's 2'-FL has been tested in a series of toxicological studies, including a bacterial reverse mutation assay (Ames test), an *in vitro* chromosomal aberration test in Chinese hamster lung cells, an *in vivo* micronucleus test in rats, an oral acute toxicity study, and a subchronic oral toxicity study in rats. The genotoxicity studies and oral acute toxicity study were all conducted with a single lot of the 2'-FL ingredient (Lot No. 2'-FL-CG-008; purity 97.56%), while the subchronic oral toxicity study was performed using a composite mixture of 7 lots of the 2'-FL (Lot Nos. 2'-FL-CG-007, 2'-FL-CG-008, 2'-FL-CG-007.5-3, 2'-FL-CG-009, 2'-FL-CG-010, 2'-FL-CG-007-02, and 2'-FL-CG-011; purity ranging from 96.31 to 97.67%). The test articles used in the toxicology studies were representative of the 2'-FL of commerce. All studies were conducted in accordance with appropriate Organisation for Economic Co-operation and Development (OECD) test guidelines and OECD Principles of Good Laboratory Practice (GLP). Detailed descriptions of these studies are presented in Sections C.3.1.1 and C.3.2.1. The full study reports are provided in Annex G.

To identify studies reporting on relevant safety outcomes for 2'-FL, a comprehensive literature search was conducted using the electronic search tool ProQuest Dialog™ from August 2021 to March 2023 to identify relevant safety studies on 2'-FL that were not evaluated in previous applications for 2'-FL produced by microbial fermentation (FSANZ Application A1251 – FSANZ, 2022f). The databases searched included AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National

Technical Information Service, Toxicology Abstracts, and ToxFile®. The relevant studies that were identified are summarised and discussed in Sections C.3.1.2, C.3.2.2, and C.4.1 and further corroborate the established safety of 2'-FL.

C.3.1 Mutagenicity/Genotoxicity

C.3.1.1 Studies Conducted with APTech's 2'-FL Obtained by Fermentation

C.3.1.1.1 Bacterial Reverse Mutation Assay

The potential mutagenicity of 2'-FL (purity 97%) was evaluated in a bacterial reverse mutation assay (Ames test) with *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, as well as *E. coli* strain WP2*uvrA* (pKM101), using the pre-incubation method (Case *et al.*, 2021). The study was performed according to the OECD Test Guideline 471 (*Bacterial Reverse Mutation Test* – OECD, 1997), *Good Laboratory Practice Regulation for Nonclinical Laboratory Studies* (KMFDS, 2017a), and *Standards for Toxicity Studies of Drugs* (KMFDS, 2017b). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

In the presence of metabolic activation, 2-aminoanthracene (1.0 to 3.0 µg/plate) served as the positive control, while in the absence of metabolic activation, the positive controls were sodium azide (1.5 µg/plate), 2-nitrofluorene (5 µg/plate), 9-aminoacridine (80 µg/plate), and 4-nitroquinoline-*N*-oxide (0.1 µg/plate). Water served as the negative control.

A concentration range–finding study was conducted to determine the highest concentration level to be used in the main study. The study was conducted using the same strains, methods, and conditions as the main study. 2'-FL was tested in duplicate at concentrations of 4.88, 19.5, 78.1, 313, 1,250, and 5,000 µg/plate. No growth inhibition or precipitation of the test article was observed at any test concentrations in the presence or absence of metabolic activation. Based on the results of the concentration range–finding study, the highest concentration, 5,000 µg/plate, was selected for the main study. The main study was conducted in duplicate at concentrations of 313, 625, 1,250, 2,500, and 5,000 µg/plate in the presence and absence of metabolic activation.

The mean number of revertant colonies was less than twice that of the negative control at all test concentrations, and no growth inhibition or precipitation of the test substance was observed. Conversely, the mean number of revertant colonies in the positive control group was greater than 2 times of those of the negative control group.

Based on the results of this study, it was concluded that 2'-FL does not have mutagenic potential at concentrations up to 5,000 µg/plate in the presence or absence of metabolic activation, the highest concentration tested.

C.3.1.1.2 *In Vitro* Chromosomal Aberration Test

The clastogenic potential of 2'-FL (purity 97%) was evaluated in an *in vitro* chromosomal aberration test conducted with mammalian Chinese hamster lung (CHL/IU) cells (Case *et al.*, 2021). This study was conducted in accordance with OECD Test Guideline 473 (*In Vitro Mammalian Chromosomal Aberration Test* – OECD, 2016a) and *Good Laboratory Practice Regulation for Nonclinical Laboratory Studies* (KMFDS, 2017a). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

CHL/IU cells were plated and pre-incubated at 37°C for 24 hours and were then subject to short-term treatment for 6 hours or continuous treatment for 24 hours. Short-term treatment was performed with and without metabolic activation, while continuous treatment was conducted without metabolic

activation. In the assays conducted in the absence of metabolic activation, mitomycin C (10 µg/mL) served as the positive control; in the presence of metabolic activation, benzo[a]pyrene (20 µg/mL) served as the positive control. Water served as the negative control in all treatment assays. A concentration range–finding study was conducted at concentrations of 19.5, 39.1, 78.1, 156, 313, 625, 1,250, 2,500, and 5,000 µg/mL. No cytotoxicity or precipitation of the test article was observed at any concentration. Therefore, the main study was conducted in duplicate at concentrations of 1,250, 2,500, and 5,000 µg/mL.

Three-hundred metaphases per concentration were observed for chromosomal aberrations. Structural aberrations were categorised as chromatid break, chromatid exchange, chromosome break, chromosome exchange, chromatid gap, chromosome gap, and fragmentation. Several gaps and breaks were observed in metaphase, and they were recorded as fragmentation. A gap was described as a lesion narrower than the width of a chromatid and any cell with 1 or more observed aberrations was considered an aberrant cell. The results of this study were considered positive if (i) the frequency of cells with chromosome aberrations were statistically significantly increased at more than 1 dose level in test groups compared to the negative control group; (ii) the increase was dose dependent; and (iii) the increase was over the 95% control limits of distribution of historical data in the negative control group.

The incidence of chromosomal aberrations in the short-term treatments and in the continuous treatment was not statistically significantly increased compared to the negative control group. Conversely, the positive control resulted in a statistically significant increase in the incidence of structural chromosomal aberrations in all treatment assays compared to the negative control group.

Based on the results of this study, it was concluded that 2'-FL does not have clastogenic potential at concentrations up to 5,000 µg/mL, the highest concentration tested, in the presence and absence of metabolic activation in CHL/IU cells.

C.3.1.1.3 *In Vitro* Micronucleus Test

The potential genotoxicity of 2'-FL (Lot No. SP2F20019; purity >94%) was evaluated in an *in vitro* mammalian cell micronucleus test (GenEvolutioN, 2021 [unpublished]). This study was conducted in accordance with OECD Test Guideline 487 (*In Vitro Mammalian Cell Micronucleus Test* – OECD, 2016c) and OECD Principles of GLP (OECD, 1998a). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

2'-FL was tested at concentrations up to 5,000 µg/mL in short-term (3-hour) and long-term (24-hour) treatment periods. The short-term treatment was tested with and without S9 metabolic activation, while the long-term treatment was tested without S9 metabolic activation. The negative control was the vehicle, while mitomycin C, vinblastine, and cyclophosphamide were employed as the clastogenic and aneugenic positive controls. No cytotoxicity was observed in either treatment period, and concentrations of 2,500, 4,000, and 5,000 µg/mL were selected for micronuclei analysis. No precipitation of the test article was observed in either treatment period. No significant increases in the frequency of micronucleated cells were observed compared to the negative control in the short-term treatment assays. In the long-term treatment, a significant increase in micronucleated cells were observed at 2,500 µg/mL; however, this effect was not deemed to be concentration related, as similar findings were not observed at 4,000 or 5,000 µg/mL. One culture out of the 2 tested cultures at 2,500 µg/mL had a frequency of micronucleated cells within the historical negative control data of the testing laboratory; therefore, the observed elevated frequency was not considered to be a cytogenetic effect but a variability of the cell culture in spontaneous micronucleated cell frequency. Based on the results of this study, it was concluded that 2'-FL did not induce micronuclei in cultured human peripheral blood lymphocytes under the conditions of this assay when tested at concentrations up to 5,000 µg/mL.

C.3.1.1.4 *In Vivo* Micronucleus Test

The potential genotoxicity of 2'-FL (purity 97%) was evaluated in an *in vivo* micronucleus test with ICR mice (Case *et al.*, 2021). This study was conducted in accordance with OECD Test Guideline 474 (*Mammalian Erythrocyte Micronucleus Test* – OECD, 2016b) and *Good Laboratory Practice Regulation for Nonclinical Laboratory Studies* (KMFDS, 2017a). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

A dose range–finding study was conducted to determine the dose levels for the main study. Male and female ICR mice (n=3/sex/group) were administered 2'-FL by gavage at doses of 0, 1,250, 2,500, 5,000, or 7,500 mg/kg. The highest test dose was set at 7,500 mg/kg based on consultation with the test sponsor. No clinical signs or mortality were observed at any test doses, and no significant changes in body weight were observed. Therefore, the main study was conducted at doses of 2,500, 5,000, and 7,500 mg/kg. Male ICR mice (n=5/group) were administered 2'-FL by gavage twice (24-hour interval) at doses of 2,500, 5,000, or 7,500 mg/kg. Mitomycin C (2 mg/kg) served as the positive control and the vehicle (water) was used as the negative control. Clinical signs were recorded immediately after administration of the first dose (Day 0), second dose (Day 1), and 2 hours after the second dosing (Day 2). Body weights were recorded on Day 1 after the second dosing. Animals were euthanised by cervical dislocation and bone marrow cells were harvested for analysis. Samples were observed for polychromatic erythrocytes (PCE) (4,000/animal) and the ratio of micronucleated PCE to PCE was calculated. A total of 500 erythrocytes per animal were observed and the ratio of PCE to total erythrocytes was calculated. A finding was considered to be positive if (i) the incidence of micronucleated polychromatic erythrocytes (MNPCE) increased in a dose-dependent manner; (ii) the increase was outside the historical control range; and (iii) at least 1 dose resulted in a statistically significant increase in MNPCE compared to the negative control.

No clinical signs or abnormalities were observed at any dose group, and no statistically significant changes in the body weights of any animal were observed compared to the negative control group. No significant changes in MNPCE or ratio of PCE to total erythrocytes were observed in any test group. Conversely, the incidence of MNPCE in PCE was significantly increased in the positive control compared to the negative control, thus confirming the results of the study.

Based on the results of this study, 2'-FL was concluded to be non-genotoxic in ICR mice at doses up to 7,500 mg/kg.

C.3.1.2 Studies Conducted with Other 2'-FL Preparations

A summary of genotoxicity studies conducted with various 2'-FL ingredients produced *via* chemical synthesis or fermentation is presented in Table C.3.1.2-1 below. The results from these studies have already been evaluated by FSANZ and are included for completeness to support the safety of APTech's 2'-FL from *C. glutamicum* (strain APC199). The results of these studies are consistent with the absence of genotoxic or mutagenicity concluded in the product-specific studies summarised in Section C.3.1.1 above.

Table C.3.1.2-1 Summary of Mutagenicity and Genotoxicity Data for Other 2'-FL Preparations

Test Material	Toxicology Study	Concentration/Dose	Results	Reference
2'-FL produced by chemical synthesis	Bacterial Reverse Mutation (OECD TG 471)	Up to 5,000 µg/plate (±S9)	Negative	Coulet *et al.* (2014) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
	In Vitro Mammalian Cell Gene Mutation (OECD TG 476)	Up to 5,000 µg/mL (±S9)	Negative	
2'-FL/DFL mixture	Bacterial Reverse Mutation (OECD TG 471)	Up to 5,000 µg/plate (±S9)	Negative	Phipps *et al.* (2018) – reviewed in

Table C.3.1.2-1 Summary of Mutagenicity and Genotoxicity Data for Other 2'-FL Preparations

Test Material	Toxicology Study	Concentration/Dose	Results	Reference
produced by fermentation	*In Vitro* Mammalian Cell Micronucleus (OECD TG 487)	Up to 2,000 µg/mL (±S9)	Negative	FSANZ Application A1155 (FSANZ, 2018)
Mixture of 5 HMOs produced by fermentation	Bacterial Reverse Mutation (OECD TG 471)	Up to 246 mg 2'-FL/plate (±S9)	Negative	Parschat *et al.* (2020) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
	In Vitro Mammalian Cell Micronucleus (OECD TG 487)	Up to 24.6 mg 2'-FL/mL (±S9)	Negative	
2'-FL produced by fermentation	*In vitro* Mammalian Cell Micronucleus (OECD TG 487)	Up to 2,000 µg/mL (±S9)	Negative	Verbaan *et al.* (2015) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
2'-FL produced by fermentation	Bacterial Reverse Mutation (OECD TG 471)	Up to 5,000 µg/plate (±S9)	Negative	Verspeek-Rip *et al.* (2015) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
2'-FL produced by fermentation	Bacterial Reverse Mutation (OECD TG 471)	Up to 5,000 µg/plate (±S9)	Negative	Launstein (2014a) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
	In Vivo Mammalian Erythrocyte Micronucleus (OECD TG 474)	Up to 2,000 mg/kg bw	Negative	Launstein (2014b) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
	In Vitro Micronucleus (OECD TG 487)	Up to 5,000 µg/plate (±S9)	Negative	Spruth (2015) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
2'-FL produced by fermentation	Bacterial Reverse Mutation (OECD TG 471)	Up to 5,000 µg/plate (±S9)	Negative	van Berlo *et al.* (2018) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
	In Vitro Mammalian Cell Micronucleus (OECD TG 487)	Up to 2,000 µg/mL (±S9)	Negative	

2'-FL = 2'-fucosyllactose; bw = body weight; DFL = difucosyllactose; FSANZ = Food Standards Australia New Zealand; HMO = human milk oligosaccharide; OECD = Organisation for Economic Co-operation and Development; TG = Test Guideline.

C.3.2 Repeated-dose Toxicity Studies

C.3.2.1 Studies Conducted with APTech's 2'-FL Obtained by Fermentation

C.3.2.1.1 Acute Toxicity in Rats

The acute oral toxicity of 2'-FL (purity 97%) was investigated in juvenile (7-day-old) male and female Sprague-Dawley rats (Case *et al.*, 2021). This study was conducted in accordance with *Good Laboratory Practice Regulation for Nonclinical Laboratory Studies* (KMFDS, 2017a) and OECD Principles of GLP (OECD, 1998a). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

Sprague-Dawley rats (n=5/sex/group) were administered 2'-FL by gavage at doses of 0, 2,500, 5,000, or 7,500 mg/kg. The control animals received the vehicle (water). Animals were evaluated for clinical signs, including mortality and general condition, at least once for 30 minutes following dosing and 1, 2, 4, and 6 hours after dosing on Day 0. Clinical observations were performed once per day on Days 1 through 14. Body weights were measured before dosing on Days 0, 1, 3, 7, 14, and on the day of necropsy. Animals were euthanised by exsanguination from the abdominal aorta, and gross examination was performed. No significant findings were observed upon necropsy; therefore, no histopathological examination was conducted.

One female rat in the 7,500 mg/kg group was found dead on Day 2 after dose administration; however, this finding was determined to be due to natural causes, as there were no treatment-related clinical signs and other female rats in the same group were unaffected by treatment. No abnormalities were observed in clinical signs in any animal. A significant decrease in body weight and body weight gain was observed in male rats of the 7,500 mg/kg/day dose group on Days 1 to 14 of the study. No significant changes in body weight or body weight gain were reported in any other group. No macroscopic findings were reported in any treatment group upon necropsy.

Based on the results of this study, the approximate lethal dose of 2'-FL was concluded to be greater than 7,500 mg/kg in juvenile male and female Sprague-Dawley rats.

C.3.2.1.2 90-Day Repeated Oral Toxicity in Rats

The subchronic toxicity of 2'-FL (purity ranging from 96.3 to 97.7%) was investigated in juvenile Sprague-Dawley rats (7-day-old) in a 90-day repeated oral toxicity study with a 4-week recovery period (Case *et al.*, 2021). This study was conducted in accordance with OECD Test Guideline 408 (*Repeated Dose 90-Day Oral Toxicity Study in Rodents* – OECD, 1998b) and OECD Principles of GLP (OECD, 1998a). This study was conducted with neonatal rats, as 2'-FL is intended for use in infant formula, in consideration of the requirements of the EFSA *Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age* (EFSA, 2017). The full study report is provided in Appendix A-4, and the redacted version is included in Appendix E.

Sprague-Dawley rats (n=10/sex/group) were administered 2'-FL by gavage at doses of 0 (control), 2,500 (low-dose), 5,000 (mid-dose), or 7,500 (high-dose) mg/kg body weight/day for 90 days. The control group received the vehicle (water). The control and high-dose groups included an additional 5 animals per group. Animals were observed for clinical signs daily, and body weight and food consumption were observed on a weekly basis. Animals were subject to a functional observational battery and motor activity and ophthalmology examination. Blood samples were collected from the abdominal aorta and prepared for haematology and clinical chemistry analyses. Urine samples were collected over 24 hours and subject to urinalysis. At the end of the study period, animals were euthanised by exsanguination, and organs and tissues were removed for analysis. The additional animals in the control and high-dose group were carried for another 4 weeks without treatment.

One male of the mid-dose group and 1 female of the high-dose group were found dead on Day 72 and Day 30, respectively. These deaths were not considered to be related to treatment as the male animal did not show any morphological changes upon necropsy and the female animal that died on Day 30 with a serous fluid-filled thoracic cavity and pulmonary congestion/oedema was accidental and attributable to error of gavage administration. Diarrhoea and soft stool were observed in both males and females in the high-dose group from Day 26 through the end of the dosing regimen but were not observed during the recovery period. Haematuria was observed in 1 male in the high-dose recovery group, but this finding was not considered significant, as there were no haematuria-related morphological changes during the histopathological examination.

No significant changes in body weight, food consumption, functional observational battery, ophthalmological examination, urinalysis, haematology, clinical chemistry, organ weights, macroscopic examination, or histopathology were reported in any test group. Changes in several haematology, clinical chemistry, and urinalysis parameters were reported; however, these were not observed after the 4-week study period and were not considered to be treatment related. Upon macroscopic examination, black focus was observed on the mucosa of the glandular stomach in 1 animal of the high-dose group, and upon histopathological examination, a renal mesenchymal tumour was observed on the kidney of 1 male animal of the high-dose group. These findings were unilateral, not dose dependent, and occurred only in 1 animal and were therefore considered to be spontaneous and incidental and not related to the 2'-FL treatment.

Based on the results of the study, the no-observed-adverse-effect level (NOAEL) of 2'-FL was concluded to be 7,500 mg/kg/day, the highest dose tested, in male and female Sprague-Dawley rats.

C.3.2.2 Studies Conducted with Other 2'-FL Preparations

A summary of the toxicity studies conducted on various other 2'-FL preparations manufactured *via* chemical synthesis or fermentation technologies is presented in Table C.3.2.2-1 below. Many of these studies have already been reviewed by FSANZ and are included for completeness to support the safety of APTech's 2'-FL from *C. glutamicum* (strain APC199). The results of these studies are consistent with the product-specific studies summarised in Section C.3.2.1, demonstrating the safety of 2'-FL ingredients.

Table C.3.2.2-1 Summary of Toxicological Data for Other 2'-FL Preparations

Test Material	Toxicology Study	Concentration/Dose	Results	Reference
2'-FL produced by chemical synthesis	14-Day Repeated Oral Toxicity Study in Neonatal Wistar Rats	0, 2,500, 5,000, 7,500 mg/kg bw/day	NOAEL >7,500 mg/kg bw/day	Coulet *et al.* (2014) – reviewed in FSANZ Application A1155 (FSANZ, 2020)
	90-Day Oral Toxicity Study in Neonatal Wistar Rats (OECD TG 408)	0, 2,000, 5,000, 6,000 mg/kg bw/day	NOAEL = 5,000 mg/kg bw/day	
2'-FL produced by fermentation	90-Day Oral Toxicity Study in Neonatal Wistar Rats (OECD TG 408)	2,000, 4,000, 5,000 mg/kg bw/day	NOAEL = 5,000 mg/kg bw/day	Penard (2015) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
2'-FL produced by fermentation	21-Day Oral (*via* diet) Study in Neonatal Piglets (GLP)	0, 200, 500, 2,000 mg/L (*i.e*., 29.37, 72.22, 291.74 mg/kg bw/day in males and 29.30, 74.31, 298.99 mg/kg bw/day in females)	NOAEL = 291.74 mg/kg bw/day in males and 298.99 mg/kg bw/day in females	Hanlon and Thorsrud (2014) – reviewed in FSANZ Application A1155 (FSANZ, 2018)
2'-FL/DFL mixture produced by fermentation	14-Day Repeated Oral Toxicity Study in Neonatal Sprague-Dawley Rats	0, 4,000, 5,000 mg/kg bw/day	NOAEL = 5,000 mg/kg bw/day (*i.e.*, 4,444 mg 2'-FL/kg bw/day)	Phipps *et al.* (2018) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
	90-Day Oral Toxicity Study in Neonatal Sprague-Dawley Rats (OECD TG 408)	0, 1,000, 3,000, 5,000 mg/kg bw/day	NOAEL = 5,000 mg/kg bw/day (*i.e.*, 4,444 mg 2'-FL/kg bw/day)	Phipps *et al.* (2018) – reviewed in FSANZ Application A1155 (FSANZ, 2019b)
2'-FL produced by fermentation	7-Day Oral Toxicity Study in Sprague-Dawley Rats	10% in feed (*i.e.*, 7,700 mg/kg bw/day)	NOAEL = 7,700 mg/kg bw/day	Leuschner (2013) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
	90-Day Oral Toxicity Study in Sprague-Dawley Rats (OECD 408)	10% in feed (*i.e.*, 7,700 mg/kg bw/day in males and 8,700 mg/kg bw/day in females)	NOAEL = 7,700 mg/kg bw/day in males and 8,700 mg/kg bw/day in females	Hansen (2014) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
2'-FL produced by fermentation	14-Day Repeated Oral Toxicity Study in Rats	0, 2,670, 5,050, 7,990 mg/kg bw/day	NOAEL >7,990 mg/kg bw/day (assumed)	Triskelion (2016) – reviewed in FSANZ Application A1233 (FSANZ, 2022g)
	90-Day Oral Toxicity Study in Neonatal Wistar Rats (OECD TG 408)	0, 2,170, 4,270, 7,250 mg/kg bw/day in males and 2,450, 5,220, 7,760 mg/kg bw/day in females	NOAEL = 7,250 mg/kg bw/day in males and 7,760 mg/kg bw/day in females	van Berlo *et al.* (2018) – reviewed in FSANZ Application A1190 (FSANZ, 2019b)
Mixture of 5 HMOs produced by fermentation	91-day Oral Toxicity Study in CD Rats (OECD TG 408)	0 or 10% in feed (*i.e.*, 5,670 mg/kg bw/day in males and 6,970 mg/kg bw/day in females)	NOAEL = 5,670 mg/kg bw/day in males and 6,970 mg/kg bw/day in females	Parschat *et al.* (2020)

Table C.3.2.2-1 Summary of Toxicological Data for Other 2'-FL Preparations

Test Material	Toxicology Study	Concentration/Dose	Results	Reference
Commercially available 2'-FL (manufacturing process NS)	18-Day Oral Study in Sprague-Dawley Rats	0, 1.2 g/L (2'-FL alone), or 0.6 g/L (in combination with 0.6 g/L 3'-SL) Intake volume increased throughout the study from 4.75 mL/day at study onset to 25.25 mL/day at weaning	NOAEL = 1.2 g/L at the described intake volumes (assumed)	Wang *et al.* (2022)
Mixture of 5 HMOs produced by fermentation	21-Day Oral (*via* diet) Study in Domestic Yorkshire Crossbred Piglets	0, 5.75, or 8.0 g/L (*i.e.*, 0, 2,556, or 3,576 mg/kg bw/day in males and 0, 2,604, or 3,660 mg/kg bw/day in females)	NOAEL = 3,576 mg/kg bw/day in males and 3,660 mg/kg bw/day in females (assumed)	Hanlon *et al.* (2020)
2'-FL produced by fermentation	21-Day Oral (*via* diet) Study in Neonatal Piglets	0 or 1.0 g/L Mean daily intake volume throughout the study = 317 mL/kg bw/day	NOAEL = 317 mg/kg bw/day (assumed)	Daniels *et al.* (2022)

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose; bw = body weight; DFL = difucosyllactose; FSANZ = Food Standards Australia New Zealand; GLP = Good Laboratory Practice; NOAEL = no-observed-adverse-effect level; OECD = Organisation for Economic Co-operation and Development; TG = Test Guideline.

C.4 Clinical Data

C.4.1 Human Studies Conducted with 2'-FL

There exists an extensive database of clinical studies conducted with 2'-FL, either alone or in combination with other HMOs, that have already been evaluated by FSANZ in FSANZ Applications A1155 (Prieto 2005; Kajzer *et al*., 2016 *via* Puccio *et al*., 2017; Reverri *et al*., 2018; Nowak-Wegrzyn *et al*., 2019; Storm *et al*., 2019; Palsson *et al*., 2020; Román Riechmann *et al*., 2020), A1190 (Marriage *et al*., 2015; Elison *et al*., 2016; Goehring *et al*., 2016), A1233 (Fonvig *et al.*, 2021), and A1251 (Vandenplas *et al*., 2020). From these clinical studies, including those conducted in infants, FSANZ has 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.

Additionally, 9 human clinical studies conducted to evaluate the safety-related effects and tolerability of 2'-FL, either alone or in combination with other HMOs, were identified in a search of the scientific literature since the submission of FSANZ Application A1251. The results of these studies are summarised in Table C.4.1-1. In general, the results from the newly identified human clinical studies do not indicate any safety concerns with 2'-FL at levels up to 2.9 g/L. Test formulas containing 2'-FL alone, or in combination with other HMOs, were considered by the authors to be well tolerated and the study populations exhibited normal growth with no difference in observed adverse events between the test groups.

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
Blend of 5 HMOs: 2'-FL, DFL, LNT, 3'-SL, and 6'-SL	Healthy, formula-fed infants (≥7 to ≤21 days old) N=230 in Control N=229 in Test Formula 1 N=227 in Test Formula 2	**Control**: Standard cow's milk–based infant formula **Test Formula 1:** Standard formula with 1.5 g/L HMOs **Test Formula 2:** Standard formula with 2.5 g/L HMOs	Up to 6 months of age	• Weight gain was non-inferior in test groups compared to control. • Stool frequency and consistency were similar between formula-fed groups, and test groups mainly had loose/soft stool during the feeding period. • Treatment formulas were well tolerated, and no adverse events were reported.	Bauer *et al.* (2021) (Abstract only)
2'-FL and LNnT	86 preterm infants (27–33 weeks gestation, birth weight <1,700 g) N=43/group Average 6 days of age at intervention initiation	**Control (Placebo) Supplement**: Glucose (0.140 g/kg bw/day) **Test Supplement**: 2'-FL and LNnT in 10:1 ratio (0.374 g/kg bw/day)	Enrolment to discharge from neonatal unit	• Adjusted mean time to reach full enteral feeding from birth was 2 days shorter in the treatment group compared to the control (not statistically significant). • GI tolerance measures, and incidence of GI adverse events, necrotising colitis, and other illnesses and infections were similar between groups. • No reported illnesses or infections were deemed related to the intervention. • Trend towards better feeding tolerance with HMO supplement.	Hascoët *et al.* (2021)

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
2'-FL and LNnT	32 full-term infants with CMPA between 1 and 8 months old at enrolment Open-label, single-arm, multi-centre study	**Test Formula:** AAF supplemented with 1.0 g/L 2'-FL and 0.5 g/L LNnT	4 months	• Weight- and length, and head circumference-for-age Z-scores trended close to WHO child growth standards of treatment. • Statistically significant improvement of CMPA symptoms such as crying, fussing, frequent or persistent regurgitation, vomiting, prevalence of significant issues feeding, and frequent or continuous skin issues was reported 1 month after intervention. • With increasing age, a trend towards more formed and less frequent stools was reported. • Any reported serious adverse effects were deemed to be unrelated to the study formula. • 4 "mild" adverse events were reported in 2 subjects that were deemed to be related or "probably related" to the test material.	Gold *et al.* (2022)

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
2'-FL	Infants 0–60 days of age (N=36) with suspected food protein allergy, persistent feeding intolerance, or requiring an EHF	**Test Formula**: Extensively hydrolysed casein-based formula with 0.2 g/L 2'-FL	2 months	• Formula was well tolerated. • Infants maintained their weight-for-age z-scores at Days 30 and 60. • Adverse events were reported in 15 infants. • Adverse events were mainly seborrheic dermatitis (n=5), GI reflux disease (n=3), and infantile spit-up (n=2). • No worsening of symptoms related to diarrhoea, constipation, blood in stool, vomiting, spit-up/gagging/reflux, fussiness, or rash/eczema reported at Day 30 or 60.	Ramirez-Farias *et al.* (2021)
HMO mix mimicking the natural concentrations of the top 5 HMOs (5.75 g/L total, comprising 52% 2'-FL, 13% 3-FL, 26% LNT, 4% 3'-SL, and 5% 6'-SL)	225 healthy term infants and 116 healthy breastfed infants (Reference Group) N=112–113 per formula group ≤14 days of age at enrolment	**Control Formula:** Basic infant formula **Test Formula:** Same as control, plus a target HMO content of 5.75 g/L (2.99 g/L of 2'-FL, 1.5 g/L of LNT, 0.75 g/L of 3-FL, 0.28 g/L of 6'-SL, and 0.23 g/L of 3'-SL). Average intake of HMO mix was reported to be 2.64 ± 0.79 g (Visit 1), 3.46 ± 0.77 g (Visit 2), 4.28 ± 0.94 g (Visit 3), 4.62 ± 0.87 g (Visit 4), 4.94 ± 1.09 g (Visit 5), and 5.19 ± 0.97 (Visit 6), equivalent to daily consumption of 2'-FL of approximately 1.37 g (Visit 1), 1.80 g (Visit 2), 2.23 (Visit 3), 2.40 g (Visit 4), 2.57 g (Visit 5), and 2.70 g (Visit 6), respectively. **Reference Group:** Breastfed infants	4 months	• The number and intensity of adverse events were equivalent in all groups. • A greater incidence of genital fungal infection was reported in the treatment group compared to the control, and haematochezia and plagiocephaly were more frequent in the test group compared to the breastfed reference group. • In each of the formula-fed groups, 2 of the reported serious adverse effects were treatment related. • 5-HMO mix was well tolerated.	Parschat *et al.* (2021)

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
2'-FL	349 healthy full-term infants <14 days old at the time of enrolment 145 healthy full-term infants (control group) 144 healthy full-term infants (test formula group) 60 breastfed infants (reference group) Randomized, double-blind, controlled, multi-centre, parallel study	**Control Formula:** Standard bovine milk–based whey predominant infant formula containing *Lactobacillus reuteri* (1×10^7 CFU/g) **Test Formula:** Same as control with 1.0 g/L 2'-FL **Reference group:** Breastfed infants	6 months	• Weight gain was similar between the test and control formula groups. • No significant difference in anthropometric z-scores, stooling characteristics (based on parental questionnaire), gastrointestinal effects and associated behaviours, adverse events, or medication use between the formula-fed groups. • 4 adverse events (all reported in the test material group) were considered to be related to the test material.	Alliet *et al.* (2022)

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
HMO mixture containing 2'-FL, LNT, 3-FL, 6'-SL, and 3'-SL	363 healthy full-term infants N=129 (control) N=130 (test formula) N=104 (breastfed) ≤14 days of age at enrolment Multi-centre, randomized, double-blind, controlled, parallel study	**Control Formula:** Standard cow's milk–based infant formula **Test Formula:** Same as control, with a target HiMO content of 5.75 g/L (3.0 g/L of 2'-FL, 1.5 g/L of LNT, 0.8 g/L of 3-FL, 0.3 g/L of 6'-SL, and 0.2 g/L of 3'-SL) **Human Milk Group:** Breastfed infants	4 months	• No significant difference in mean daily weight gain from Days 14–119 between groups (the test material was non-inferior to the control). • No significant difference in length and head circumference gains between the test and control group • Statistically significant decrease in head circumference gain in males fed the test formula compared to the control, but not in males fed human milk. • Growth parameters were near or greater than the 50th percentile based on WHO growth charts for males and females. • No significant difference reported in daily formula intake, average number of daily feedings, or percentage of feedings with spit-up/vomit between formula groups. • Statistically significant decrease in mean rank stool consistency (*i.e.*, softer stools) in the test group compared to the control. • Statistically significant increase in stool frequency in treatment group compared to the control. • Stools were predominantly watery and yellow in the test group compared to the control. • Based on responses to questionnaires, parents of test group infants noted greater average loose stool dimension scores and lower constipation dimension scores compared to the control. • No significant difference in serious adverse events and non-serious adverse events between groups.	Lasekan *et al.* (2022)

Table C.4.1-1 Summary of Human Studies Published Since Food Standards Australia New Zealand's Evaluation of FSANZ Application A1251

Test Substance	Study Group/Population	Dose/Concentration	Study Duration	Results	Reference
2'-FL and LNnT	Full-term infants with CMPA 0–6 months old at enrolment N=96 (control group), N=94 (test formula group) Randomized, double-blind, controlled, multi-centre, parallel study	**Control Formula:** Commercially available whey–based EHF without HMO **Test Formula:** Whey-based EHF supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of LNnT	4 months	• Daily weight gain was non-inferior between groups after 4 months of intervention. • No significant between-group differences were reported in anthropometric parameters up to 12 months of age. • A minor increase in weight gain and growth velocity was reported compared to WHO growth reference from enrolment to 12 months. • Statistically significant increase in mean formula intake volumes (10–13%) in the test group compared to controls at 3, 4, and 6 months. • No significant difference in the rate of adverse events. • Serious adverse events were considered to be related to the test material; 1 adverse event in each study group was considered to be treatment-related, while 5 and 10 events in the test and control groups were considered "probably related" to the test material, respectively.	Vandenplas *et al.* (2022)
2'-FL	221 healthy full-term infants N=66 (control) N=66 (test formula) N=89 (breastfed) ≤29 days of age at enrolment Randomized, double-blind, controlled, parallel study	**Control Formula:** Commercial whey-dominant cow's milk–based infant formula containing GOS and FOS **Test Formula:** Control formula with the inclusion of 1 g/L 2'-FL as a replacement for the same amount of GOS **Reference Group:** Breastfed infants	16 weeks	• No effect on growth or adverse effects. • Modest changes in microbiome in the direction of breastfed infants. • No significant between group differences in anthropomorphic measures in any group.	Wallingford *et al.* (2022)

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; AAF = amino acid formula; bw = body weight; CMPA = cow's milk protein allergy; DFL = difucosyllactose; EHF = extensively hydrolysed formula; FOS = fructo-oligosaccharide; FSANZ = Food Standards Australia New Zealand; GI = gastrointestinal; GOS = galacto-oligosaccharide; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; N = number of participants; n = number.

C.5 Safety of the Production Microorganism

C.5.1 History of Consumption

APTech's 2'-FL is obtained *via* fermentation with *C. glutamicum* APC199. As described in Section B.4.2, *C. glutamicum* is a non-pathogenic bacterium widely used in industrial amino acid production and has been studied for more than 60 years since its discovery as a glutamate-secreting bacterium (Vertès *et al.*, 2012). *C. glutamicum*, which is a Gram-positive and non-spore forming bacterium regarded as a GRAS strain, has been extensively used in the fermentation industries for the production of amino acids and nucleic acid (Nakayama *et al*., 1978; Date *et al.*, 2006).

C. *glutamicum* ATCC 13032 (NCIMB 10025), the parent microorganism, was originally isolated from sewage as a producer of glutamate (Kinoshita *et al*., 1958). While the strain had been taxonomically named "*Micrococcus glutamicus*," and deposited as ATCC 13032 and NCIMB 10025, it was designated as *C. glutamicum* type strain and classified as a Biosafety Level 1 organism by the ATCC. *C. glutamicum* ATCC 13032 is a strain of the *C. glutamicum* species (other names: DSM 20300, IMET 10482, NCIMB 10025, and KCTC 1445). The genome of *C. glutamicum* ATCC 13032 consists of a single circular chromosome 3.3 Mbp in size (Meyer *et al.*, 2003). The G+C content of the genome is 53.8%, which is close to that of *E. coli* and at the lower boundary for the taxonomic class of the Actinobacteria, which is referred to as high G+C Gram-positive bacteria.

Moreover, *C. glutamicum* has QPS status for the production of amino acids and other food ingredients, such as flavouring compounds (EFSA, 2019a). According to the EFSA, *C. glutamicum* does not produce toxic compounds, and there are no associated hazards to the presence of toxic metabolites in the fermentation broth of this species (EFSA, 2019a). The source organism, *C. glutamicum*, has a long history of use in food production. *C. glutamicum* was first isolated in 1956 and was characterised by its ability to produce large amounts of glutamic acid from sugar and ammonia (Vertès *et al.*, 2013). *C. glutamicum* has been identified as part of the surface microflora in cheese during ripening, indicating that this microorganism has a history of consumption as a species in cheese (Dolci *et al.*, 2009).

A number of *Corynebacterium spp.* (*C. ammoniagenes, C. casei, C. flavescens,* and *C. variabile*) are listed in the International Dairy Federation 2018 inventory of microbial species with technological beneficial roles in fermented food products (IDF, 2018). *Corynebacterium* spp. have been in use in the production of a variety of food products including cereal, bread, and alcoholic beverages, as well as in the fermentation of Korean food products (Puspito and Fleet, 1985; Shin *et al*., 2011; Cheng and Han, 2014; Jung *et al*., 2016). *Corynebacterium* spp. have also been used in the cooking of native dishes due to their ability to hydrolyse starch to organic acids (Hahn, 1988; Haard *et al*., 1999; Osungbaro, 2009; Nwagu *et al*., 2011).

C.5.2 Bioinformatic Searches for Homology with Known Toxins and Allergens

C.5.2.1 Allergenicity

The purification steps in the manufacturing process include a series of filtration steps that sufficiently remove protein (*e.g.*, any potential allergens) to levels below 10 µg/g in APTech's final 2'-FL product. Nevertheless, the presence of the inserted genes and their products were analysed in 5 production lots of the 2'-FL final product. The production microorganism used in the manufacturing process of 2'-FL is a derivative of *C. glutamicum* episomally overexpressing the genes required for 2'-FL biosynthesis (*gmd*, *wcaG*, *lacY*, and *α-1,2-ft)*. 2'-FL produced *via* APTech's production process has been thoroughly analysed for the presence of genes from the *C. glutamicum* production strain. Specifically, 5 lots of 2'-FL (Lot Nos. 2'-FL-CG-011, 2'-FL-CG-012, 2'-FL-CG-013, 2'-FL-CG-014, and 2'-FL-CG-015) were analysed using quantitative polymerase chain reaction (qPCR) for unintentional incorporation of protein (*gmd*, *wcaG*, *lacY*, and *α-1,2-ft*, *NPTII*, or 16s rDNA) in the final product (see Appendix A-2). The results of these qPCR analyses showed that there were no unintentional incorporations of protein in the final product. The proteins encoded by the overexpressed genes from the pFP110 vector inserted in *C. glutamicum* ATCC 13032 (*gmd*, *wcaG*, *lacY*, and *α-1,2-ft)* were further evaluated for their allergenic potential below.

As recommended by FAO/WHO (2001), to investigate the allergenicity potential of the proteins encoded by the genes *gmd*, *wcaG*, *lacY*, and *α-1,2-ft* (GDP-D-mannose 4,6-dehydratase [*gmd*], GDP-L-fucose synthase [*wcaG*], lactose permease [*lacY*], and fucosyltransferase [*α-1,2-ft*], respectively) from the multicloning site of the pFP110 vector, which are required for 2'-FL biosynthesis, and to demonstrate that they do not contain amino acid sequences similar to known allergens that might produce an allergenic response, sequence homology searches were conducted using the AllergenOnline database version 21 (available at http://www.allergenonline.org; updated 14 February 2021) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2021). The database contains a comprehensive list of putative allergenic proteins developed *via* a peer-reviewed process for the purpose of evaluating food safety. Full-length alignment searches of AllergenOnline were conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). No matches were identified from searching with the full amino acid sequences of *gmd*, *wcaG*, *lacY*, and *α-1,2-ft*.

A second series of homology searches was conducted according to the approach outlined by FAO/WHO (2001) and Codex Alimentarius (2009). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80–amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the amino acid sequences of *gmd*, *wcaG*, *lacY*, and *α-1,2-ft*. The 80–amino acid alignment searches were conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility (FAO/WHO, 2001). Using this search strategy, no matches were identified. A third series of homology searches conducted using the exact 8-mer approach also did not result in any matches for *gmd*, *wcaG*, *lacY*, or *α-1,2-ft*. The full search report is provided in Appendix A-2.

Based on the information provided above, no evidence exists indicating that the proteins encoded by the genes *gmd*, *wcaG*, *lacY*, and *α-1,2-ft* would produce an allergenic response following consumption of foods to which APTech's 2'-FL will be added. Therefore, the presence of these proteins is not anticipated to pose any allergenicity concerns in consumers.

C.5.2.2 Toxigenicity

The whole genome sequence of *C. glutamicum* APC199 was analysed to detect known major virulence genes of the pathogenic *Bacillus cereus*. Virulence genes include aggregation substance, cytolysin, cytotoxin K, enterococcal surface protein, endocarditis antigen, adhesion of collagen, enterotoxin, gelatinase, haemolysin, hyaluronidase, and cereulide. As the whole genome sequencing results of *C. glutamicum* with *B. cereus* ATCC 14579 were compared, no toxigenic genes were found in *C. glutamicum* APC199, while various toxigenic genes were detected in *B. cereus* ATCC 14579, implying the safety of *C. glutamicum* APC199 and the absence of potentially toxigenic genes.

As stated by Pearson (2013) with regard to protein alignments, homologous sequences sharing more than 40% identity are very likely to share functional similarity, E-values of <0.001 can reliably be used to infer homology, and a bit-score of 50 is "*almost always significant*," while a bit-score of 40 is only significant (E-value <0.001) in searches of protein databases with less than 7,000 entries.

To determine whether APTech's gmd, wcaG, lacY, and α-1,2-ft proteins share significant sequence homology with known protein toxins, sequence homology searches were conducted using the Basic Local Alignment Search Tool (BLAST), hosted by the NCBI, with the amino acid sequence of APTech's gmd, wcaG, lacY, and α-1,2-ft against protein sequences obtained from a curated databases of 7,563 animal venom proteins and toxins[9] maintained by UniProt. The BLAST searches were conducted using default algorithm (word size of 6 and expect threshold of 0.05) and scoring parameters (BLOSUM62 scoring matrix with default gap costs and composition adjustments). Sequences were considered to share structural homology/similarity based on the criteria by Pearson (2013) described above (*i.e.*, percent identities >40%, E-values <0.001, and bit scores >40 or >50 for databases of less than or greater than 7,000 entries, respectively). No significant sequence homology to any of the venom proteins or toxins were identified from the sequence alignment searches. The findings of this search suggest that APTech's gmd, wcaG, lacY, and α-1,2-ft do not harbour any overtly toxigenic potential.

C.5.3 *In Vitro* Safety Tests for *Corynebacterium glutamicum* APC199 Production Strain

The analysis of whole genomic sequencing of APTech's *C. glutamicum* APC199 revealed that the strain is absent of virulence genes. In addition, *C. glutamicum* APC199 was shown to (i) have no haemolytic activity; (ii) have no gelatinase activity; and (iii) not produce biogenic amines.

In Vitro Haemolysis Test

C. glutamicum APC199 was grown at 30°C for 24 hours in brain heart infusion (BHI) broth then streaked onto 5% sheep blood agar (Hanil Komed) and incubated for 24 hours at 30°C. alpha-Haemolysis was considered as the partial decomposition of the haemoglobin of the red blood cells (but does not represent true haemolysis), *beta*-haemolysis as the complete breakdown of the haemoglobin of the red blood cells observed as a clear zone in the agar plate, and *gamma*-haemolysis as a lack of haemolysis. *Staphylococcus aureus* ATCC 6538 was used as a positive control. *C. glutamicum* showed a negative reaction for haemolysis (see Table C.5.3-1).

[9] UniProt Animal toxin annotation project; available at: https://www.uniprot.org/program/Toxins.

Table C.5.3-1 Haemolysis Activity of *Corynebacterium glutamicum* APC199 and *Staphylococcus aureus* ATCC 6538

Strain	Haemolysis Activity
Corynebacterium glutamicum APC199	*Gamma*
Staphylococcus aureus ATCC 6538 (positive control)	*Beta*

In Vitro Gelatinase Activity Test

Basic glutaminase activity test protocol was followed according to ASM Science Recommendation (dela Cruz and Torres, 2012). *C. glutamicum* APC199, grown at 30°C for 24 hours in BHI broth, was inoculated in a gelatine medium with a loop and incubated at 30°C for up to 1 week; it was checked daily for gelatine liquefaction. Gelatine normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes were placed in a refrigerator for 15 to 30 minutes. Afterwards, the tubes were tilted to observe whether the gelatine had been hydrolysed. Hydrolysed gelatine will result in a liquified medium even after exposure to cold temperature. *Bacillus cereus* ATCC 11778 was used as a positive control. *C. glutamicum* APC199 showed a negative reaction for the gelatinase test (see Table C.5.3-2).

Table C.5.3-2 Gelatinase Test for *Corynebacterium glutamicum* APC199 and *Bacillus cereus* ATCC 11778

Strain	Gelatinase Test
Corynebacterium glutamicum APC199	Negative
Bacillus cereus ATCC 11778 (Positive control)	Positive

In Vitro Biogenic Amine Test

C. glutamicum, grown at 30°C for 24 hours in BHI broth, was streaked out onto special medium with lysine, tyrosine, histamine, and ornithine as precursor amino acids using the method described in Bover-Cid and Holzapfel (1999) and was incubated for 48 hours at 30°C to detect biogenic amine production (cadaverine, tyramine, histamine, and putrescine, respectively). *E. coli* ATCC 25922 was used as a positive control. *C. glutamicum* was shown to be negative for 4 different biogenic amine productions (histamine, cadaverine, tyramine, and putrescine) at 30°C (see Table C.5.3-3).

Table C.5.3-3 Biogenic Amine Production Activity of *Corynebacterium glutamicum* APC199 and *Escherichia coli* ATCC 25922

Strain	Histamine	Cadaverine	Tyramine	Putrescine
Corynebacterium glutamicum APC199	Negative	Negative	Negative	Negative
Escherichia coli ATCC 25922 (positive control)	Positive	Positive	Positive	Positive

Conclusions

C. glutamicum APC199 did not produce biogenic amines and had no haemolytic and gelatinase activities. The whole genome sequencing analysis also revealed that *C. glutamicum* APC199 was negative for major virulence genes. Thus, it is concluded that *C. glutamicum* APC199 is non-toxigenic and non-pathogenic.

C.6 Assessments by Authoritative Bodies

C.6.1 European Union

In the EU, 2'-FL produced by chemical synthesis or microbial fermentation has been authorised for use as a novel food ingredient. The safety of 2'-FL (produced from chemical synthesis and identical in structure to the ingredient produced by fermentation described herein) was first evaluated by EFSA in 2015, at the request of the European Commission (EFSA, 2015). As a result of this evaluation, the EFSA NDA Panel concluded that 2'-FL is safe for infants up to 1 year of age when added to infant and follow-on formulae at concentrations up to 1.2 g/L and that it is also safe for young children older than 1 year of age when added to follow-on and young-child formulae at concentrations up to 1.2 g/L. 2'-FL use as an ingredient in other foods was also concluded to be safe.

Since the publication of that safety opinion in 2015, the safety of additional 2'-FL ingredients (alone or in combination with other HMOs) produced from fermentation with various microbial sources (including APTech's *C. glutamicum* APC199) has been evaluated by EFSA in 2015, 2019, and 2022 (EFSA, 2015, 2019b, 2022a-c). Based on these safety evaluations, 2'-FL from both chemical synthesis and microbial fermentation has been approved for use in the EU in a number of food applications under Commission Implementing Regulation (EU) 2017/2470 (EU, 2017). In the most recent safety evaluation of microbially-derived 2'-FL, conducted at the request of the European Commission, the EFSA NDA Panel delivered the scientific opinion that APTech's 2'-FL from microbial fermentation with *C. glutamicum* APC199 is safe for its intended uses as a novel food ingredient (EFSA, 2022a).

C.6.2 United States

APTech's 2'-FL ingredient obtained from fermentation with a *C. glutamicum* APC199–derived strain has self-GRAS status in the U.S. for use as an ingredient at a level of 2.4 g/L of formula as consumed in milk- and soy-based, non-exempt infant formula for term infants. APTech's 2'-FL ingredient is also GRAS for use at a level of 2.4 g/L in drinks for toddlers and children ages 1 to 3 years; at levels ranging from 0.24 to 1.2 g/serving in infant and toddler foods; and at levels ranging from 0.28 to 1.2 g/serving in beverage and beverages bases, breakfast cereals, dairy product analogues, frozen dairy desserts and mixes, gelatins, puddings, fillings, grain products and pastas, jams and jellies, milk and milk products, processed fruits and fruit juices, and sweet sauces, toppings and syrup. The GRAS status of this ingredient was notified to the U.S. FDA in 2020 as GRN 932, which received a "no questions" response to the conclusion that use of APTech's 2'-FL in term infant formula at a maximum level of 2.4 g/L is GRAS, as consumed (U.S. FDA, 2021a).

Moreover, a wide range of other 2'-FL ingredients produced by microbial fermentation (or chemical synthesis) have GRAS status for use in term infant formula at levels up to 2.4 g/L (GRNs 546, 571, 650, 735, 749, 815, 852, 897, 929 – U.S. FDA, 2015a,b, 2016, 2018a,b, 2019b,c, 2020, 2021b), as well as other food products, such as baby foods, drinks for infants and young children, such as toddler formula, beverages and beverage bases, dairy product analogues, grain products and pasta, milk (whole and skim), milk products, processed fruits and juices, processed vegetables and juices, baked goods and baking mixes, non-alcoholic beverages and beverage bases, breakfast cereals, and tube-feeding formulas at levels ranging from 1.2 to 40 g/kg (GRNs 650, 897 –U.S. FDA, 2016, 2020).

C.6.3 Other Authoritative Bodies

Other authoritative bodies have evaluated the safety of 2'-FL from fermentation. In 2018, Health Canada evaluated the safety of 2'-FL derived from a genetically modified strain of *E. coli* BL21 (DE3) (strain #1540) for use in Canadian infant formula products (Health Canada, 2018a,b). In its public notification, Health Canada stated that it has no objection to the use of the bacterially synthesised 2'-FL ingredient (≥90% purity) when used in infant formula for term infants to a maximum use level of 1.2 g 2'-FL/L of formula. Furthermore, in 2017, the Ministry of Health of Israel concluded that 2'-FL obtained from microbial fermentation using strains derived from *E. coli* BL21 (DE3) or *E. coli* K-12 DH1 is authorised as a novel food for use in milk-based infant formulas (ages 0 to 6 months) and follow-on formulas (ages 6 to 12 months), at levels of up to 2.0 g/L of the ready-to-feed product (Israel MOH, 2020).

D. INFORMATION ON DIETARY INTAKE OF THE NUTRITIVE SUBSTANCE

Information on the proposed uses and use levels for APTech's 2'-FL, and accordingly, the anticipated level of exposure, is provided in this section. This section has been completed in accordance with the requirements outlined in the relevant sections of *Guideline 3.3.3 – Substances used for a nutritive purpose* and *Guideline 3.6.2 – Special purpose food – Infant formula products* of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a). The pertinent requirements of each guideline have been addressed in the subsections below.

D.1 Proposed Food Uses and Maximum Use Levels

APTech's 2'-FL is intended for use as a nutritive substance for use in infant formula. Currently, 2'-FL is approved for use in Australia and New Zealand as a nutritive substance in infant formula products at a maximum use level of 96 mg 2'-FL/100 kJ (*i.e.*, 2.4 g/L) per *Schedule 29 – Special purpose foods* (FSANZ, 2022b). APTech intends to market 2'-FL from *C. glutamicum* APC199 in infant formula products at levels that meet established use levels and therefore proposes no changes to currently permitted levels.

D.2 Natural Occurrence of 2'-FL in the Diet

HMOs all contain lactose at their reducing end. Approximately 200 individual milk oligosaccharides have been identified, based on the extension of lactose (ten Bruggencate *et al*., 2014). Of the over 200 HMOs that have been identified, 2'-FL is the most abundant (Castanys-Munoz *et al*., 2013). 2'-FL is an HMO that exists in small amounts in beestings (cow's foremilk) but not in commercialised milk products, whereas it is known to be abundant in human milk.

Table D.2-1 summarises 2'-FL concentrations of human milk collected from various cohorts (Grollman and Ginsburg, 1967; Thurl *et al*., 1996, 2010; Chaturvedi *et al*., 1997, 2001; Coppa *et al*., 1999, 2011; Kunz *et al*., 1999; Nakhla *et al*., 1999; Erney *et al*., 2000, 2001; Sumiyoshi *et al*., 2003; Morrow *et al*., 2004; Musumeci *et al*., 2006; Asakuma *et al*., 2008, 2011; Leo *et al*., 2009, 2010; Gabrielli *et al*., 2011; Galeotti *et al*., 2012, 2014; Bao *et al*., 2013; Castanys-Munoz *et al*., 2013; Smilowitz *et al*., 2013; Goehring *et al*., 2014; Hong *et al*., 2014; Marx *et al*., 2014; Balogh *et al*., 2015; Austin *et al*., 2016; Donovan and Comstock, 2016; McGuire *et al*., 2017). The mean concentrations of 2'-FL in human milk range from 0.22 to 8.4 g/L, depending on the genotype of the mother and stage of lactation, as indicated by the studies summarised in Table D.2-1.

Table D.2-1 2'-FL Content in Human Milk

Reference	Location	Days, Weeks, or Months Postpartum	2'-FL Content (g/L)
Grollman and Ginsburg (1967)	NS	1–3 d	0.24–0.36
		5 wk	0.46
		6 wk	0.031
Thurl *et al.* (1996)	Europe	Mature milk	1.84
Chaturvedi *et al.* (1997)	Mexico City	30–60 d	1.21
Coppa *et al.* (1999)	Europe	4 d	3.93
		10 d	3.02
		30 d	2.78
		60 d	1.84
		90 d	2.46
Kunz *et al.* (1999)	Germany	2–28 d	0.45
Nakhla *et al.* (1999)	U.S.	0–33 d	1.13
		4–128 d	1.27

Table D.2-1 2'-FL Content in Human Milk

Reference	Location	Days, Weeks, or Months Postpartum	2'-FL Content (g/L)
Erney *et al.* (2000)	Asia	0–2 d	2.29
		3–10 d	2.26
		11–30 d	2.36
		>31 d	1.50
	Europe	0–2 d	3.40
		3–10 d	2.69
		11–30 d	2.38
		>31 d	2.36
	Latin America	3–10 d	2.79
		11–30 d	2.61
		>31 d	1.91
	U.S.	3–10 d	2.78
		11–30 d	2.56
		>31 d	1.69
Erney *et al.* (2001)	America and Europe	1–100 d	2.38
Chaturvedi *et al.* (2001)	U.S.	1 d	2.8
		2 d	3
		3 d	3.5
		>10 d	3.6
Sumiyoshi *et al.* (2003)	Japan	4 d	0.20
		10 d	0.34
		30 d	0.29
		100 d	0.05
Morrow *et al.* (2004)	Latin America	1–100 d	3.95
Musumeci *et al.* (2006)	Burkinabe, Africa	1 d	1.80
		2 d	4.50
		3 d	8.40
	Italy	1 d	1.00
		2 d	2.10
		3 d	4.20
Asakuma *et al.* (2008)	Japan	1 d	2.49
		2 d	2.01
		3 d	1.58
Leo *et al.* (2009)	Samoa	5–10 d	0.22
		>10 d	0.69
Leo *et al.* (2010)	Samoa	5–10 d	0.22
		22–155 d	0.69
Thurl *et al.* (2010)	Germany	5–10 d	3.37
		>10 d	2.96
Gabrielli *et al.* (2011)	Italy	4 d	7.3
		5–10 d	6.05
		>10 d	5.25
Asakuma *et al.* (2011)	Japan	30–120 d	1.48
Coppa *et al.* (2011)	Europe	25–35 d	0–2.66
Galeotti *et al.* (2012)	Europe	4–30 d	0–7.15

Table D.2-1 2'-FL Content in Human Milk

Reference	Location	Days, Weeks, or Months Postpartum	2'-FL Content (g/L)
Bao *et al.* (2013)	U.S.	3 d	1.12
		14–29 d	1.08
Castanys-Munoz *et al.* (2013)	Asia	NS	2.1
	Europe		2.6
	Latin America		2.48
	U.S.		2.0
Smilowitz *et al.* (2013)	U.S.	90 d	1.22
Galeotti *et al.* (2014)	Europe	4–30 d	0–7.80
Goehring *et al.* (2014)	NS	14 d	2.87
Hong *et al.* (2014)	U.S.	35 d	0.48–2.50
Marx *et al.* (2014)	California, U.S.	NS	2.40–3.70
Balogh *et al.* (2015)	NS	1 wk of lactation	4.53–6.27
Austin *et al.* (2016)	China – Urban	5–11 d	2.00
		12–30 d	1.90
		1–2 mo	1.70
		2–4 mo	1.30
		4–8 mo	1.10
Donovan and Comstock (2016)	NS	NS	2.7
McGuire *et al.* (2017)	Ethiopia – Rural	71 d	1.11
	Ethiopia – Urban	59 d	1.39
	Gambia – Rural	65 d	1.44
	Gambia – Urban	62 d	2.06
	Ghana	58 d	0.70
	Kenya	73 d	1.65
	Peru	60 d	3.19
	Spain	70 d	1.91
	Sweden	49 d	2.77
	Washington, U.S.	68 d	2.03
	California, U.S.	62 d	3.44

2'-FL = 2'-fucosyllactose; d = days; mo = months; NS = not specified; U.S. = United States; wk = weeks.

D.3 Consumption Information for Foods Not Included in the Most Recent Australian or New Zealand National Nutrition Surveys

Consumption data for infant formula products in individuals younger than 2 years of age are not included in the most recent Australia and New Zealand National Nutrition Surveys (NNS) (*i.e.*, 2011–12 National Nutrition and Physical Activity Survey component of the 2011–13 Australian Health Survey for ages 2 years and above; the 2008–09 New Zealand NNS for ages 15 years and above; and the 2002 New Zealand Children's NNS for ages 5 to 14 years). Information on likely consumption for this group can instead be based on consumption data from similar markets in the U.S. or EU (see Section D.6.2).

Consumption of infant formula products is unlikely to have changed significantly since previous intake estimates were last conducted.

D.4 Percentage of Foods Likely to Contain 2'-FL

The use of APTech's 2'-FL from *C. glutamicum* APC199 is expected to be fully substitutional to the already-approved use of 2'-FL as an ingredient in infant formula products. Previously, in deriving the estimated intake of 2'-FL from its proposed use in infant and follow-on formulae as part of FSANZ Application A1155 (see Section D.6.1, below), it was assumed that 2'-FL would be added to all infant formula products marketed in Australia and New Zealand (including both powdered and ready-to-feed formulations) as a highly conservative measure. This level of market penetration is highly unlikely, despite its utility in the derivation of a conservative exposure estimate. More realistically, market penetration estimates for infant and follow-on formulae in Australia and New Zealand were presented and ranged from 3.4 to 11%, based on expected use in specific brands and market share (Application A1155; FSANZ, 2018). The percentage of infant formula on the Australian and New Zealand markets that will contain APTech's 2'-FL is unknown; however, using a similar approach, APTech expects a market penetration of approximately 4 to 5% in infant formula products for these markets.

D.5 Approved Uses of 2'-FL in Other Countries

D.5.1 European Union

In the EU, 2'-FL from both chemical synthesis and microbial fermentation have been approved for use in a number of food applications under Commission Implementing Regulation (EU) 2017/2470 (EU, 2017). The specifications and list of permitted food uses and use levels reproduced from the Union list are presented in Tables D.5.1-1 and D.5.1-2, respectively.

Table D.5.1-1 Specifications for 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)]

Authorised Novel Food	Specification
2'-Fucosyllactose (synthetic)	**Definition:** Chemical name: α-l-Fucopyranosyl-(1→2)-β-d-galactopyranosyl-(1→4)-d-glucopyranose Chemical formula: $C_{18}H_{32}O_{15}$ CAS No.: 41263-94-9 Molecular weight: 488,44 g/mol **Description:** 2'-Fucosyllactose is a white to off-white powder that is produced by a chemical synthesis process and is isolated by crystallisation. **Purity:** **2'-Fucosyllactose: ≥ 95 %** D-Lactose: ≤ 1,0 w/w % L-Fucose: ≤ 1,0 w/w % Difucosyl-d-lactose isomers: ≤ 1,0 w/w % 2'-Fucosyl-d-lactulose: ≤ 0,6 w/w % pH (20°C, 5 % solution): 3,2-7,0 Water (%): ≤ 9,0 % Ash, sulphated: ≤ 0,2 % Acetic acid: ≤ 0,3 % Residual solvents (methanol, 2-propanol, methyl acetate, acetone): ≤ 50,0 mg/kg singly, ≤ 200,0 mg/kg in combination) Residual proteins: ≤ 0,01 %

Table D.5.1-1 Specifications for 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)]

Authorised Novel Food	Specification		
	Heavy Metals: Palladium: ≤ 0,1 mg/kg Nickel: ≤ 3,0 mg/kg **Microbiological Criteria:** Aerobic mesophilic bacteria total count: ≤ 500 CFU/g Yeasts and Moulds: ≤ 10 CFU/g Residual endotoxins: ≤ 10 E.U./mg		
2'-Fucosyllactose (microbial source)	**Definition:** Chemical name: α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose Chemical formula: $C_{18}H_{32}O_{15}$ CAS No.: 41263-94-9 Molecular weight: 488,44 g/mol		
	Source: Genetically modified strain of *Escherichia coli* K-12 **Description:** 2'-Fucosyllactose is a white to off-white crystalline powder that is produced by a microbial process. **Purity:** **2'-Fucosyllactose: ≥ 90 %** D-Lactose: ≤ 3,0 % L-Fucose: ≤ 2,0 Difucosyl-D-lactose: ≤ 2,0 % 2'-Fucosyl-D-lactulose: ≤ 1,0 % pH (20°C, 5 % solution): 3,0-7,5 Water: ≤ 9,0 % Ash, sulphated: ≤ 2,0 % Acetic acid: ≤ 1,0 % Residual proteins: ≤ 0,01 % **Microbiological Criteria:** Aerobic mesophilic bacteria total count: ≤ 3,000 CFU/g Yeasts: ≤ 100 CFU/g Moulds: ≤ 100 CFU/g Endotoxins: ≤ 10 E.U./mg	**Source:** Genetically modified strain of *Escherichia coli* BL21 **Description:** 2'-Fucosyllactose is a white to off white powder and the liquid concentrate (45 % ± 5 % w/v) aqueous solution is a colourless to slight yellow clear aqueous solution. 2'-Fucosyllactose is produced by a microbiological process. 2'-Fucosyllactose is isolated by spray drying. **Purity:** **2'-Fucosyllactose: ≥ 90 %** Lactose: ≤ 5,0 % Fucose: ≤ 3,0 % 3-Fucosyllactose: ≤ 5,0 % Fucosylgalactose: ≤ 3,0 % Difucosyllactose: ≤ 5,0 % Glucose: ≤ 3,0 % Galactose: ≤ 3,0 % Water: ≤ 9,0 % (powder) Ash, sulphated: ≤ 0,5 % (powder and liquid) Residual proteins: ≤ 0,01 % (powder and liquid) **Heavy Metals:** Lead: ≤ 0,02 mg/kg (powder and liquid); Arsenic: ≤ 0,2 mg/kg (powder and liquid) Cadmium: ≤ 0,1 mg/kg (powder and liquid) Mercury: ≤ 0,5 mg/kg (powder and liquid)	**Source:** Genetically modified strain of *Corynebacterium glutamicum* ATCC 13032 **Description:** 2'-Fucosyllactose is a white to off white/ivory powder that is produced by a microbiological process. **Purity:** **2'-Fucosyllactose (w/w dry matter): ≥ 94,0 %** D-Lactose (w/w dry matter): ≤ 3,0 % L-Fucose (w/w dry matter): ≤ 3,0 % 3-Fucosyllactose (w/w dry matter): ≤ 3,0 % Difucosyllactose (w/w dry matter): ≤ 2,0 % D-Glucose (w/w dry matter): ≤ 3,0 % D-Galactose (w/w dry matter): ≤ 3,0 % Water: ≤ 9,0 % Ash: ≤ 0,5 % Residual proteins: ≤ 0,005 % **Contaminants:** Arsenic: ≤ 0,03 mg/kg Aflatoxin M1: ≤ 0,025 µg/kg Ethanol: ≤ 1 000 mg/kg **Microbiological Criteria:** Total plate count: ≤ 500 CFU/g Yeasts and Moulds: ≤ 100 CFU/g Enterobacteriaceae: absence in 10 g *Salmonella*: absence in 25 g *Cronobacter* spp.: absence in 10 g Endotoxins: ≤ 100 E.U./g

Table D.5.1-1 Specifications for 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)]

Authorised Novel Food	Specification	
		Microbiological Criteria: Total plate count: ≤ 10^4 CFU/g (powder); ≤ 5000 CFU/g (liquid) Yeasts and Moulds: ≤ 100 CFU/g (powder); ≤ 50 CFU/g (liquid) Enterobacteriaceae/Coliforms: absence in 11 g (powder and liquid) *Salmonella*: negative/100 g (powder), negative/200 ml (liquid) *Cronobacter*: negative/100 g (powder), negative/200 ml (liquid) Endotoxins: ≤ 100 E.U./g (powder), ≤ 100 E.U./ml (liquid) Aflatoxin M1: ≤ 0,025 µg/kg (powder and liquid)

2'-FL = 2'-fucosyllactose; CAS = Chemical Abstracts Service; CFU = colony-forming units; E.U = endotoxin units.

Table D.5.1-2 Permitted Uses of 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)]

Authorised Novel Food	Conditions Under which the Novel Food May Be Used	Additional Specific Labelling Requirements	Other Requirements
2'-Fucosyllactose	***Specified Food Category***	***Maximum Levels***	1. The designation of the novel food on the labelling of the foodstuffs containing it shall be "2'-fucosyllactose." 2. The labelling of food supplements containing 2'-fucosyllactose shall bear a statement that the supplements should not be used if other foods with added 2'-fucosyllactose are consumed the same day. 3. The labelling of food supplements containing 2'-fucosyllactose intended for young children shall bear a statement that the supplements should not be used if breast milk or other foods with added 2'-fucosyllactose are consumed the same day
	Unflavoured pasteurised and sterilised (including UHT) milk-based products	1,2 g/l	
	Unflavoured fermented milk-based products	1,2 g/l beverages	
		19,2 g/kg products other than beverages	
	Flavoured fermented milk-based products including heat-treated products	1,2 g/l beverages	
		19,2 g/kg products other than beverages	
	Dairy analogues, including beverage whiteners	1,2 g/l beverages	
		12 g/kg for products other than beverages	
		400 g/kg for whitener	
	Cereal bars	12 g/kg	
	Table-top sweeteners	200 g/kg	
	Infant formula as defined in Regulation (EU) No 609/2013 (EU, 2013)	1,2 g/l alone or in combination with up to 0,6 g/l of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	

Table D.5.1-2 Permitted Uses of 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)]

Authorised Novel Food	Conditions Under which the Novel Food May Be Used	Additional Specific Labelling Requirements	Other Requirements
	Follow-on formula as defined in Regulation (EU) No 609/2013 (EU, 2013)	1,2 g/l alone or in combination with up to 0,6 g/l of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Processed cereal-based food and baby food for infants and young children as defined in Regulation (EU) No 609/2013 (EU, 2013)	12 g/kg for products other than beverages	
		1,2 g/l for liquid food ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Milk-based drinks and similar products intended for young children	1,2 g/l for milk-based drinks and similar products added alone or in combination with up to 0,6 g/l lacto-*N*-neotetraose, at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Foods for special medical purposes as defined in Regulation (EU) No 609/2013 (EU, 2013)	In accordance with the particular nutritional requirements of the persons for whom the products are intended	
	Total diet replacement for weight control as defined in Regulation (EU) No 609/2013 (EU, 2013)	4,8 g/l for drinks	
		40 g/kg for bars	
	Bread and pasta products bearing statements on the absence or reduced presence of gluten in accordance with the requirements of Commission Implementing Regulation (EU) No 828/2014 (EU, 2014)	60 g/kg	
	Flavoured drinks	1,2 g/l	
	Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; tea, herbal and fruit infusions, and chicory extracts; tea, plant, fruit, and cereal preparations for infusions, as well as mixes and instant mixes of these products	9,6 g/l - the maximum level refers to the products ready to use	
	Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants (EC, 2002)	3,0 g/day for general population	
		1,2 g/day for young children	

2'-FL = 2'-fucosyllactose; UHT = ultra-high temperature.

D.5.2 United States

APTech's 2'-FL ingredient, as described herein, has self-GRAS status in the U.S., and its GRAS status has been notified to the U.S. FDA (GRN 859 – U.S. FDA, 2019d). Several data deficiencies were noted by the FDA in this original submission, and the GRAS Notice was withdrawn (U.S. FDA, 2019b); however, APTech has since addressed the data deficiencies and a second GRAS Notice was submitted in April 2020. APTech has since received a "no questions" letter from the FDA for the 2'-FL ingredient, which has been filed under GRN 932 (U.S. FDA, 2021a). The food uses and maximum use levels of APTech's 2'-FL obtained by fermentation that are considered GRAS in the U.S. are presented in Table D.5.2-1 below.

Table D.5.2-1 Individual Food Uses and Use Levels for APTech's 2'-FL from Microbial Fermentation that are Generally Recognized as Safe in the United States (GRN 932 – U.S. FDA, 2021a)

Food Category	Food Uses	Maximum Use Level (g/serving)	Serving Size (g or mL)	Maximum Use Level (g/kg unless noted otherwise)[a]
Beverages and beverage bases	Energy drinks	0.28	360 mL	0.80
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360 mL	0.80
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1.20	15 g (puffed) 40 g (high-fibre) 60 g (biscuit-types)	80.0 30.0 20.0
	Hot cereals for adults and children	1.20	40 g (dry) 250 g (prepared)	4.8 (as consumed)
Dairy product analogues	Milk substitutes such as soy milk and imitation milks	0.28	240 mL	1.2.0
Frozen dairy desserts and mixes	Frozen desserts including ice creams and frozen yogurts, frozen novelties	1.20	70 g	17.0
Gelatins, puddings, and fillings	Dairy-based puddings, custards, and mousses	1.20	70 g	17.0
	Fruit pie filling	1.20	85 g	14.1
	Fruit filling in bars, cookies, yogurt, and cakes	1.20	40 g	30.0
Grain products and pastas	Bar, including snack bars, meal-replacement bars, and breakfast bars	0.48	40 g	12.0
Jams and jellies, commercial	Jellies and jams, fruit preserves, and fruit butters	1.20	20 g	60.0
Milk, whole, and skim[b]	All Acidophilus or fortified and skim milks, non-fat and low-fat fluids, including fluid milk and reconstituted milk powder	0.28	240 mL	1.2
Milk products	Flavoured milks, including milk, coffee drinks, cocoa, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir	0.28	240 mL	1.2
	Milk-based meal replacement beverages or diet beverages	0.28	240 mL	1.2
	Yogurt	1.20	225 g	5.3
	Formula intended for pregnant women	1.20	200 mL	6.0

Table D.5.2-1 Individual Food Uses and Use Levels for APTech's 2'-FL from Microbial Fermentation that are Generally Recognized as Safe in the United States (GRN 932 – U.S. FDA, 2021a)

Food Category	Food Uses	Maximum Use Level (g/serving)	Serving Size (g or mL)	Maximum Use Level (g/kg unless noted otherwise)[a]
Processed fruits and fruit juices	Fruit drinks, including vitamin and mineral fortified products	0.28	240 mL	1.2
	Fruit juices	0.28	240 mL	1.2
Sweet sauces, toppings, and syrups	Syrups used to flavour milk beverages	0.28	40 g	7.0
Non-exempt infant and follow-on formula	Infant formula* (0 to 6 months), including ready-to-drink formula or reconstituted formula prepared from powder	N/A	N/A	2.4 g/L
	Follow-on formula* (6 to 12 months), including ready-to-drink formula or formula prepared from powder	N/A	N/A	2.4 g/L
	Infant meal replacement products	0.24	100 g	2.4
Baby foods	Milk formula for toddlers and children aged 12 to 36 months*	N/A	N/A	2.4 g/L
	Ready-to-eat, ready-to-serve, hot cereals	1.20	15 g (dry) 110 g (ready-to-serve)	10.9 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.20	129	10.0
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.20	110	10.9
	Baby crackers, pretzels, cookies, and snack items	0.40	7	57.0

2'-FL = 2'-fucosyllactose; GRAS = Generally Recognized as Safe; GRN = Generally Recognized as Safe (GRAS) Notice.
[a] The proposed maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.
[b] Milk is a standardised food in the United States. When the milk is fortified with 2'-FL, it would be classified as a milk product.

In addition, a number of other 2'-FL ingredients produced by chemical synthesis or microbial fermentation have GRAS status for use in term infant formula at levels up to 2.4 g/L (GRNs 546, 571, 650), as well as other food products including as baby foods, drinks for infants and young children, such as toddler formula, beverages and beverage bases, dairy product analogues, grain products and pasta, milk (whole and skim), milk products, processed fruits and juices, and processed vegetables and juices at maximum levels ranging from 0.084 to 2.4 g of 2'-FL per serving (GRN 650 – U.S. FDA, 2015a,b, 2016).

GRNs 735, 749, 815, 852, 897, 929, 1014, 1034, (1040, 1051, and 1060 pending) for 2'-FL have also received "no questions" letters (U.S. FDA, 2023).[10]

[10] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices.

D.5.3 Other Jurisdictions

2'-FL obtained from microbial fermentation of a strain derived from *E. coli* BL21 (DE3) or *E. coli* K-12 DH1 is authorised as a novel food for use in milk-based infant formulas (ages 0 to 6 months) and follow-on formulas (ages 6 to 12 months), at levels of up to 2.0 g/L of the ready-to-feed product in Israel (Israel MOH, 2020). Furthermore, 2'-FL derived from a genetically modified strain of *E. coli* BL21 (DE3) (strain #1540) is approved for use in Canadian infant formula products (Health Canada, 2018a,b). In Vietnam, 2'-FL is also permitted for sale.

D.6 Estimated Intake of 2'-FL from Proposed Uses

D.6.1 Australia/New Zealand

APTech's 2'-FL is intended for use as a nutritive substance in Australia and New Zealand under the same conditions of use as those presently authorised for 2'-FL in infant formula products. Dietary intakes of 2'-FL are not expected to change from current levels as a result of a successful application to permit *C. glutamicum* APC199 as a source of 2'-FL, as the use of this ingredient is intended to be fully substitutional to the 2'-FL preparations currently marketed.

As part of FSANZ's evaluation of Application A1155 for the addition of 2'-FL to infant formula products, a dietary intake assessment of 2'-FL was conducted. This assessment was conducted through the construction of model diets to estimate intakes for children below the age of 2 years, as the 2011–12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS) did not contain these data.

The level of 2'-FL intake from use in these products was estimated to be within the range of 2'-FL present in human breast milk. In application A1155, the estimated mean and 90th percentile dietary intakes of 2'-FL were 0.33 g/kg body weight/day and 0.66 g/kg body weight/day for 3-month-old infants, 0.16 g/kg body weight/day and 0.32 g/kg body weight/day for 9-month-old infants, and 0.10 to 0.20 g/kg body weight/day and 0.20 to 0.40 g/kg body weight/day for 12-month-old infants.

D.6.2 Exposure Assessments that were Previously Conducted

Dietary exposure assessments have also been conducted to estimate the intake levels of 2'-FL from a range of other food use categories, including infant formula products, in the EU and U.S. These intake estimates are higher than what has been estimated from uses of 2'-FL in Australia and New Zealand due to the much broader permissible food use categories. In the EU and the U.S., the highest all-user estimated intake of 2'-FL in infants from all food uses was 1,110 mg/kg body weight/day (4 to 11 months; 95th percentile) and 532 mg/kg body weight/day (0 to 5.9 months; 90th percentile), respectively.

D.6.2.1 European Union

In 2015, EFSA evaluated the safety of proposed food uses for 2'-FL that would result in estimated daily intake levels of 2.5 g 2'-FL/day (418 mg/kg body weight per day) in the 95th percentile of infants 0 to 6 months old from its use in infant formula alone in the United Kingdom (EFSA, 2015). EFSA (2015) noted, "*It was determined that infants aged 4 to 6 months have the greatest mean all-user intakes of 2'-FL on an absolute basis of 2.75 g per day, while infants aged 7 to 12 months had the highest 95th percentile all-user intakes at 6.12 g per day, respectively*." On a body weight basis, the mean and 95th percentile all-user intakes for infants 4 to 6 months old were identified as 338 and 668 mg/kg body weight per day, respectively, from all proposed uses in the foods for infant and young children categories. Furthermore, when the maximum use levels were utilised to estimate intakes in the general European population for all other intended food uses, the high-level intakes of 2'-FL in the total population, as reported in the EFSA Food Additive Intake Mode tool (EFSA, 2015), ranged from 89 to

286 mg/kg body weight per day in the elderly and up to 822 to 1,928 mg/kg body weight per day in toddlers (EFSA, 2011, 2015).

More recently, in an evaluation of the safety of a 2'-FL/DFL mixture conducted by the EFSA in 2019, individual data from the EFSA Comprehensive Food Consumption Database (EFSA, 2019b, 2021b) were utilised to estimate the dietary intake of 2'-FL from its wide range of accepted food uses in the general European population (EFSA, 2019b). The estimated intake values for 2'-FL, from the use of this novel food mixture in a variety of foods and beverages, are comparable to those discussed in the initial scientific opinion for 2'-FL issued by the EFSA in 2015 (EFSA, 2015) and are summarised in Table D.6.2.1-1, below. The EFSA NDA Panel noted that all of the proposed uses included in this estimate are in line with already authorised maximum use levels for 2'-FL in the EU.

Table D.6.2.1-1 Summary of the Estimated Daily Intakes of 2'-FL (mg/kg body weight/day) from Novel Food Uses in the European Union Based on the Individual Data from the EFSA Comprehensive Food Consumption Database

Population Group	Age Group	Number of EU Dietary Surveys	Estimated Daily Intake of 2'-FL – All Subjects (mg/kg body weight/day)	
			Range of Means (lowest and highest) Among EU Dietary Surveys	Range of 95th Percentile Among EU Dietary Surveys
Infants	4–11 months	11	53–339	133–1,110
Young children or toddlers	12–35 months	14	58–250	186–697
Other children	3–9 years	19	28–150	77–378
Adolescents	10–17 years	18	11–39	30–99
Adults	18–64 years	19	6–23	28–73
Elderly	≥65 years	18	6–21	25–60
Pregnant women	-	2	4–19	11–61
Lactating women	-	2	16–23	60–62

2'-FL = 2'-fucosyllactose; EU = European Union; EFSA = European Food Safety Authority.
This table was adapted from Tables 6 and 7 in the EFSA Opinion "*Safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283*," published in June 2019.

D.6.2.2 United States

The estimated daily intakes of 2'-FL based on its intended use in the U.S. are published in several GRAS Notices (GRNs 546, 571, 650, 735, 749, 815, 852, 897, 929, 932 – U.S. FDA, 2015a,b, 2016, 2018a,b, 2019b,c, 2020, 2021a,b). Data utilised in the derivation of estimated daily intake levels as part of GRN 932, which was notified to the U.S. FDA for APTech's 2'-FL ingredient produced from *C. glutamicum* APC199, are presented below in Tables D.6.2.2-1 to D.6.2.2-4.

Table D.6.2.2-1 presents the data on infant formula intakes by age, which range from 1,077 to 1,219 g/person/day. On a body weight basis, these intakes correspond to 118 to 226 g/kg body weight/day.

Table D.6.2.2-1 Estimated Daily Intakes of Infant Formula

Population Group	All-person Intake		All-users Intake			
	Mean	90th Percentile	% Users	n	Mean	90th Percentile
g/person/day						
0 to <3	509	1,095	66.5	140	766	1,212
3 to <6	609	1,128	71.8	151	849	1,219
6 to <9	629	1,069	81.2	162	775	1,077
9 to <12	495	1,012	68.6	115	721	1,156
0 to <12	563	1,096	72.2	568	780	1,157
g/kg body weight/day						
0 to <3	96.3	204.4	66.5	140	144.9	225.8
3 to <6	85.6	170.4	71.8	151	119.2	175.5
6 to <9	74.0	133.4	81.2	162	91.1	140.8
9 to <12	52.8	76.6	68.6	115	76.6	118.3
0 to <12	77.9	168.3	72.2	568	107.8	179.7

2'-FL = 2'-fucosyllactose; n = number.

Table D.6.2.2-2 presents the estimates for the daily intake of 2'-FL from its use in only term-infant formula. From the use of 2'-FL in only infant formula (a maximum level of 2.4 g/L of ready-to-use or reconstituted formula), the estimated mean and 90th percentile intakes of 2'-FL were determined to be 1.87 and 2.78 g/person/day, respectively, in all-user infants 0 to 11.9 months old. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg body weight/day, respectively. The all-user estimated mean and 90th percentile intakes of 2'-FL were greatest in infants 3 to 5.9 months old, at 2.04 and 2.93 g/person/day, respectively. On a body weight basis, the greatest intake was observed to occur in infants 0 to 2.9 months old, at 347.8 and 541.9 mg/kg body weight/day, respectively.

Table D.6.2.2-2 Estimated Daily Intakes of 2'-FL from the Proposed Use in Infant Formula Only

Population Group	All-person Intake		All-users Intake			
	Mean	90th Percentile	% Users	n	Mean	90th Percentile
g/person/day						
0 to <3	1.22	2.63	66.5	140	1.84	2.91
3 to <6	1.46	2.71	71.8	151	2.04	2.93
6 to <9	1.51	2.57	81.2	162	1.86	2.58
9 to <12	1.18	2.43	68.6	115	1.73	2.77
0 to <12	1.35	2.63	72.2	568	1.87	2.78
g/kg body weight/day						
0 to <3	231.1	490.6	66.5	140	347.8	541.9
3 to <6	205.4	409.0	71.8	151	286.1	421.2
6 to <9	177.6	320.2	81.2	162	218.6	337.9
9 to <12	126.7	183.8	68.6	115	183.8	283.9
0 to <12	187.0	403.9	72.2	568	258.7	431.3

2'-FL = 2'-fucosyllactose; n = number.

As presented in GRN 735, an earlier GRAS Notice incorporated in GRN 932 by reference, the mean and 90th percentile estimated daily intakes (EDIs) of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively (see Table D.6.2.2-3). The mean and 90th percentile intakes in all-user infants were estimated to be 1.91 to 2.28 and 3.00 to 3.86 g/person/day, respectively. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day.

Table D.6.2.2-3 Summary of the Estimated Daily Intakes of 2'-FL from Proposed Combined Use in Infant Formula and Other Foods and Beverages (g/day)

Population Group	Age Group	All-person (or *per capita*) Intake (g/day)		All-users Intake (or consumers only, g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0–5.9 mo	1.10	2.75	57.5	107	1.91	3.00
	6–11.9 mo	2.14	3.86	94.1	160	2.28	3.86
Toddlers	12–35 mo	1.83	2.97	100.0	348	1.83	2.97
Children	3–11 y	1.96	3.53	99.7	1,277	1.97	3.53
Female teenagers	12–19 y	1.47	2.95	94.7	544	1.55	2.95
Male teenagers	12–19 y	1.85	4.16	92.5	526	2.00	4.29
Women of child-bearing age	16–45 y	1.22	2.82	89.9	1,219	1.36	2.87
Female adults	20+ y	1.32	2.96	91.9	2,169	1.44	3.05
Male adults	20+ y	1.59	3.81	86.8	1,842	1.84	3.97
Elderly	65+ y	1.76	3.74	92.8	939	1.90	3.91
Total population	All ages	1.55	3.41	91.2	6,973	1.70	3.54

2'-FL = 2'-fucosyllactose; mo = months; n = number; y = years.

On a body weight basis, the mean and 90th percentile EDIs were 36 and 80 mg/kg body weight/day, respectively, in all-users of all ages (see Table D.6.2.2-4). Of all-users, infants 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs, at 315 and 532 mg/kg body weight/day, respectively.

Table D.6.2.2-4 Summary of the Estimated Daily Intakes of 2'-FL from Proposed Combined Use in Infant Formula and Other Foods and Beverages (mg/kg body weight/day)

Population Group	Age Group	All-person (or *per capita*) Intake (mg/kg body weight/day)		All-users Intake (mg/kg body weight/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0–5.9 mo	181	477	57.5	107	315	532
	6–11.9 mo	244	441	94.1	160	259	447
Toddlers	12–35 mo	148	243	100.0	346	148	243
Children	3–11 y	75	147	99.7	1,268	76	147
Female teenagers	12–19 y	24	52	94.7	536	26	52
Male teenagers	12–19 y	29	67	92.5	524	31	67
Women of child-bearing age	16–45 y	18	42	89.9	1,209	20	43
Female adults	20+ y	19	42	91.9	2,156	20	43
Male adults	20+ y	19	46	86.7	1,833	22	48
Elderly	65+ y	24	53	92.6	928	26	54
Total population	All ages	32	76	91.1	6,930	36	80

2'-FL = 2'-fucosyllactose; mo = months; n = number; y = years.

These EDIs are within safe intake levels as described in Section C of this application dossier. This exposure assessment was conducted based on the assumption that APTech's 2'-FL would only replace 2'-FL that was currently available on the U.S. market at the time of filing with the U.S. FDA and was not anticipated to alter cumulative exposures. In addition, it is important to note that these EDIs are amplified estimates since it is unlikely that 2'-FL will be used at the maximum levels for all intended use food categories.

E. INFORMATION RELATED TO THE NUTRITIONAL IMPACT OF A NUTRITIVE SUBSTANCE OTHER THAN VITAMINS OR MINERALS

Inclusion of *C. glutamicum* (strain APC199) as a permitted source of 2'-FL preparation *via* fermentation is not expected to significantly alter the nutritional value of infant formula products that already contain 2'-FL. Multiple 2'-FL ingredients produced from fermentation are already approved for use in infant formula products in Australia and New Zealand, and the use of APTech's 2'-FL ingredient is expected to be fully substitutional to other 2'-FL ingredients currently on the market.

F. INFORMATION RELATED TO THE POTENTIAL IMPACT ON CONSUMER UNDERSTANDING AND BEHAVIOUR

No changes in consumer understanding and behaviour are to be expected in response to the proposed modifications to the Code, as multiple 2'-FL ingredients produced from fermentation are already approved for sale in infant formula products in Australia and New Zealand.

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