APPLICATION FOR THE APPROVAL OF 2'-FUCOSYLLACTOSE (2'-FL) FROM *ESCHERICHIA COLI* BL21(DE3) AS A SUBSTANCE USED FOR A NUTRITIVE PURPOSE TO BE INCLUDED IN THE AUSTRALIA AND NEW ZEALAND FOODSTANDARDS CODE

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Application for the Approval of 2'-Fucosyllactose (2'-FL) from *Escherichia coli* BL21(DE3) as a Substance Used for a Nutritive Purpose to Be Included in the *Australia and New Zealand Food Standards Code*

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Application for the Approval of 2'-Fucosyllactose (2'-FL) from *Escherichia coli* BL21(DE3) as a Substance Used for a Nutritive Purpose to Be Included in the *Australia and New Zealand Food Standards Code*

INTRODUCTION

This application has been prepared in accordance with the information requirements outlined in the relevant sections of the *Food Standards Australia New Zealand Application Handbook* (updated 1 July 2024), specifically:

- *Chapter 3.1 - General Requirements for Applications (all sections)*
- *Guideline 3.3.2 - Processing Aids (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 Product (all sections)*

GENERAL REQUIREMENTS

In accordance with **Section 3.1 - General Requirements** of the *Food Standards Australia New Zealand Application Handbook* (updated 1 July 2024), the following general information is provided:

1. *Format of the application*
2. *Applicant details*
3. *Purpose of the application*
4. *Justification for the application*
5. *Information to support the application.*
6. *Assessment procedure*
7. *Confidential commercial information*
8. *Other confidential information*
9. *Exclusive capturable commercial benefit*
10. *International and other national standards*
11. *Statutory declaration*
12. *Checklist*

Each point is addressed in the following subsections

1 Form of the Application

This application is in English, and prepared according to relevant format requirements.

2 Application Details

(a) applicant (individual or organisation's) name: Suzhou Yixi Biotech Co., Ltd.
(b) name of contact person: [redacted]
(c) address (street and postal): 2.5 Industrial Park, No. 88 Dongchang Road, Suzhou, 215000 China
(d) telephone number: [redacted]
(e) email address: [redacted]
(f) nature of applicant's business: Supplier of food ingredients

3 Purpose of the application

The applicant, Suzhou Yixi Biotech Co., Ltd. (Yixi) submit this application to Food Standards Australia New Zealand (FSANZ) seeking approval for the use of 2'-fucosyllactose (2'-FL), a purified human milk oligosaccharide (HMO) ingredient, produced via fermentation of *Escherichia coli* EC102 (genetically modified strain of *Escherichia coli* BL21(DE3) encoded with the gene for α-1,2-fucosyltransferase from *Akkermansia muciniphila*), in infant formula products as a nutritive substance.

At present, several applications for the use of 2'-FL in Australia and New Zealand have been submitted to FSANZ and get approved (specifically, Applications A1155, A1190, A1233, A1277 and A1283). The permitted 2'-FL is regulated by several Standards and their associated Schedules of the Food Standards Code (the Code), mainly as follows:

- *Standard 1.5.2 - Food Produced Using Gene Technology*
- *Standard 2.9.1 - Infant Formula Products*
- *Schedule 3 - Identity and purity*
- *Schedule 26 - Food produced using gene technology*
- *Schedule 29 - Special purpose foods*

Yixi's 2'-FL is structurally and chemically identical to the 2'-FL ingredients currently permitted in the Code, as well as to the 2'-FL naturally present in human breast milk. Given that the microbial origin of Yixi's 2'-FL can not match the source currently described in the Code, the purpose of this application is to amend *Schedules 3*, *Schedules 26*, and *Schedules 29* to:

- include this *Escherichia coli* BL21(DE3) encoded with the gene for α-1,2-fucosyltransferase from *Akkermansia muciniphila* as a source for 2'-FL; and
- include a specification for this source of 2'-FL.

Yixi is NOT request any change to the food categories and use levels of 2'-FL (only be added to infant formula products at a maximum use level of **96 mg/100 kJ**, equivalent to **2.4 g/L**) as permitted following FSANZ's current assessments of 2'-FL.

4 Justification for the application

Breastfeeding remains the globally recommended and promoted gold standard for infant nutrition. Human milk contains hundreds of bioactive compounds, including unique oligosaccharides found exclusively in human milk - known as human milk oligosaccharides (HMOs). Among these, 2'-FL is one of the most abundant HMOs to which the majority of infants have been naturally exposed. The inclusion of 2'-FL in infant formula helps achieve a composition that more closely resembles that of human milk.

As demonstrated in this Application, Yixi's 2'-FL:

- is chemically and structurally identical to naturally occurring 2'-FL in human milk, which has been shown to provide optimal nutritional and immunological benefits with a documented history of safe infant consumption;
- is produced via microbial fermentation using a recombinant *Escherichia coli* BL21(DE3) strain, which is not currently approved as a permitted source of 2'-FL. Comprehensive data for the production microorganism have been provided to support its safety;
- is verified to be free from endotoxins, residual recombinant DNA, and viable cells of production strain;
- is not seeking any change to the use conditions currently permitted. Thus, the safety conclusions derived from the previous assessments (specifically, Applications A1155, A1190, A1233, A1277 and A1283) are applicable to Yixi's 2'-FL.

The approval and introduction of Yixi's 2'-FL is projected to provide substantial advantages for both consumers and manufacturers in Australia and New Zealand by facilitating the development of premium infant formula that better replicates human milk composition. This regulatory approval would create valuable opportunities for manufacturers to diversify their product offerings, provide consumers with enhanced nutritional choices, and meet the increasing market demand for authentic HMO ingredients in infant nutrition products.

The Australia-New Zealand region offers significant commercial opportunities for domestic infant formula production and export markets, particularly Southeast Asia and China. Yixi is strategically positioned to leverage these market prospects with its 2'-FL product.

4.1 Regulatory impact information

It is well-notified and accepted that the requested permission for Yixi's 2'-FL for use as a nutritive substance in infant formula products applies to **Australia only**.

4.1.1 Costs and benefits of the application

- **For consumer:** This application is not expected to impose any additional economic costs on consumers. Consumers may benefit from foods containing greater availability of 2'-FL through the approval of an additional source organism. The introduction of Yixi's 2'-FL as nutritive substances in infant formula is not expected to have any negative impact on the consumers.

- **For industry:** This application is not expected to impose any additional economic costs on industry. Food manufacturers may benefit from the availability to source 2'-FL from an alternative supplier.
- **For government:** This application is not expected to impose any additional economic costs on governments beyond the normal costs of ensuring compliance with food laws.

4.1.2 Impact on international trade

This application will align Australian and New Zealand with the United States and China, which permit the addition of Yixi's 2'-FL to food products. This has the potential to enhance international trade in respect of both the import and export of fortified food products.

5 Information to support the application

This application is prepared in accordance with the relevant sections within the *Food Standards Australia New Zealand Application Handbook* (updated 1 July 2024), including the following:

- *Chapter 3.1 - General Requirements for Applications (all sections)*
- *Guideline 3.3.2 - Processing Aids (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 Product (all sections)*

In addition, Yixi also has provided the following information as supporting documents:

Annex 1 - Statutory Declaration

Annex 2 - Checklist

Annex A.1 - Report on Molecular Characteristics of Recombinant *Escherichia Coli* Strain EC102 [Confidential]

Annex A.2 - Report on Gene Manipulation in Recombinant *Escherichia Coli* Strain EC102 [Confidential]

Annex A.3 - Integration Stability of Target Gene in Recombinant *Escherichia Coli* Strain EC102 [Confidential]

Annex A.4 - Transcription Level Stability of Target Gene in Recombinant *Escherichia Coli* Strain EC102 [Confidential]

Annex A.5 - Growth Rate Stability of Recombinant *Escherichia Coli* Strain EC102 [Confidential]

Annex A.6 - 2'-FL Yield Stability [Confidential]

Annex A.7 - Detection Method of Residual Exogenous Genes in the Final 2'-FL Product

Annex A.8 - CoA for Residual Exogenous DNA and Proteins in the Final 2'-FL Product [Confidential]

Annex A.9 - Detection Method of Residual Exogenous Protein in the Final 2'-FL Product - Coomassie Brilliant Blue Method

Annex A.10 - CoA for Residual Viable Cells of the Production Strain in the Final 2'-FL Product [Confidential]

Annex A.11 - Bioinformatics Analysis for Toxicity and Anti-nutrition of Exogenous Proteins [Confidential]

Annex A.12 - Bioinformatics Analysis for Allergenicity of Exogenous Proteins [Confidential]

Annex A.13 - Species Identification Report [Confidential]

Annex B.1 - Chemical Structure Verification [Confidential]

Annex B.2 - Stability Studies on Yixi's 2'-FL (Third-party) [Confidential]

Appendix B.2-1 - CoA for Stability of Yixi's 2'-FL (third-party) [CONFIDENTIAL]

Appendix B.2-2 - CoA for Stability of Yixi's 2'-FL in Food Matrice (third-party) [CONFIDENTIAL]

Annex B.3 - Stability Studies on Yixi's 2'-FL (In-house) [Confidential]

Annex B.4 - Test Method for Determining the Content of 2'-FL in Milk Powder

Annex B.5 - Validation Report of the Test Method for Determining the Content of 2'-FL in Milk Powder

Annex B.6 - Manufacturing Process [Confidential]

Appendix B.6-1 - ISO 22000 certificate [Confidential]

Appendix B.6-2 CoAs for Raw Materials [Confidential]

Annex B.7 - 3-Batch Analyses [Confidential]

Appendix B.7 - CoA for 3-Batch Analysis of Yixi's 2'-FL [Confidential]

Annex B.8 - Determination of 2'-Fucosyllactose, D-lactose, and Difucosyllactose

Annex B.9 - Validation Report of the Methods for Determination of Contents of 3-Fucosyllactose

Annex B.10 - CoA for Acute Oral Toxicity Study of Yixi's 2'-FL [Confidential]

Annex B.11 - CoA for 90-Day Subchronic Oral Toxicity Study of Yixi's 2'-FL [Confidential]

Annex B.12 - CoA for Genotoxicity Studies of Yixi's 2'-FL [Confidential]

Annex B.13 - CoA for Teratogenicity Study of Yixi's 2'-FL [Confidential]

6 Assessment procedure

2'-FL has been already assessed comprehensively in its safety and benefit by FSANZ and is approved in *the Code*. This application is not seeking any changes to the currently use condition of 2'-FL, consequently, the General Procedure, level 1 or 2, is considered the appropriate procedure to be adopted in assessing this application.

7 Confidential commercial information (CCI)

Yixi requests the information contained within the following Appendices and Annexes be considered confidential commercial information (CCI):

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The above CCI has been developed with significant financial resources and time investment by Yixi, which is not expected to be made public. The release of CCI would incur a significant negative commercial impact for Yixi and may provide commercial advantage to competitors.

Non-confidential summaries are provided in the relevant section in this dossier.

8 Other confidential information

The document ***DECLARATION [Confidential]*** is a data sharing statement and is requested to remain confidential aside from what has been outlined in the section above.

9 Exclusive capturable commercial benefit (ECCB)

Given the nature of Yixi's production strain technology, it can be reasonably expected that only Yixi will commercially benefit from the inclusion of *Escherichia coli* BL21(DE3) encoded with the gene for α-1,2-fucosyltransferase from *Akkermansia muciniphila* 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.

The following questions listed in the Application Handbook have been addressed.

Factors Considered	Response
Why are you making this application? **What are you hoping to get out of its approval?**	There is a profitable/viable market in Australia and New Zealand for the manufacturing of infant formula products for use in Australia and New Zealand, as well as for export to other countries, specifically in South East Asia and China. Furthermore, there is an increasing public interest in high quality infant formula with a composition more similar to human milk. Australia and New Zealand manufacturers of infant formula are generally recognised by their higher quality products that are attractive to the Asian markets, as well as the local market. The conferring of an exclusive permission will allow Yixi to market to manufacturers of infant formula in Australia and New Zealand and to establish early access to these markets relative to other competitors. This may result in greater returns on the investment that these infant formula manufacturers have committed to by developing the new formula products.
How will you benefit from the approval of your application?	If ECCB is conferred on Yixi's 2'-FL, it provides Yixi the opportunity to market 2'-FL to infant formula manufacturers in Australia and New Zealand, and gain access to a broad number of Asian markets with increasing competitiveness.
Who besides you, will benefit from the approval of your application? How and why will they benefit?	Yixi's business partners (i.e. manufacturers purchasing Yixi's 2'-FL) will benefit from potentially higher sales and greater market shares by manufacturing higher-quality products in the global market.
If your application is approved, whose permission will be required before anyone can derive a benefit from that approval?	As the applicant and producer/supplier (only produce according to the technology and specifications described in the application), Yixi will provide the permission to its partners to market and sell 2'-FL. Permission will be provided in form of specific supply agreements before anyone else can benefit financially.
Who holds the intellectual property in the subject matter of your application?	Yixi holds the intellectual property rights for the information relating to Yixi's 2'-FL of this application.

10 International and other national standards

10.1 International Standards

There is no Codex Alimentarius Commission (Codex) Standard that explicitly names the use of 2'-FL as an ingredient in foods. However, the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants states “other ingredients may be added in order to provide substances ordinarily found in human milk” (CXS 72-1981-Section 3.2.1).

10.2 Other national standards or regulations

The national and international standards that are relevant to the current application or approval of Yixi's 2'-FL are listed below with regulatory process summary, respectively.

10.2.1 China

In China, 2'-FL is regulated as food additive (sub-category: nutritional fortifier).

Under the *Administrative Measures on New Food Additives* released in 2010, to used a new food additive in in China, applicants shall submit new food additive application to the National Health Commission of P.R. China (NHC) and assessed by China National Center for Food Safety Risk Assessment (CFSA). Only after the new food additive has passed the safety evaluation by the CFSA, can it be used in food production.

The following items are commonly required to be submitted to NHC:

- Information about the applicant;
- Information proving technical necessity and effectiveness of use;
- Specification and detection methods;
- Description of production process (including quality and safety standards for raw materials);
- The safety report of the production microorganism;
- CoAs of structural verification, compositional analyses, stability studies, and toxicological studies.

Several 2'-FL produced from different production strains have passed the the technical review by CFSA and are permitted to be used as nutritional fortifier with **0.7-2.4 g/L**, limited to the following food categories: milk powder (for children only), **infant formula**, **formula for older infants and toddlers**, and **infant formula for special medical purposes**.

10.2.2 United States

In U.S., substances added to food need to be approved as either a food additive or regarded by the United States Food and Drug Administration (US FDA) as Generally Recognized as Safe (GRAS).

For a substance to be recognized as GRAS, there must be a consensus among qualified experts that the scientific data and information support the safety of the substance under the conditions of intended use. Manufacturers apply for GRAS status by providing comprehensive supporting documentation of safety to the US FDA for their evaluation. Decision of no objections is usually given as “no question”.

In 2024, Yixi composed a safety dossier and convened a panel of experts that are suitably qualified by scientific training and experience to evaluate the safety of substances added to food (the Panel). The Panel independently and critically evaluated the identity, manufacturing process, specifications, estimated dietary exposure and published information supporting the safety of Yixi's 2'-FL and concluded that Yixi's 2'-FL is self-determined to be GRAS under the conditions of intended use (in **non-exempt infant formula** for term infants at a maximum use level of **2.4 g/L** as consumed).

The complete safety dossier was submitted to the U.S. FDA in February 2025 and currently awaiting FDA's response.

10.2.3 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** (**3,0 g/L** in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer)
- **follow-on formula** (**3,64 g/L** in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer)
- food supplements (3,0 g/day for general population, 1,2 g/day for young children)

Yixi's 2'-FL is preparing for EU novel food application following the procedure described in *Administrative guidance for the preparation of novel food applications in the context of Article 10 of Regulation (EU) 2015/2283* and *Guidance on the scientific requirements for an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283*.

A summary of 2'-FL approved in other country/region currently (March, 2025) is listed below. Details of the approved specification are summarized in ***Section B.2.5.***

Jurisdiction	Authority	Approval	Approval date	Production strain
Australia and New Zealand	FSANZ	A1155	2019.12.20	*Escherichia coli* K-12
		Review for A1155	2020.11.27	
		A1190	2021.11.08	*Escherichia coli* BL21
		A1233	2022.05.06	*Escherichia coli* K-12
		A1277	2024.05.31	*Escherichia coli* K-12
		A1283	2024.06.19	*Corynebacterium glutamicum*
European Union	EFSA	Decision 2016/376	2016.03.11	*Escherichia coli* K-12

Jurisdiction	Authority	Approval	Approval date	Production strain
		Regulation 2017/2201	2017.11.27	*Escherichia coli* BL21
		Regulation 2023/859	2023.04.26	*Corynebacterium glutamicum* ATCC 13032
		Regulation 2024/2036	2024.07.29	*Escherichia coli* W (ATCC 9637)
United States	FDA	GRN No. 546	2015.09.16	/
		GRN No. 571	2015.11.06	*Escherichia coli* BL21 (DE3) #1540
		GRN No. 650	2016.11.23	*Escherichia coli* K-12 DH1
		GRN No. 735	2018.04.06	*Escherichia coli* K-12 GI724
		GRN No. 749	2018.04.23	*Escherichia coli* K-12 MG1655
		GRN No. 852	2019.11.15	*Escherichia coli* LU20297
		GRN No. 897	2020.06.12	*Escherichia coli* K-12 INB000846
		GRN No. 929	2021.02.26	*Escherichia coli* BL21 (DE3)
		GRN No. 932	2021.02.18	*Corynebacterium glutamicum* KCTC 13735BP
		GRN No. 1014	2022.07.15	*Escherichia coli* BL21(DE3)
		GRN No. 1034	2022.10.21	Escherichia coli K-12 DH1 MDO
		GRN No. 1051	2023.11.21	*Escherichia coli* W ATCC 9637
		GRN No. 1060	2023.04.04	*Escherichia coli* K-12 DH1 MDO
		GRN No. 1091	2023.12.01	*Escherichia coli* K-12 MG1655
		GRN No. 1157	2024.08.07	*Escherichia coli* BL21 (DE3)
China	NHC	NHC Announcement 2023 No.8	2023.10.08	*Escherichia coli* K-12 DH1 MDO
				Escherichia coli K-12 MG1655
				Escherichia coli BL21 (DE3)
		NHC Announcement 2024 No.2	2024.03.13	*Escherichia coli* BL21 (DE3)
		NHC Announcement 2024 No.3	2024.08.05	*Escherichia coli* BL21 star (DE3)
				Corynebacterium glutamicum ATCC 13032

Jurisdiction	Authority	Approval	Approval date	Production strain
		NHC Announcement 2024 No.5	2024.10.10	*Escherichia coli* K-12 GI724
		NHC Announcement 2024 No.6	2024.12.13	*Escherichia coli* BL21 (DE3)
				Escherichia coli BL21 (DE3)
		NHC Announcement 2025 No.1	2025.02.10	*Escherichia coli* BL21 (DE3)
				Escherichia coli W
Canada	Health Canada	Completed safety assessments of novel foods	2018.12.05	*Escherichia coli* BL21 (DE3) Strain #1540
			2021.06.09	Escherichia coli BL21 (DE3) Strain #1242
			2021.11.03	*Escherichia coli* K12 MG1655 strain (sINB000846)
			2022.07.22	*Escherichia coli* K-12 (DH1) MDO MAP1001d strain
EFSA = The European Food Safety Authority FDA = The United States Food and Drug Administration NHC = National Health Commission of the P.R.China				

11 Statutory Declaration

A signed Statutory Declaration is provided in **Annex 1**.

12 Checklist

Completed checklists relating to the information required for submission with this application are provided in **Annex 2**.

A REQUIREMENTS OF PROCESSING AIDS

In accordance with **Section 3.3.2 - Processing Aids** of the *Food Standards Australia New Zealand Application Handbook* (updated 1 July 2024), the following technical information is provided:

1. Additional information related to the safety of an enzyme processing aid derived from a microorganism (Section 3.3.2 - Part D of the Handbook);
2. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism (Section 3.3.2 - Part E of the Handbook).
3. Information on the potential toxicity of the enzyme processing aid (Section 3.3.2 - Part C.2 of the Handbook);
4. Information on the potential allergenicity of the enzyme processing aid (Section 3.3.2 - Part C.3 of the Handbook).

Each point is addressed in the following subsections.

A.1 Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism

A.1.1 Information on the Source Microorganism

Yixi's 2'-FL is produced through microbial fermentation using the recombinant *Escherichia coli* strain EC102, which is derived from *Escherichia coli* BL21(DE3).

A.1.1.1 Taxonomic status on the source microorganism

The host strain (source microorganism) is classified within the *Escherichia coli* species, identical to those described in A1155, A1190, A1233, and A1277. Its taxonomic classification is as follows:

Kingdom: *Bacteria*
Phylum: *Proteobacteria*
Class: *Gammaproteobacteria*
Order: *Enterobacterales*
Family: *Enterobacteriaceae*
Genus: *Escherichia*
Species: *Escherichia coli*
Stain: *Escherichia coli* BL21(DE3)

A.1.1.2 Origin of the source microorganism

Escherichia coli BL21(DE3), belonging to *Escherichia coli* strain B, is a gram-negative *Brevibacterium* with blunt round ends.

In 1885, Theodor Escherich first described in detail the microscopic characteristics of *Escherichia coli* in

the feces of breastfed infants, hence the name *Escherichia coli*. *Escherichia coli* is widely distributed in nature, from soil and air to animal intestines. It is an important bacterial species living in the large intestine of warm-blooded animals (including birds and mammals) and plays an important role in the normal digestion of food.

Escherichia coli strain B was separated from a human gut symbiont by D'Herelle in 1918 (Daegelen *et al.*, 2009), which is one of the four *Escherichia coli* laboratory strains, and the other three are *Escherichia coli* K12, *Escherichia coli* W, and *Escherichia coli* C. Due to the absence of adverse effects on humans and mammals and ability to colonize in human intestine (Bauer *et al.*, 2008), these four models of *Escherichia coli* strains are classified as laboratory microorganisms with safety level I (Archer *et al.*, 2011).

As a protease-deficient strain of *Escherichia coli* strain B, *Escherichia coli* BL21 is commonly used to express heterologous proteins. DE3, a DNA fragment that is inserted into the *Escherichia coli* genome and stably inherited, is mainly composed of the promoter of lactose operon, operator gene, and T7 phage RNA polymerase-encoding gene. Under the induction of lactose or isopropyl β-D-1-thiogalactopyranoside (IPTG), T7 phage RNA polymerase can be expressed, which specifically recognizes the reading frame behind the T7 phage promoter on the pET32a (+) vector and transcribes it in large quantities.

In 1986, researchers constructed *Escherichia coli* BL21 (DE3) through multiple rounds of gene manipulations including UV mutagenesis, introduction of methionine-deficient genetic element, and integration of T7 phage RNA polymerase gene (Daegelen *et al.*, 2009).

The pedigree chart of the construction process is shown in ***Figure A.1.1.2***.

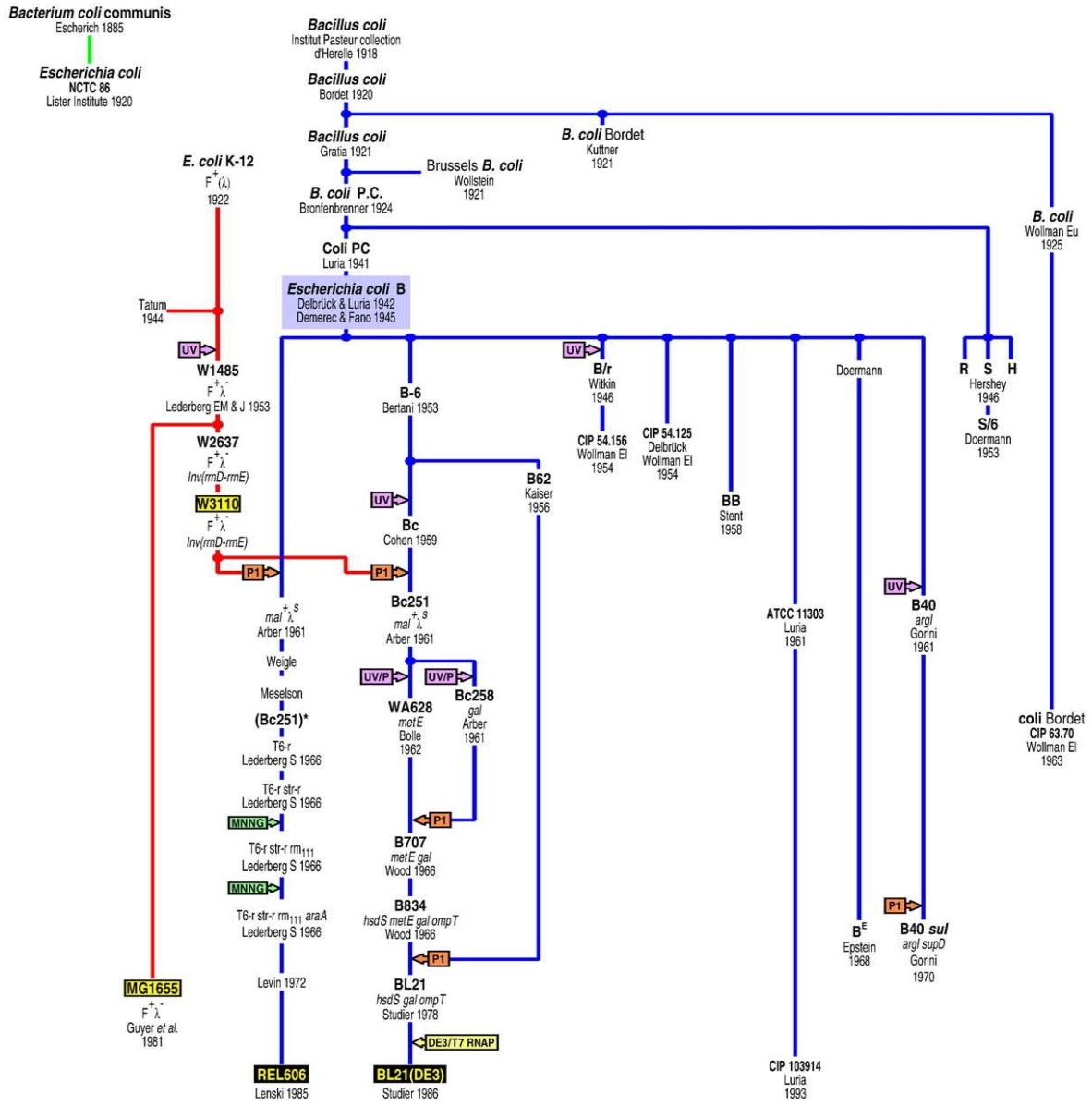


Figure A.1.1.2 **Pedigree chart of *Escherichia coli* BL21 (DE3)**

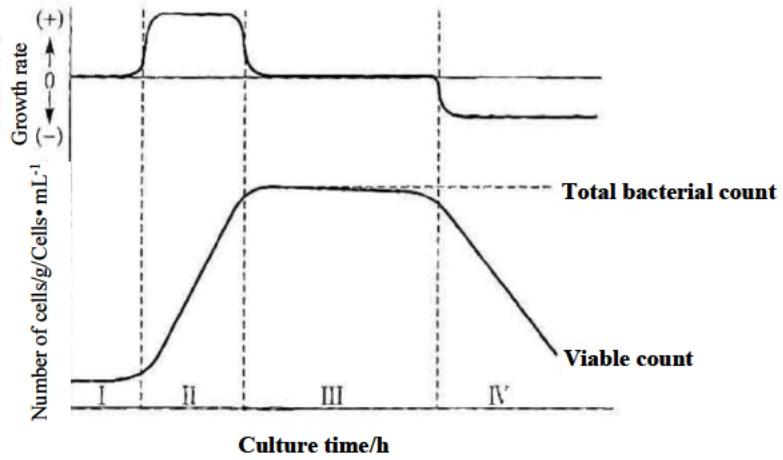
The whole-genome sequence of *Escherichia coli* BL21 (DE3) is available at: https://www.ncbi.nlm.nih.gov/nuccore/CP001509.3?report=fasta#opennewwindow

A.1.1.3 Biological characteristics of the source microorganism

A.1.1.3.1 Multiplication period and generation time

The generation time of *Escherichia coli* is 20 minutes. Theoretically, under optimal growth conditions, a single *Escherichia coli* cell will multiply and reach a cell density of over 1 billion after 10 hours, and the number of bacteria will be huge and beyond counting after 24 hours. But in fact, due to the consumption of nutrients, the accumulation of toxic products, and the change in environmental pH, bacteria can't keep multiplying at the same rate forever. After a certain period, the active proliferation rate of bacteria gradually slows down, with the number of dead bacteria gradually increasing and the viable rate gradually decreasing.

When *Escherichia coli* cells are cultured in a certain amount of liquid medium under appropriate conditions (e.g., culture temperature, oxygen, and pH), the size and number of the cells will change regularly with time. The growth curve of *Escherichia coli* shows its population growth, and the growth and reproduction of *Escherichia coli* cells involve changes in cell volume and number. A typical *Escherichia coli* growth curve is shown in ***Figure A.1.1.3.1***, which is divided into four phases.



I Lag phase II Exponential phase III Stationary phase IV Death phase

Figure A.1.1.3.1. **Schematic diagram of a typical growth curve of *Escherichia coli***

A.1.1.3.2 Reproductive mode and capability

Escherichia coli typically reproduces by binary fission and divides along the transverse axis. *Escherichia coli* exhibits vigorous metabolism and rapid reproduction rate, with a generation time of 20 minutes.

When an *Escherichia coli* cell divides, it elongates first, and genome duplication occurs. *Escherichia coli*, a gram-negative bacterium, has no mesosome. When it divides, the genome is attached directly to the cell membrane, and the newly duplicated genome is attached to a nearby point. A new cell membrane is formed between the two points, separating the two clusters of genomes to the two ends. Finally, the cell wall invaginates along the diaphragm, and the whole cell divides into two daughter cells.

Theoretically, under optimal growth conditions, a single *Escherichia coli* cell can multiply and reach a cell density of over 1 billion in 10 hours, and the number of bacteria will be huge and beyond counting after 24 hours. But in fact, due to the consumption of nutrients, the accumulation of toxic products, and the change in environmental pH, bacteria can't keep multiplying at the same rate forever. After a certain period, the active proliferation rate of bacteria gradually slows down, with the number of dead bacteria gradually increasing and the viable rate gradually decreasing.

A.1.1.3.3 Mode, capability, and influencing factors of colonization, survival, spread, and dissemination of Escherichia coli in the environment

Escherichia coli is more resistant to heat than other enterobacteria, and some *Escherichia coli* cells still survive after being heated at 55°C for 60 minutes and at 60°C for 15 minutes. It can survive for weeks to months in natural water and longer in feces at lower temperatures. Bile salts and brilliant green can inhibit *Escherichia coli*.

A.1.1.4 Use history of the source microorganism

Laboratory strains of *Escherichia coli* were among the first organisms for which complete genome sequences were published, and they are widely used in genetics, biochemistry, molecular biology, and systems biology modeling. *Escherichia coli* is in broad usage as a host for gene replication and expression in bioengineering. In the past 30 years, a variety of new strategies and technologies for metabolic engineering have been adopted to design, construct, and optimize cell factories for *Escherichia coli* chemicals, greatly improving the production rate and yield of biologically synthesized chemicals.

Like microorganisms such as *Bacillus subtilis* and yeast, non-pathogenic *Escherichia coli* strains are engineered into cell factories with various functions and uses, allowing for the efficient production of enzymes, intermediates, polyols, organic acids, and antibody drugs. Engineered microorganisms can also be used to catalyze various biological and chemical reactions, and synthesize complex compound molecules that were previously difficult to synthesize, accelerating the innovation and development of new drugs.

Furthermore, *Escherichia coli* has a gene expression regulatory network with a pyramid-shaped hierarchical structure that can manage the autoregulation, co-regulation, and cross-regulation of factors, constituting a complex and sophisticated transcriptional regulatory network. Microorganisms adapt to new tolerance conditions by responding to environmental changes in fast via perturbing and optimizing this efficient regulatory network. Microbial tolerance is a complex phenotype controlled by multiple genes. With the engineering of *Escherichia coli* regulatory factors, the regulatory network can be reconstructed on a large scale and strain tolerance can be significantly improved, which has become a research hotspot in recent years.

In recent years, scholars have engineered *Escherichia coli*, i.e., changing its metabolic pathways through gene knockout and plasmid introduction, thereby producing biochemical products such as a variety of amino acids, virus-like particle vaccines, and protein/peptide biopharmaceuticals in a green and efficient manner. *Escherichia coli* BL21 (DE3) used in this application is currently the most widely used strain for recombinant protein expression.

Escherichia coli strain BL21(DE3) is used worldwide by universities, research institutes, and industrial laboratories, e.g., used extensively in the biopharmaceutical industry for producing recombinant pharmaceutical proteins, and used for producing a variety of food ingredients recognized as GRAS by the U.S. FDA.

A.1.2 Information on the Pathogenicity and Toxicity of the Source Microorganism

Escherichia coli is a normal resident bacterium in animal intestines and does not cause disease under normal living conditions. The pathogenicity of *Escherichia coli* is closely related to the virulence factors it carries, such as adhesins, toxins, pili, and iron transport systems. When bacteria invade the body, the various virulence genes of *Escherichia coli* interact with each other, enabling it to evade and destroy the host's defense mechanism, leading to an inflammatory response in the host.

Currently, there are mainly six internationally recognized categories of *Escherichia coli*, namely,

enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), enteroinvasive *Escherichia coli* (EIEC), enterohemorrhagic *Escherichia coli* (EHEC), enteroaggregative *Escherichia coli* (EAEC), and enteric Shiga-like-toxin-producing *Escherichia coli* (ESIES). In addition, uropathogenic *Escherichia coli* (UPEC) and enteroaggregative adherent *Escherichia coli* (EAggEC) are also included.

However, *Escherichia coli* BL21 (DE3) used in this application is not a pathogenic strain, and its genome sequences do not contain genes encoding virulence factors such as invasion factors, adhesion molecules, and enterotoxins (Jeong *et al.*, 2009), thus having no potential to survive in host tissues or cause diseases.

A.1.3 Information on the Genetic Stability of the Source Microorganism

A.1.3.1 Genetic stability of the source microorganism

Escherichia coli, which is one of the most well-studied microorganisms, has a clear genetic background as well as stable heritability and variability like other microorganisms. Generally, the mutation rate of a microorganism is related to its metabolic rate. The more vigorous the metabolic activity of a microorganism, the faster the mutation rate, and mutations may accumulate during multiple asexual reproductions. Based on this rule, people can create various conditions to make microbial activities in a weak or inactive state, so as to reduce the occurrence of mutation.

The host strain *Escherichia coli* BL21 (DE3) from which the recombinant *Escherichia coli* EC102 is obtained does not contain transposon elements or other mobile genetic elements such as plasmids and episomal vectors, therefore gene transfer through bacterial conjugation will not occur. Even in microbial fermentation, which is carried out in a closed and sterile environment, the strains used are inactivated after batch fermentation, thus the possibility of the genetic material being transferred to other organisms not under the closed fermentation is extremely low.

A.1.3.2 Method and possibility for monitoring the source microorganism

Although *Escherichia coli* BL21 (DE3) is not pathogenic, certain strains of *Escherichia coli* are important indicator bacteria of food contamination and must be strictly controlled during food processing. During years of application, mature detection, control, and sterilization methods have been accumulated.

It is recommended to use peroxide-based bactericides for food sterilization, which are characterized by a fast and strong action that kills all microorganisms. Peroxide-based disinfectants are widely used and can be used as bactericides without drug resistance. In particular, food-grade hydrogen peroxide is a novel, safe, efficient, broad-spectrum disinfectant without “carcinogenic, teratogenic, and mutagenic effects” and is a Class A disinfection product designated by the World Health Organization.

A.2 Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Genetically-modified Microorganism

Yixi developed recombinant *Escherichia coli* strain EC102 for efficient production of 2'-FL by performing several gene manipulations, like gene insertion and gene deletion, in the genome of the host strain *Escherichia coli* BL21 (DE3) using genetic modification technology, whose e safety was assessed.

A.2.1 Information on the Methods Used in the Genetic Modification of the Source Organism

To elucidate the molecular characteristics of recombinant *Escherichia coli* EC102 and describe the genetic modification method, detailed reports have been constructed respectively. Please see **Annex A.1 [Confidential]** and **Annex A.2 [Confidential]**.

A.2.1.1 Genetic manipulations of *Escherichia coli* EC102

The recombinant *Escherichia coli* strain EC102 was developed by performing three gene manipulations (partial knockout of *lacZ* gene, substitution of *wca*KLM gene with exogenous gene cluster *FRS-GRS-CRS-BRS*, and substitution of *wca*J gene with exogenous gene *AKTRS*) in the genome of *Escherichia coli* BL21 (DE3) using the CRISPR/Cas9 technology.

The function, size, and donor organism of the genes introduced or modified in recombinant *Escherichia coli* strain EC102 are shown in the table below.

Table A.2.1.1. **Information of the modified genes of recombinant *Escherichia coli* strain EC102**

Gene	Insertion/Deletion	Function	Size	Donor
lacZ	Knockout	Encode β-galactosidase	1509 bp	Not applicable
wcaKLM	Knockout	Engage in the production of the exopolysaccharide colanic acid	3493 bp	Not applicable
wcaJ	Knockout	UDP-glucose lipid carrier transferase	1457 bp	Not applicable
FRS	Substituting *wca*KLM	Encode GDP-L-fucose synthase	966 bp	*E. coli* K12
GRS		Encode GDP-D-mannose-4,6-dehydratase	1122 bp	*E. coli* K12
CRS		Encode α-D-mannose-phosphate guanylyltransferase	1437 bp	*E. coli* K12
BRS		Encode phosphomannomutase	1371 bp	*E. coli* K12
AKTRS	Substituting *wca*J	Encode α-1,2-fucosyltransferase	903 bp	*Akkermansia muciniphila*

The genetic manipulation process of the recombinant *Escherichia coli* EC102 is summarized as follows:

A.2.1.1.1 Construction of plasmid vector

The target gene was inserted into the plasmid pET28a (+) to obtain *FRS-GRS-CRS-BRS*.pET28a (+). The sgRNA of the gene to be knocked out/substituted/inserted was inserted into the plasmid pTargetF to obtain Δ*lacZ*.pTargetF, *FRS-GRS-CRS-BRS*.pTargetF, and *AKTRS*.pTargetF.

A.2.1.1.2 CRISPR/Cas9 editing

Escherichia coli BL21 (Δ*lacZ*)$^{+}$, BL21(Δ*lacZ*, *FRS-GRS-CRS-BRS*)$^{+}$, and BL21 (Δ*lacZ*, *FRS-GRS-CRS-BRS*, *AKTRS*)$^{+}$ strains were constructed, the plasmid vector was eliminated, and the production strain recombinant *Escherichia coli* EC102 was finally obtained.

A.2.2 Information on the Safety of the Production Microorganism

To confirming the taxonomic identity of the production microorganism, Yixi has conducted the species identification for its production strain from its morphological characteristics, biochemical identification results and gene sequence analysis (please find the report in **Annex A.13 [Confidential]**). The report confirms the taxonomic identity of the production strain as follows:

- **Genus:** *Escherichia*
- **Species:** *coli*

A.2.2.1 Genetic stability studies

A.2.2.1.1 Integration stability of target gene in recombinant *Escherichia Coli* Strain EC102

Yixi established a method of polymerase chain reaction (PCR) & gel electrophoresis for detection of each gene editing site in the genome of 8 generations of recombinant *Escherichia coli* strain EC102 and individual target genes in the exogenous gene cluster. The method can be found in **Annex A.10 [Confidential]**.

The test results showed that the sizes of the PCR products were as expected for all generations. Therefore, all knockout, substitution, or insertion sites in the genome of recombinant *Escherichia coli* strain EC102 were stably inherited in at least 8 generations.

Please find details in **Annex A.3 [Confidential]**.

A.2.2.1.2 Transcription level stability of target gene in recombinant *Escherichia Coli* Strain EC102

Yixi conduct the transcription level study for all exogenous target genes (including *FRS*, *GRS*, *CRS*, *BRS*, and *AKTRS*) in 8 generations of recombinant *Escherichia coli* strain EC102 using reverse transcription-qPCR, where *Escherichia coli* 16S was used as an internal reference.

The study results showed that the transcript levels of all exogenous target genes were within the normal fluctuation range of *Escherichia coli* mRNAs from the 1st- to the 8th-generation. Therefore, the

transcription level of all exogenous target genes of recombinant *Escherichia coli* strain EC102 was stable (CV < 12%) in at least 8 generations.

Please find details in **Annex A.4 [Confidential].**

A.2.2.1.3 Growth rate stability of recombinant *Escherichia Coli* Strain EC102

Yixi determined the absorbance value of microbial suspension of recombinant *Escherichia coli* strain EC102 for 8 consecutive generations after different incubation time under the same conditions, plotted the growth curve, and compared it with the recipient strain *Escherichia coli* BL21 (DE3).

The test results showed that the growth pattern of 8 consecutive generations of recombinant *Escherichia coli* EC102 was the same as that of the recipient strain, and there was no significant difference in the growth characteristics between the production strain and the recipient strain. Under the same culture conditions, the growth trend of 8 consecutive generations of recombinant *Escherichia coli* EC102 generally remained stable, with no significant difference (CV < 15%), indicating that the growth rate was stable.

Please find details in **Annex A.5 [Confidential].**

A.2.2.1.4 2'-FL yield stability

Yixi utilized high-performance liquid chromatography (HPLC) to measure the 2'-FL content produced by the 1st- to 8th-generation strain of recombinant *Escherichia coli* EC102. The analysis result confirmed the stability of 2'-FL production by the recombinant *Escherichia coli* EC102 strain.

Please find details in **Annex A.6 [Confidential].**

A.2.2.2 Residual tests for exogenous DNA, proteins and viable cells of the production strain in the 2'-FL

A.2.2.2.1 Residual exogenous DNA

Yixi established a method based on PCR and gel electrophoresis for the detection of all exogenous target genes (*FRS, GRS, CRS, BRS,* and *AKTRS*) of recombinant *Escherichia coli* EC102. The method as well as an internal 3-batch residual exogenous DNA detection can be found in **Annex A.7**.

A third-party testing facility (CMA-certified*) was commissioned to conduct the residual exogenous DNA detection by this method, and the result showed that no residual DNA fragment was detected in the final 2'-FL product. Please find the CoA in **Annex A.8 [Confidential]**.

**CMA: China Inspection Body and Laboratory Mandatory Approval, is a mandatory certification system established by the Chinese government to regulate the quality of inspections and testing conducted by laboratories and inspection bodies.*

A.2.2.2.2 Residual exogenous proteins

The final 2'-FL product is of extremely high purity. The purification process (including microfiltration

and ultrafiltration) during production can completely remove exogenous proteins.

To verify the absence of residual exogenous proteins, Yixi detected the protein residue in the final 2'-FL product for 3 batches by Coomassie Brilliant Blue method (**Annex A.9**). The detection results showed that no protein residue was detected in the final product (the residue was equal to or lower than the LOD of 17 mg/kg). Please find the CoA in **Annex A.8 [Confidential]**.

A.2.2.2.3 Residual viable cells of the production strain

The production strain, recombinant *Escherichia coli* strain EC102, belongs to the family *Enterobacteriaceae*. So, Yixi entrusted a CMA-certified third-party testing facility to conduct the viable cell residual detection in 3 independent batches of final 2'-FL product in accordance with the Chinese national standards *National Food Safety Standard—Microbiological Examination of Food Hygiene - Examination of Enterobacteriaceae* (GB 4789.41-2016) (Method I). Please find the CoA in **Annex A.10 [Confidential]**.

The results showed that there is no detectable *Enterobacteriaceae* in the final 2'-FL products from 3 batches.

Table 2.2.3.3*. Detection results of *Enterobacteriaceae

Lot No.	Sample No.	Recombinant *Escherichia coli* EC1021
20220708	BJ1531001	ND
20220801	BJ1531002	ND
20220802	BJ1531003	ND

Note: The limit is < 10 CFU/g; ND (not detected) indicates that the count of *Enterobacteriaceae* detected is lower than the limit.

Therefore, it can be considered that there is no detectable recombinant *Escherichia coli* strain EC102 in the final 2'-FL products.

A.3 Information on the potential toxicity of the enzyme processing aid

A.3.1 Information on the Enzyme's Prior History of Human Consumption and its Similarity to Proteins with a History of Safe Human Consumption

As previously stated, residual exogenous proteins, residual exogenous proteins and viable cells of the production strain are confirmed absent in Yixi's final 2'-FL product. Consequently, consumer exposure to microbial processing aids used in Yixi's manufacturing process is not expected to occur.

A.3.2 Information on Any Significant Similarity Between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins

Yixi commissioned a CMA-certified third-party testing facility to conduct bioinformatics analysis on the toxicity and anti-nutrition of exogenous FRS, GRS, CRS, BRS and AKTRS proteins expressed by recombinant *Escherichia coli* EC102 according to the *Food Safety Detection of Genetically Modified Organisms and Derived Products - The Analytical Method of the Toxicity and Anti-nutritional Effect of Foreign Protein by Bioinformatics Tools* (Ministry of Agriculture Announcement No. 2630-16-2017).

The results of bioinformatics analysis of toxicity and anti-nutrition (**Annex A.11 [Confidential]**) showed that when sequence alignment was performed on the test proteins FRS, GRS, CRS, BRS, and AKTRS by protein BLAST in the NCBI, UniProt, and T3DB databases, there was no high sequence similarity between the test proteins and the known toxic proteins or anti-nutritional factors.

A.4 Information on the potential allergenicity of the enzyme processing aid

A.4.1 An Analysis of Similarity Between the Amino Acid Sequence of the Enzyme and that of Known Allergens

Yixi commissioned a CMA-certified third-party testing facility to conduct bioinformatics analysis on the allergenicity of exogenous FRS, GRS, CRS, BRS and AKTRS proteins expressed by recombinant *Escherichia coli* EC102 according to the *Food Safety Detection of Genetically Modified Organisms and Derived Products - The Analytical Method of the Allergenicity of Foreign Protein by Using Bioinformatics Tools* (Ministry of Agriculture Announcement No. 1485-18-2010).

The results of bioinformatics analysis of allergenicity in test proteins FRS, GRS, CRS, BRS, and AKTRS showed (**Annex A.12 [Confidential]**) that (1) no high sequence homology (< 35%) was found in the 80-amino acid sequence alignment; (2) no eight consecutive amino acids were found to be identical. Therefore, the proteins do not have a high sequence homology with known allergens and their potential allergenicity is low.

It should be emphasized that the final 2'-FL product of recombinant *Escherichia coli* EC102 is extremely pure. The final 2'-FL product was verified containing no detectable residual protein, therefore the 2'-FL was also free of all the exogenous proteins (including FRS, GRS, CRS, BRS, and AKTRS). Any potential toxicity, anti-nutrition, or allergenicity of the exogenous proteins has no effect on the final 2'-FL product.

B SUBSTANCES USED FOR A NUTRITIVE PURPOSE

In accordance with **Section 3.3.3 - Substances used for a nutritive purpose** of the Food Standards Australia New Zealand Application Handbook (updated 1 July 2024), the following general information is provided:

1. Information on the use of the nutritive substance (Section 3.3.3 - Part A of the Handbook)
2. Technical information on the use of the nutritive substance (Section 3.3.3 - Part B of the Handbook)
3. Information related to the safety of the nutritive substance (Section 3.3.3 - Part C of the Handbook)
4. Information on dietary intake of the nutritive substance (Section 3.3.3 - Part D of the Handbook)
5. Information related to the nutritional impact of a nutritive substance other than vitamins and minerals (Section 3.3.3 - Part F of the Handbook)
6. Information related to potential impact on consumer understanding and behaviour (Section 3.3.3 - Part G of the Handbook)

Each point is addressed in the following subsections.

B.1 Information on the Use of the Nutritive Substance

FSANZ has previously evaluated the nutritive purpose of 2'-FL as a component in infant formula products under applications A1155, A1190, A1233, A1277, and A1283. It was concluded that the beneficial physiological effects of 2'-FL as a component of infant formula include promoting a healthy intestinal microbiota (specifically, by enhancing a bifidogenic effect), inhibiting the binding of pathogenic strains of *Campylobacter jejuni* to intestinal epithelial cells, and playing a positive role in infant growth and development. And *Schedule 26* of the Code permits the addition of 2'-FL produced by several genetically modified organisms to infant formula products at levels up to 96 mg/100 kJ (2.4 g/L).

In this application, Yixi's intended nutritive purpose for incorporating 2'-FL into infant formula products aligns with the nutritive purpose of 2'-FL previously assessed by FSANZ. Yixi is not requesting any changes to the currently permitted uses or use levels of 2'-FL but seeking permission for the inclusion of a new genetically modified source organism for 2'-FL.

B.2 Technical Information on the Use of the Nutritive Substance

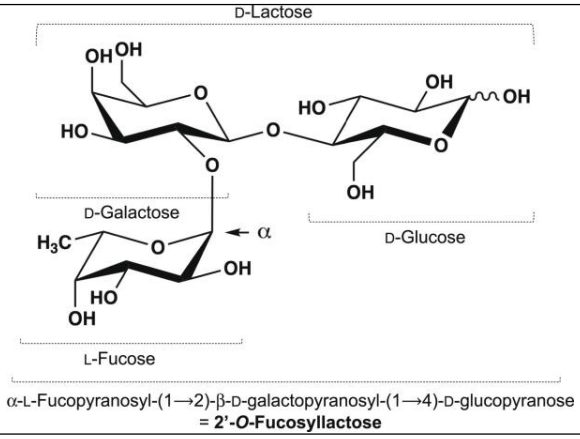
Yixi intends to market 2'-FL for use as a nutritive substance in infant formula products.

Yixi is not requesting any changes to the currently permitted uses or use levels of 2'-FL but seeking permission for the inclusion of a new genetically modified source organism and specification for 2'-FL.

B.2.1 Information to Enable Identification of the Nutritive Substance

2'-Fucosyllactose (2'-FL) is a trisugar composed of L-fucose, D-galactose and D-glucose groups. The monosaccharide L-fucose is linked to the galactosyl group of the disaccharide lactose by the α-1, 2 glycoside bond. The characteristic and chemical structure of 2'-FL is summarized in ***Table B.2.1***.

Table B.2.1. **General Descriptive Characteristics of 2'-FL**

Parameter	Description
Chemical Name	2'-Focusllactose
IUPAC Name	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Synonyms	2'-O-Fucosyllactose; 2'-Fucosidolactose; 2-Fucosyl-D-Lactose 2'-O-L-Fucosyl-D-lactose; Fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc-α-(1→2)-Gal-β-(1→4)-Glc
Abbreviation	2'-FL; 2-FL; 2FL
CAS Number	41263-94-9
Structural Formula	$C_{18}H_{32}O_{15}$
Molecular Weight	488.44 g/mol
Chemical Structure	

B.2.2 Information on the Chemical and Physical Properties of the Nutritive Substance

B.2.2.1 Chemical and Structural Identity of the Nutritive Substance

To confirm the composition characteristics of Yixi's 2'-FL, the chemical structure of 3-batch of Yixi's 2'-FL samples were confirmed against standard 2'-FL isolated from human milk by hydrogen spectrum (^{1}H-MR), carbon spectrum (^{13}C-NMR), DEPT-45°, DEPT-90°, DEPT-135°, two-dimensional spectrum ^{1}H-^{1}HCOSY, two-dimensional spectrum ^{1}H-^{13}CHSQC, two-dimensional spectrum ^{1}H-^{13}CHMBC, high resolution mass spectrometry (HRMS), and liquid-phase mass spectrometry (LC-MS).

A comparative analysis of the above test results demonstrates that the three batches of Yixi's samples are identical with the standard 2'-FL derived from human milk. The full analysis report and the Certificates of Analysis for the standards are provided in **Annex B.1 [CONFIDENTIAL].**

3-batch analysis of Yixi's 2'-FL produced by genetically modified strain *Escherichia coli* EC102 demonstrate that it is a high-purity product (2'-FL ≥ 94%) with low levels of other structurally related saccharides (see ***Section B.2.5.2***).

- **Solubility**

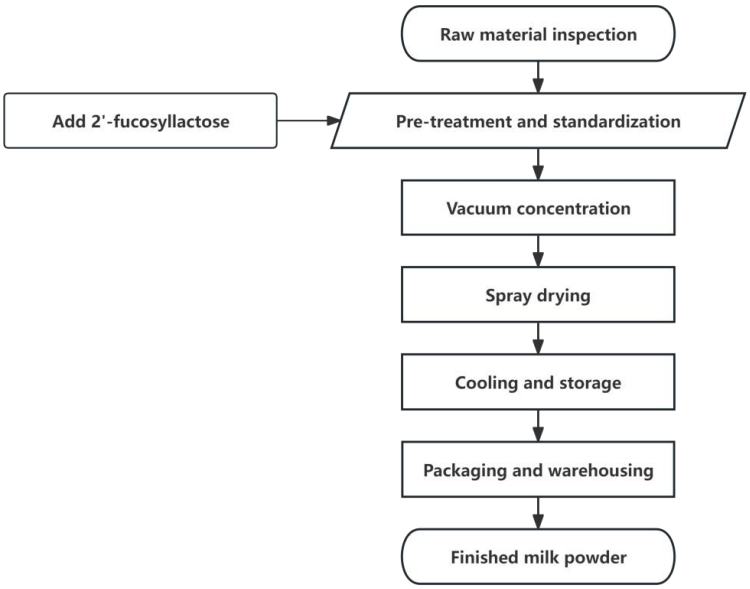
As evaluated by regulatory authorities across multiple jurisdictions—including the European Food Safety Authority (EFSA NDA Panel, 2022; 2023) and the U.S. FDA's GRAS determination panel (FDA, 2021a; 2021b; 2023)—2′-FL has been confirmed to exhibit high water solubility, with measured values ranging from 445 to 716.6 g/L at 20 °C. This solubility profile eliminates the risk of nanoparticle formation, consistent with the EFSA Guidance (EFSA Scientific Committee, 2021).

The high solubility ensures full compatibility of 2′-FL with the matrix of infant formula products. During preparation, 2′-FL dissolves rapidly and completely, enabling its uniform distribution in reconstituted infant formula products. Yixi's 2′-FL has been verified to be structurally identical to the 2′-FL naturally present in human breast milk. Therefore, the solubility properties described above can be equally applicable to Yixi's 2′-FL.

B.2.2.2 The incorporation manner of 2'-FL in the intended uses

This application proposes the use of 2′-FL as a nutritive substance in infant product, including infant formula, follow-on formula and special medical purpose product for infant to compensate for the absence of this component in the aforementioned food categories and align with the nutritional and functional benefits provided by breastfeeding. The proposed use is identical to the nutritive purpose of the 2'-FL ingredients previously assessed by FSANZ.

As a raw ingredient, 2′-FL is incorporated using similar production processes in its intended uses, typically added during the ingredient blending stage. The general production workflow is as follows: raw material inspection → pretreatment and standardization → vacuum concentration → spray drying → cooling and storage → packaging and warehousing → finished milk powder. The flowchart is shown below.



B.2.2.3 Stability

To validate the stability of Yixi's 2′-FL, Yixi carried out a series of stability studies on multiple lots through both a third-party testing facility (CMA-certified) and in-house testing. Including:

- 6-month accelerated storage stability of Yixi's 2'-FL (third-party)
- 15-month accelerated storage stability of Yixi's 2'-FL (in-house)
- 12-month normal storage stability of Yixi's 2'-FL (in-house)
- 6-month accelerated storage stability of Yixi's 2'-FL in infant formula milk powder (third-party)

B.2.2.3.1 6-month accelerated storage stability of Yixi's 2'-FL (third-party)

A 6-month accelerated storage stability on 3-batch Yixi's 2'-FL was conducted by a CMA-certified third-party testing facility. The monitored parameters included physical and chemical indicators, microbiological indicators, 2'-FL content and other components. No increase in microbial contaminants or heavy metal contaminants were reported, and there was no significant alteration in moisture content over the duration of the study.

The study results demonstrate that Yixi's 2'-FL can keep stable throughout the 6-month test period at a temperature of 40℃ and relative humidity (RH) of 75%.

The data are provided in **Annex B.2** **[CONFIDENTIAL]**. The Certificates of Analysis for this study are provided in **Appendix B.2-1** **[CONFIDENTIAL]**.

B.2.2.3.2 Storage stability studies of Yixi's 2'-FL (in-house)

The 2'-FL samples underwent accelerated stability testing at 37 °C ± 2 °C and 75% ± 5% relative humidity for 15 months, alongside ambient storage stability testing for 12 months.

Detailed analysis of the 15-month accelerated and 12-month long-term stability results for three-batch product demonstrated that all sensory (color and physical state), physicochemical (including 2'-FL content), and microbiological parameters remained consistent with baseline (0-month) values. All indicators complied with established specifications, and the purity variation coefficient remained within 1%, confirming product stability.

These results support the conclusion that storage under ambient conditions for up to 24 months does not adversely affect the critical quality attributes of the product.

The full study reports are provided in **Annex B.3** **[CONFIDENTIAL]**.

B.2.2.3.3 Stability of Yixi's 2'-FL in food matrices

Yixi engaged a CMA-certified third-party testing facility to conduct accelerated storage stability on 3-batch infant formula milk powder containing 1.6% Yixi's 2'-FL over a 6-month period. One batch of

2'-FL-free infant formula milk powder served as the blank control. The monitored parameters included 2'-FL content, sensory indicators, and microbiological indicators.

Stability data revealed that Yixi's 2'-FL remains relatively stable for a duration of 6 month at a temperature of 40°C and RH of 75 %.The blank control showed no detectable 2'-FL, confirming that all measured 2'-FL in test samples originated from the new additive rather than endogenous sources.

The data are provided in **Annex B.2 [CONFIDENTIAL]**. The Certificates of Analysis for this study are provided in **Appendix B.2-2 [CONFIDENTIAL].**

The method used to determine the content of 2'-FL in milk powder is provided in **Annex B.4**. A third-party laboratory was engaged to verify the method, please find the report in **Annex B.5**.

As discussed above, Yixi's proposed use of the 2'-FL is identical to the nutritive purpose of 2'-FL ingredients previously assessed by FSANZ. The chemical structure and specification of Yixi's 2'-FL have been verified to be comparable to those 2'-FL permitted in *the Code*. Due to the existence of previously evaluated stability data demonstrating the stability of 2'-FL under the intended conditions, the stability data conducted with Yixi's 2'-FL provided in this section is considered sufficient to support the its stability in other food matrices.

B.2.3 Information on the Impurity Profile

The manufacturing process of Yixi's 2'-FL utilizes various ingredients including sucrose and lactose. Potential metabolic byproducts from the production microorganism during fermentation may include difucosyllactose and lactose. These significant carbohydrate components are effectively controlled through further purification process.

Yixi has established product specifications for the byproducts of 2'-FL. Analysis of 3 production lots of Yixi 2'-FL demonstrate the high-purity of 2'-FL (≥ 94%) as well as the absence of specified carbohydrate compounds (see ***Section B.2.5.2*** and relevant appendices).

The confirmation of absence of viable cells and residual DNA from the production strain have also been conducted in 3 lots (Same batches used for specification analyses). Results from these analyses indicate that there the final 2'-FL product is absent of viable cells and that there is no detectable level of residual DNA (see ***Section A.2.2.3*** and relevant appendices).

With Yixi's 2'-FL characterized to no less than 94.0%, any potential contaminants would constitute only a minor fraction of the final 2'-FL product, representing negligible safety concern.

B.2.4 Manufacturing Process

Yixi's 2'-FL produced by genetically engineered *E. coli* EC102 strain is manufactured according to good manufacturing practices. All raw materials including media components, processing aids, and equipment

used in the manufacture of 2'-FL are food-grade quality sourced from qualified vendors with certificates of analyses.

The manufacturing process of Yixi's 2'-FL includes 2 main steps: fermentation and purification. The manufacturing flow scheme, details of the manufacturing process and the list of raw materials are presented in **Annex B.6 [CONFIDENTIAL]**.

For the safety of the production strain construction, please find details in ***Section A.2.2*** and relevant appendices.

B.2.5 Specification for Identity and Purity

B.2.5.1 Specifications

Considering that the Yixi's 2'-FL produced using genetically modified *E. coli* EC102 strain derived from *E. coli* BL21(DE3) is highly purified with higher 2'-FL content and lesser carbohydrate by-products compared to the current specification for 2'-FL sourced from *E.coli* BL21 in *Schedule 3* of the Code, Yixi is requesting to include the specification for Yixi's 2'-FL into *Schedule 3* or amend the provision of S3-45.

The comparison among the proposed specification of Yixi's 2'-FL, *E.coli* BL21 sourced 2'-FL, and permitted 2'-FL in Australia and New Zealand is presented in ***Table B.2.5.1*** below.

Table B.2.5.1. **Specification comparison between Yixi's 2'-FL, *E.coli* BL21 sourced 2'-FL, and permitted 2'-FL in Australia and New Zealand**

Parameter		Yixi's 2'-FL *E. coli* BL21(DE3)	Australia and New Zealand			China	EU	US (*E.coli* BL21)		
			***E.coli* BL21**	*E.coli* K-12	*C. glutamicum*	***E.coli* BL21**	***E.coli* BL21**	**GRAS 571**	**GRAS 929**	**GRAS 1014**
2'-Fucosyllactose (dried basis)	≥	**94.0%**	**90.0%**	83.0%	94.0%	94.0%	90.0%	90.0%	90.0%	90.0%
D-lactose	≤	**5.0%**	**5.0%**	10.0%	3.0%	3.0%	5.0%	5.0%	5.0%	5.0%
L-fucose	≤	**3.0%**	**3.0%**	2.0%	3.0%	/	3.0%	3.0%	3.0%	3.0%
3-fucosyllactose	≤	**5.0%**	**5.0%**		3.0%	/	5.0%	5.0%	5.0%	5.0%
Difucosyllactose	≤	**5.0%**	**5.0%**	5.0%	2.0%	2.0%	5.0%	5.0%	5.0%	5.0%
2'-fucosyl-D-lactulose	≤	/	/	1.5%	/	/	/	/	/	/
Fucosyl-galactose	≤	/	**3.0%**	/	/	/	3.0%	3.0%	3.0%	3.0%
Glucose	≤	**3.0%**	**3.0%**	/	3.0%	/	3.0%	3.0%	3.0%	3.0%
Galactose	≤	**3.0%**	**3.0%**	/	3.0%	/	3.0%	3.0%	3.0%	3.0%
Sum of saccharides	≥	/	/	90.0%;	/	/	/	/	/	/
pH (20°C, 5% solution)		/	/	3.0-7.5	/	/	/	/	/	/
Water	≤	**9.0%**	**9.0%**	9.0%	9.0%	9.0%	9.0%	9.0%	9.0%	9.0%
Ash, sulphated	≤	**0.5%**	**0.5%**	2.0%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%
Residual proteins	≤	**0.01%**	**0.01%**	0.01%	0.005%	100 mg/kg	0.01%	0.01%	100 µg/g	100 µg/g
Acetic acid		/	/	1.0%	/	/	/	/	/	/
Ethanol	≤	/	/	/	1.000 mg/kg	/	/	/	/	/
Lead	≤	**0.02 mg/kg**	**0.02 mg/kg**	/	0.02 mg/kg	0.05 mg/kg	0.02 mg/kg	0.02 mg/kg	0.02 mg/kg	0.02 mg/kg
Arsenic	≤	**0.2 mg/kg**	**0.2 mg/kg**	/	0.03 mg/kg	0.2 mg/kg	0.2 mg/kg	0.2 mg/kg	0.2 mg/kg	0.2 mg/kg
Cadmium	≤	**0.1 mg/kg**	**0.1 mg/kg**	/	0.01 mg/kg	/	0.1 mg/kg	0.1 mg/kg	0.1 mg/kg	0.1 mg/kg
Mercury (Hg)	≤	**0.5 mg/kg**	**0.5 mg/kg**	/	0.05 mg/kg	/	0.5 mg/kg	0.5 mg/kg	0.5 mg/kg	0.5 mg/kg

Parameter	Yixi's 2'-FL *E. coli* BL21(DE3)	Australia and New Zealand			China	EU	US (*E.coli* BL21)		
		***E.coli* BL21**	*E.coli* K-12	*C. glutamicum*	***E.coli* BL21**	***E.coli* BL21**	**GRAS 571**	**GRAS 929**	**GRAS 1014**
Salmonella	**Absent/100 g**	**Absent/100 g**	/	/	Absent/25 g	Absent/100 g	Absent/100 g	Absent/100 g	Absent/100 g
Total plate count ≤	**10^4 CFU/g**	**10^4 CFU/g**	3000 CFU/g	500 CFU/g	500 CFU/g	10^4 CFU/g	10^4 CFU/g	10^4 CFU/g	10^4 CFU/g
Enterobacteriaceae	**< 10 CFU/g**	**Absent/11 g**	/	/	< 10 CFU/g	Absent/11 g	Absent/11 g	Absent/11 g	Absent/11 g
Califorms	/	/	/	/	< 10 CFU	/	/	/	/
Cronobacter sakazakii	**Absent/100 g**	**Absent/100 g**	/	/	/	Absent/100 g	Absent/100 g	Absent/100 g	Absent/100 g
Yeast ≤	**100 CFU/g**	**100 CFU/g**	100 CFU/g	100 CFU/g	/	100 CFU/g	100 CFU/g	100 CFU/g	100 CFU/g
Mould ≤			100 CFU/g		/				
Aflatoxin M_1 ≤	**0.025 μg/kg**	**0.025 μg/kg**	/	0.025 μg/kg	/	0.025 μg/kg	0.025 μg/kg	0.025 μg/kg	0.025 μg/kg
Endotoxins ≤	**10 EU/mg**	**10 EU/mg**	10 EU/mg	10 EU/mg	10 EU/mg	10 EU/mg	300 EU/g	300 EU/g	300 EU/g
GMO detection	/	**Not detected**	/	/	/	/	Negative	Negative	≤ 0.01%

- **Justification for increasing 2'-FL content to 94%**

a. Global regulatory precedents

- EU: Regulation (EU) 2023/859 recognizes 2'-FL of purity ≥94% as technologically achievable and safe;
- USA: FDA issued "no question" letter to GRN 897, whose 2'-FL content is limited as no less than 96%, demonstrating that higher purity is internationally achievable and recognized;
- China: China requires the purity of 2'-FL be no less than 94% from the very start when 2'-FL got approved in 2023.

With advancements in purification process technology, global regulators are converging on higher purity (94–96%) standards for 2'-FL driven by technological capabilities. Yixi believes that a higher purity of the primary component (2'-FL) will not raise any safety concern but be in line with international trend and deliver a superior product for consumers.

b. Safety equivalence

FSANZ has previously evaluated the safety of 2'-FL under the currently permitted conditions of use (specifically, applications A1155, A1190, A1233, A1277, and A1283). 2'-FL) with purity not less than 94% has been recognized as safe for current use condition. In this application, Yixi is not requesting any changes to the currently permitted uses or use levels of 2'-FL. Therefore, the safety conclusions regarding 2'-FL established in previous applications remain applicable to Yixi's 2'-FL (purity ≥94%).

- **Justification for exclusion of fucosyl-galactose**

Based on 3-batch composition analyses (***Section B.2.5.2***), Yixi's 2'-FL product currently demonstrates a near-100% composition. As previously noted, while 90% purity meets established safety standards, the achieved 94% purity level provides additional risk mitigation against impurities. Consequently, inclusion of fucosyl-galactose in Yixi's 2'-FL specifications is deemed unnecessary.

- **Justification for exclusion of GMO detection**

a. The absence of viable strain residues has been verified in 3-batch analysis

As described in ***Section A.2.2.3***, Yixi's 2'-FL has been verified that no viable cells or DNA remains in the final 2'-FL product. The current specifications comprehensively address microbial safety through stringent parameters (including total viable count, pathogens, foreign protein and endotoxin limits), rendering additional GMO testing redundant.

b. Regulatory consistency

A comparative analysis of current quality specifications for 2'-FL approved in Australia-New Zealand and internationally reveals that GMO detection is not universally require. The exemption rationale applied by Australia-New Zealand to equivalent products (e.g., those derived from *E. coli* K-12) should equally apply to 2'-FL sourced from *E. coli* BL21.

B.2.5.2 Batch Analyses

Yixi commissioned a third-party testing facility (CMA-certified) to conduct 3-batch analysis of the 2'-FL produced by genetically modified *E. coli* EC102 strain.

The quantitative compositional data on Yixi's 2'-FL are provided in **Annex B.7 [CONFIDENTIAL]**, as well as data on chemical and microbiological contaminants. Certificates of Analysis are provided in **Appendix B.7 [CONFIDENTIAL]**.

The analysis results show that Yixi's 2'-FL is a purified carbohydrate ingredient consisting primarily of 2'-FL, with lesser amounts of carbohydrate by-products (e.g., D-lactose, L-fucose, difucosyllactose, and galactose), indicating that the manufacturing process as described in ***Section B.2.4*** produces a consistent product that meets the defined specifications, which are listed in ***Table B.2.5.1***.

B.2.6 Analytical Method for Detection

The methods of analysis are Chinese standard methods obtained from Chinese Pharmacopoeia and GB (Chinese national standards), or were developed internally by Yixi and confirmed to be suitable when no standardised methods were available. The methods used are listed below (***Table B.2.6)***.

Table B.2.6. **Analytical Method for Yixi's 2'-FL**

Parameter	Limit	Method
Color	White to off-white	Visual observation
Form	Powder	
2'-Fucosyllactose (dried basis)	≥ 94.0%	HPLC, Bulletin No. 8 of NHC (2023) **(Annex B.8)** *
D-lactose	≤ 5.0%	
Difucosyllactose	≤ 2.0%	
L-fucose	≤ 3.0%	NY/T 2279 (Chinese agricultural standard)
Galactose	≤ 3.0%	
3-fucosyllactose	≤ 5.0%	HPLC (internal) **(Annex B.9)****
Glucose	≤ 3.0%	GB 5009.8 (Method I)
Water	≤ 9.0%	GB 5009.3 (Method II)
Ash, sulphated	≤ 0.5%	GB 5009.4 (Method I)
Residual proteins	≤ 100 μg/g	Coomassie Brilliant Blue Method (**Annex A.9**)***
Lead	≤ 0.02 mg/kg	GB 5009.268
Arsenic	≤ 0.2 mg/kg	
Cadmium	≤ 0.1 mg/kg	
Mercury	≤ 0.5 mg/kg	
Salmonella	Absent in 25 g	GB 4789.4
Total plate count	≤ 10000 CFU/g	GB 4789.2
Enterobacteriaceae	＜ 10 CFU/g	GB 4789.41 (Method I)
Cronobacter sakazakii	Absent in 100 g	GB 4789.40 (Method I)

Parameter	Limit	Method
Yeast and mould	≤ 100 CFU/g	GB 4789.15 (Method I)
Aflatoxin M_1	≤ 0.025 μg/kg	GB 5009.24 (Method I)
Endotoxins	≤ 10 EU/mg	General Rule 1143 Gel method, ChP (2020)

ChP = Chinese Pharmacopoeia; GB = Guo Biao (Chinese national food safety standards); CFU = colony forming units; MPN = most probable number; EU = endotoxin unit; NHC = Chinese National Health Commission.

*The detection methods of 2'-Fucosyllactose, Difucosyllactose and D-Lactose was permitted by Chinese National Health Commission (2023), available at: http://www.nhc.gov.cn/sps/s7892/202310/db51a70c84ce46f684ffe7be226dcdf1.shtml. The method are provided in **Annex B.8** in English.

The detection method of 3-Fucosyllactose as well as the validation report is provided in **Annex B.9.

***The detection method of residual proteins is provided in **Annex A.9**.

B.2.7 Information on the Proposed Food Label

As Yixi is not seeking to change the uses or use level of 2'-FL in infant formula products, no change to the labelling of these products is expected. Yixi's 2'-FL shall be identified on infant formula product labels as “2'-Fucosyllactose”, and the labeling of infant formula products containing Yixi's 2'-FL will continue to be compliant with Standard 2.9.1 of the Code.

B.3 Information Related to the Safety of the Nutritive Substance

FSANZ has previously evaluated the safety of 2'-FL under the currently permitted conditions of use (specifically, applications A1155, A1190, A1233, A1277, and A1283).

In this application, Yixi is not requesting any changes to the currently permitted uses or use levels of 2'-FL. Therefore, the safety conclusions regarding 2'-FL established in previous applications remain applicable to Yixi's 2'-FL.

B.3.1 Information on the Toxicokinetics and Metabolism of the Nutritive Substance

The metabolic fate of 2'-FL has been thoroughly detailed in multiple previous applications and assessed by FSANZ.

Large number of *in vivo* and *in vitro* studies indicates that 2'-FL is resistant to human digestive enzymes and remains intact as it passes through the upper gastrointestinal tract. It is subsequently metabolized by colonic microbiota into short-chain fatty acids, with less than 1% of an oral dose of 2'-FL being absorbed into the systemic circulation.

Yixi's 2'-FL has been structurally validated to be identical to the 2'-FL naturally occurred in human milk and previously evaluated 2'-FL ingredients. Therefore, Yixi's 2'-FL will have the same metabolic fate and toxicokinetic profile as 2'-FL previously evaluated by FSANZ.

B.3.2 Information from Studies in Animals or Humans that is Relevant to the Toxicity of the Nutritive Substance

B.3.2.1 Specific Toxicity Studies of Yixi's 2'-FL

Although 2'-FL is already permitted to be used as nutritive substance under the conditions specified in the Code, and Yixi does not seek changes to the current approved uses, additional studies demonstrating the safety and suitability of Yixi's 2'-FL for use in infant formula products have been conducted to satisfy the requirements of other jurisdictions, including an acute toxicity study, a 90-day sub-chronic toxicity study, *in vivo* and *in vitro* genotoxicity studies, and a teratogenicity study.

All the studies were carried out in third party CMA-certified laboratory in China.

B.3.2.1.1 Acute toxicity study of Yixi's 2'-FL

Yixi conducted an acute oral toxicity study to examine potential toxicological effects of its 2'-FL. The study was performed according to the Chinese Standard GB 15193.3-2014, which is generally equivalent to the Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 420 (Acute Oral Toxicity - Fixed Dose Procedure). Please find the test report in **Annex B.10 [CONFIDENTIAL]**.

ICR mice (10/sex/group) received a single oral dose (gavage) 2'-FL of 10.0 g/kg bw. The mice were monitored for 14 days after test item administration for clinical changes, and then a pathological analysis was conducted on all subjects. All animals showed no clinical abnormalities or significant signs of toxicity throughout the study. No death was observed in the animals. The acute median lethal dose (LD_{50}) of 2'-FL was identified as 10.0 g/kg bw.

B.3.2.1.2 90-day sub-chronic toxicity study of Yixi's 2'-FL

Yixi conducted a repeated-dose 90-day toxicity study of its 2'-FL. The study was conducted according to Chinese Standard GB 15193.13-2015, which is generally equivalent to OECD TG 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents). Please find the test report in **Annex B.11 [CONFIDENTIAL]**.

SPF Sprague-Dawley (SD) rats (10/sex/group) were dosed via gavage daily at 0, 2.00, 4.00 and 8.00 g/kg bw/day for 90 days. In addition, both control group (0 g/kg bw) and high-dose group (8 g/kg bw) had an extra mid-term satellite group (5/sex/group), respectively. The animals in the mid-term satellite groups were given the test article for 45 days, then fasted to collect blood and urine samples, and necropsied the next day for pathological examination. During the study period, the general clinical manifestations of animals were observed at least once a day, and the signs, extent, and duration of the toxicity and death were recorded. Body weight and food consumption of the animals were recorded. Other parameters included ophthalmology, hematology, blood biochemistry, urinalysis and histopathological examination.

In all dose groups and the two mid-term satellite groups, no abnormalities were observed in general physiological signs, behavior, appearance, eye examination, urine and bowel movements. No signs of toxicity or deaths were observed in any of the animals.

No adverse effect of the test article on the body weight, body weight gain, food consumption, and food utilization of the rats were observed. In the mid-term satellite group examination (45 days into the study), although the total food utilization of the high-dose mid-term group were statistically significantly higher than that of the control group ($p < 0.05$), but the results were all within the historical control data (HCD) range and therefore not determined to be biologically significant or toxicologically significant. In the 90-day dose groups, the food consumption and the total food utilization of the mid-dose (4 g/kg bw) group were significantly lower, but there was no dose-response relationship, and the data were all within the HCD range and therefore not determined to be biologically significant as well.

At all dose of the test groups and the mid-term satellite groups, there was no statistically significant difference in hematological, blood biochemical, or urinalysis parameters ($P > 0.05$). The gross anatomical examination and organ weighing showed no obvious abnormalities in the organs, and no statistically significant difference in the organ wet weight and the organ/body weight ratio of the animals ($P > 0.05$).

In the high-dose and control groups and their mid-term satellite groups, the histopathological examination of the main organs showed no test article-induced significant pathological changes, including brain, pituitary gland, thyroid gland, thymus, lung, heart, liver, spleen, kidney, adrenal gland, stomach, duodenum, jejunum, ileum, colon, rectum, pancreas, mesenteric lymph node, ovary, uterus, testis, epididymis, prostate, or bladder of the animals.

Overall, there is no adverse effects of the test article on SD rats under the test conditions. The No-Observable Adverse Effect Level (NOAEL) for Yixi's 2'-FL was determined to be the high dose of 8.00 g/kg bw/day.

B.3.2.1.3 Genotoxicity Study of Yixi's 2'-FL

Yixi carried out a series of unpublished genotoxicological tests on its 2'-FL, including a bacterial reverse mutation test (AMES test), an *in vivo* mammalian erythrocyte micronucleus test and an *in vivo* mouse spermatogonia chromosome aberration test. Please find the test reports in **Annex B.12 [CONFIDENTIAL]**.

a. Bacterial reverse mutation test

The bacterial reverse mutation test (Ames test) was conducted according to Chinese Standard GB 15193.4-2014, which is generally equivalent to OECD TG 471 (Bacterial Reverse Mutation Test).

Yixi's 2'-FL was incorporated into plates of the histidine deficient strains of Salmonella typhimurium (S. typhimurium) TA97a, TA98, TA100, TA102, and TA1535. Five dose groups were exposed to either 50.0, 158.1, 500.0, 1581.0, or 5,000 µg/plate 2'-FL. There was also an untreated control group, and two solvent control groups, including sterile water and dimethyl sulfoxide (DMSO). Positive control groups were exposed to 2-aminofluorene (2-AF), sodium azide, Dexon, 1, 8-dihydroxyanthraquinone, or cyclophosphamide (CTX).

In addition, the second Ames test were conducted according to the same study design, with five dose groups of 8, 40, 200, 1,000, or 5,000 µg/plate 2'-FL.

In both Ames tests, no treated concentration increased the number of reverting mutant colonies and within the HCD range, either with or without metabolic activation (S9 mix). All positive control groups produced the predicted increase in revertant, validating the test. This Ames test showed 2'-FL was not mutagenic under the testing conditions.

b. *In vivo* mammalian erythrocyte micronucleus test

The *in vivo* mammalian erythrocyte micronucleus test was performed according to Chinese Standard GB 15193.5-2014, which is generally equivalent to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test).

SPF mice (5/sex/group) were administered twice oral doses of 2'-FL via gavage at 1.25, 2.50, or 5.00 g/kg bw within 30 h. The second dose was administered 24 h after the first dose, and the animals were euthanized 6 h after the second dose. A negative control group received DI water, and a positive control group received 40 mg/kg bw of cyclophosphamide. Polychromatic erythrocytes (PCE) from the sternal bone marrow were examined for changes. The positive control had the expected rise in micronuclei.

There was no significant difference in the rate of micronucleus-containing cells in any dose group compared with the negative control group, indicating that 2'-FL was not genotoxic under the test conditions.

c. *In vivo* mouse spermatogonial chromosomal aberration test

The *in vivo* mouse spermatogonial chromosomal aberration test was performed according to Chinese Standard GB 15193.8-2014, which is generally equivalent to OECD TG 483 (Mammalian Spermatogonial Chromosomal Aberration Test).

Male SPF mice (5/low or mid dose group, 10/high dose group) were administered single oral doses of 2'-FL via gavage at 2.50, 5.00, or 10.00 g/kg bw. A negative control group received DI water via gavage, and a positive control group received 40 mg/kg bw of cyclophosphamide via intraperitoneal injection. Animals in the high-dose group were euthanized and sampled at 24 h and 48 h after administration, while those in other dose groups were euthanized at 24 h after administration. Animals were injected intraperitoneally with 5 mg/kg bw colchicine 4 h before death. The changes of chromosome structure, number and aberration types were observed and analyzed. The positive control group produced the predicted increase in aberrations. There was no significant difference in the rate of chromosomal aberrations between all test item dose groups and negative control group. The findings showed that Yixi's 2'-FL was not clastogenic under the testing conditions.

B.3.2.1.4 Teratogenicity Study of Yixi's 2'-FL

Yixi conducted a teratogenicity study to assess the potential reproductive toxicity of 2'-FL in SPF SD rats according to Chinese Standard GB 15193.14-2015. Please find the test report in **Annex B.13 [CONFIDENTIAL]**.

Pregnant SPF SD rats were dosed 2.00 g/kg bw (n=18), 4.00 g/kg bw (n=18), or 8.00 g/kg bw (n=17) of 2'-FL by gavage, on the 6th to 15th day of conception. There was also a negative control group that received DI water (n=19). The pregnant rats were weighed on day zero of pregnancy, the first day of administration, and every 3 days until the day of euthanasia. During the experiment, the general clinical manifestations of pregnant rats were observed once a day, and the signs, degree and duration of toxicity, and any animal mortality were recorded. The pregnant rats were euthanized on the 20th day of conception, the uterus was removed by laparotomy, and the weight of the uterus and fetuses was measured. All the pregnant rats were dissected, and the sex, weight, body length and appearance of the fetal rats were recorded. After the gross examination of fetuses, approximately half of the fetuses in each litter were fixed with 95% ethanol, and bones were stained with alizarin red solution for bone examination. Additionally, approximately half of the fetuses were fixed with Bouins fluid for internal organs examination.

All dose groups showed no maternal and fetal toxicity. The body weight, gestational weight gain, weight of uterus and fetus, and net weight gain of pregnant rats in each dose group were not significantly different compared with the control group. Compared with the control group, there were no significant differences in the luteal number, implantation number, mean number of live births, absorption number, and fetal mortality, as well as the length and body weight of fetuses in each dose group. Fetus examination showed no significant differences in teratogenesis of appearance, viscera, skeleton, and fetal malformation rate between each dose group and the control group. In summary, the NOAEL of 2'-FL was determined to be 8.00 g/kg bw.

B.3.2.2 Other Toxicity Studies of 2'-FL

FSANZ has already reviewed multiple toxicity studies in other 2'-FL application and confirming that 2'-FL is not genotoxic and is safe for use at currently permitted doses. A summary of toxicity studies conducted with various 2'-FL ingredients evaluated in previous application is provided in ***Table B.3.2.2***.

To identify studies reporting relevant safety outcomes for 2'-FL not previously evaluated in prior applications—following the FSANZ review of A1283 (approved on 19 June 2024)—a comprehensive literature search was conducted in June 2025. The following outlines the systematic approach to retrieve relevant toxicological studies on 2'-FL published between January 2022 and June 2025.

Substance: 2'-fucosyllactose, 2'-FL

Terms: toxicity, toxicology, toxicological, genotoxicity, subchronic, NOAEL, LOAEL

Search Queries:
“2’-fucosyllactose” OR “2'-FL” AND “toxicity” OR “toxicology”
“2’-fucosyllactose” OR “2'-FL” AND “toxicity test”
“2’-fucosyllactose” OR “2'-FL” AND “toxicological study”
“2’-fucosyllactose” OR “2'-FL” AND “genotoxicity” OR “subchronic”
“2’-fucosyllactose” OR “2'-FL” AND “no observed adverse effect level” OR “NOAEL”

Databases Searched: PubMed, PMC, ScienceDirect, Google Scholar, EFSA Journal. Safety assessment reports issued by regulatory authorities such as EFSA and Health Canada were also considered.

Exclusion Criteria:
1. Irrelevant focus: Studies solely on the efficacy of 2'-FL (e.g., prebiotic function, immune modulation), synthesis, or analytical methods.
2. Incomplete data: The full study description is unavailable/unpublished, or studies with incomplete data.

Number of Records Identified: n=0

Given no articles were identified as valid or pertinent to this search, and no toxicologically relevant findings emerged, the overall safety conclusions from previous assessments remain unchanged consequently.

Table B.3.2.2. Animal Toxicity Studies of 2'-FL in Previous Application

Study type	Test Article & Route	Cell type/Spices	Concentration/Dose	Results/NOAEL	Reference
Genotoxicity Study					
Bacterial Reverse Mutation (OECD TG 471)	99% 2'-FL	*S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	Plate incorporation: 52, 164, 512, 1600, or 5000 µg/mL (±S9); Pre-incubation: 492, 878, 1568, 2800, or 5000 µg/plate (±S9)	Negative	Coulet et al., 2014 (reviewed in A1155)
In Vitro Mammalian Cell Gene Mutation (OECD TG 476)		Mouse lymphoma cells (TK-locus)	Short-term treatment for 4h (±S9): 492, 878, 1568, 2800, or 5000 µg/mL; Long-term treatment for 24h (-S9): 1.7, 5.4, 17, 52, 164, 512, 1600, or 5000 µg/mL	Negative	
Bacterial Reverse Mutation (OECD TG 471)	94% 2'-FL	*S. typhimurium* (TA1535, TA1537, TA98, and TA100); *E. coli* strain WP2*uvr*A	Plate incorporation: 0, 62, 195, 556, 1667, or 5000 µg/plate (±S9)	Negative	van Berlo et al., 2018 (reviewed in A1190)
in Vitro Mammalian Cell Micronucleus (OECD TG 487)		Cultured binucleated human lymphocytes	Short-term treatment for 4h (±S9):0, 500, 1000, or 2000 µg/mL; Long-term treatment for 24h (-S9): 0, 500, 1000, or 2000 µg/mL	Negative	
Bacterial Reverse Mutation (OECD TG 471)	75% 2'-FL/DFL mixture	S.*typhimurium* (TA98, TA100, TA1535, and TA1537); *E. coli* WP2*uvr*A	Plate incorporation: 0, 5, 15, 50, 150, 500, 1500 or 5000 µg/plate (±S9); Pre-incubation: 0, 50, 150, 500, 1500 or 5000 µg/plate (±S9)	Negative	Phipps et al., 2018 (reviewed in A1190)
in Vitro Mammalian Cell Micronucleus (OECD TG 487)		Human peripheral blood lymphocytes	Short-term treatment for 4h (±S9): 0, 500, 1000 or 2000 µg/mL; Long-term treatment for 24h (-S9): 0, 500, 1000 or 2000 µg/mL	Negative	
Bacterial Reverse Mutation (OECD TG 471)	5 HMOS Mixture (47.1% 2'-FL)	*S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537)	Plate incorporation: 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (±S9); Pre-incubation: 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (±S9)	Negative	Parschat et al., 2020 (reviewed in A1283)
In Vitro Mammalian Cell Micronucleu (OECD TG 487)		Cultured human peripheral lymphocytes	Short-term treatment for 4h (±S9): 0, 7.5, 15, 30, or 60 mg/mL; Long-term treatment for 24h (-S9): 0, 7.5, 15, 30, or 60 mg/mL	Negative	

Study type	Test Article & Route	Cell type/Spices	Concentration/Dose	Results/NOAEL	Reference
Animal Toxicity Study					
14-day Oral Toxicity	99% 2'-FL, gavage	Neonatal Wistar Rats	2000, 5000, 7500 mg/kg bw/day	Two animals in the 7500 dose group died; necropsy revealed no macroscopic abnormalities, and the cause of death was not determined.	Coulet et al., 2014 (reviewed in A1155)
90-day Oral Toxicity (OECD TG 408)		Neonatal Wistar Rats	0, 2000, 5000, 6000 mg/kg bw/day	5000 mg/kg bw/day	
Neonatal Piglets Repeated-dose Oral Toxicity	97.9% 2'-FL, diet	Neonatal Piglets	0, 200, 500, 2000 mg/L (i.e., 29.37, 72.22, 291.74 in males; 29.30, 74.31, 298.99 in females)	291.74 for males; 298.99 for females	Hanlon and Thorsrud, 2014 (reviewed in A1155)
90-day Oral Toxicity (OECD TG 408)	97.6% 2'-FL, gavage	Neonatal Wistar Rats	0, 2000, 4000, 5000 mg/kg bw/day	5000 mg/kg bw/day	Penard, 2015 (reviewed in A1155)
90-day Oral Toxicity (OECD TG 408)	94% 2'-FL	Neonatal Wistar Rats, diet	0, 3, 6, or 10% in feed (i.e., 2.17, 4.27, or 7.25 g/kg/day for males and 2.45, 5.22, or 7.76 g/kg/day for females)	7.25 g/kg/day in male rats and 7.76 g/kg/day in female rats.	van Berlo et al., 2018 (reviewed in A1190)
90-day Oral Toxicity (OECD TG 408)	75% 2'-FL/DFL mixture, diet	Neonatal Sprague-Dawley Rats	0, 1000, 3000, 5000 mg/kg bw/day	5000 mg/kg bw/day	Phipps et al., 2018 (reviewed in A1190)
91-day Oral Toxicity (OECD TG 408)	5 HMOs Mixture (47.1%2'-FL), diet	CD Rats	0 or 10% in feed (i.e., 5,670 mg/kg bw/day for males and 6,970 mg/kg bw/day for females)	5670 for males and 6970 for females	Parschat et al., 2020 (reviewed in A1283)
21-Day Oral Toxicity in Piglets (OECD TG 408)	5 HMOs Mixture (49.1%2'-FL), diet	Domestic Yorkshire Crossbred Piglets	0, 5.75, 8.0 g/L (i.e., 0, 2556, or 3576 mg/kg bw/day in males and 0, 2604, or 3660 mg/kg bw/day in females)	3576 mg/kg bw/day for males and 3660 mg/kg bw/day for females	Hanlon et al., 2020 (reviewed in A1283)
18-Day Oral Toxicity	96.1%2'-FL, diet	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	1.2 g/L at the described intake volumes (assumed)	Wang et al. 2022 (reviewed in A1283)
21-Day Oral Toxicity in Piglets	diet	Neonatal Piglets	0 or 1.0 g/L 2'-FL Mean daily intake volume throughout the study = 317 mL/kg bw/day	317 mg/kg bw/day (assumed)	Daniels et al. 2022 (reviewed in A1283)

B.3.2.3 Human Studies of 2'-FL

Pivotal safety and tolerability data for 2'-FL, administered either alone or in combination with other HMOs, were thoroughly evaluated in prior regulatory assessments by FSANZ. It has been 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.

To identify studies reporting relevant safety outcomes for 2'-FL not previously evaluated in prior applications—following the FSANZ review of A1283 (approved on 19 June 2024)—an updated systematic literature search was conducted in June 2025. The following outlines the systematic approach to retrieve relevant human studies on 2'-FL published between January 2022 and June 2025.

Substance: 2'-fucosyllactose, 2'-FL

Terms: infant, newborn, clinical, tolerance, RCT

Search Queries:
"2'-fucosyllactose" OR "2'-FL" AND "infant" OR "newborn" AND "clinical trial"
"2'-fucosyllactose" OR "2'-FL" AND "infant" OR "newborn" AND "tolerance"
"2'-fucosyllactose" OR "2'-FL" AND "infant" OR "newborn" AND "randomized controlled trial" OR "RCT"

Databases Searched: PubMed, PMC, ScienceDirect, Google Scholar, EFSA Journal. Safety assessment reports issued by regulatory authorities such as EFSA and Health Canada were also considered.

Exclusion Criteria:
1. Study Population: Studies towards toddlers, children, adults, or animals.
2. Non-Clinical Studies: *In vitro* experiments, animal studies (even with juvenile animals), in silico modelling.
3. Intervention/Exposure: Without a clear intervention with 2'-FL

Number of Records Identified: n=7

Seven relevant studies were identified through this literature search. These newly identified studies consistently align with the established safety profiles from previous evaluations and do not modify the existing safety conclusion for 2'-FL under approved conditions of use.

These studies are briefly summarized below in ***Table B.3.2.3***.

Table B.3.2.3 Summary of Human Studies Published Since FSANZ's Evaluation of Application A1283

Study Design	Subjects	Treatment (daily)	Duration	Safety Results	Reference
Single-blinded, randomized, prospective crossover study	55 infants (aged 8-12 months)	• 5.0 mg iron as FeFum • 5.0 mg iron as FeFum and 3.0 g GOS • 5.0 mg iron as FeFum and 2.0 g 2'-FL and 1.0 g LNnT	20 days	• No reporting of AEs in this study • Growth and other outcomes indicated that formula containing 2'-FL was safe and well-tolerated	Giorgetti et al., 2023
Prospective, randomized, double-blinded, controlled study	201 healthy singleton infants aged 0-5 days and with birth weight > 2490 g	• 0.2 g/L 2'-FL + 2.2 g/L GOS (n=54) • 1.0 g/L 2'-FL + 1.4 g/L GOS (n=48) • breastfed reference group (n=51) • control formula (2.4 g/L GOS) (n=48)	4 months	• No reporting of AEs in this study • Growth and other outcomes indicated that formula containing 2'-FL was safe and well-tolerated	Hill and Buck, 2023
Controlled, double-blind, randomized, multicenter, interventional clinical trial	194 non-breastfed infants aged between 0 and 6 months (mean 3.2 months) with CMPA	• EHF supplemented with 1.0 g/L 2′-FL and 0.5 g/L LNnT (TG, n=79) • the same EHF without HMO (CG, n=72)	4 months	• No reporting of AEs in this study • Growth and other outcomes indicated that formula containing 2'-FL was safe and well-tolerated	Boulangé et al., 2023
Non-randomized, open-label, prospective study	106 healthy, term (37-42 weeks of gestation) infants enrolled at age 7 days to 2 months	• Exclusively breastfed (BF, n=38) • exclusively formula fed with 1 g/L 2'FL and 0.5 g/L LNnT (FF, n=46) • both formula and human milk (MF, n=22)	8 weeks	• A total of 46 subjects experienced 69 AEs during the course of the study, and no serious AEs were reported • A total of 7.8% (*n*=4) of the AEs were potentially formula related and only reported in the FF group. • Growth and GI tolerance outcomes confirmed the tolerance and safety	Jochum et al., 2023
Non-randomized single-group, multicenter study	33 infants aged 0-90 days, formula fed and experiencing feeding intolerance symptoms	• HRF supplemented with 0.2 g/L of 2′-FL, DHA, ARA, and nucleotides	28 days	• No reporting of AEs in this study • Growth and other outcomes confirmed the tolerance and safety	Ramirez-Farias et al., 2024

Study Design	Subjects	Treatment (daily)	Duration	Safety Results	Reference
Randomized, controlled, single-blinded (participants) crossover study	82 infants (aged 8-14 months)	• FUF (with 2.2 mg iron) • FUF (with 2.2 mg iron) supplemented with 400 mg/100 mL GOS and *L. reuteri* DSM 17938 • FUF (with 2.2 mg iron) supplemented with 100 mg/100 mL 2′-FL	Single serving	• No reporting of AEs in this study • Seasonal influenza accounted for ~75% (n=33) of the total AE cases reported during the trial, followed by diarrhea of ~10% (n=6) and other causes of ~10% (n = 6) such as insect bites, rashes, and skin injuries due to falling. SAE occurred in 2 cases (n=2) due to gastroenteritis and herpangina.	Scheuchzer et al., 2023
Randomized, double-blind, controlled trial	90 healthy full-term infants (37–42 weeks) weighing 2500–4500 g at birth	• 5 g/L 2′-FL + GOS +FOS (n=29) • 4 g/L GOS+FOS (n=30) • breastfed group (n=29)	90 days	• No reporting of AEs in this study • Growth and other outcomes confirmed the tolerance and safety	Lazarini et al., 2025

FeFum: Fe-labeled ferrous fumarate
GOS = galacto-oligosaccharides
2'-FL = 2'-fucosyllactose
LNnT = lacto-N-neotetraose
AE = adverse events
CMPA = cow's milk protein allergy
EHF = extensively hydrolyzed formula
HRF = hydrolyzed rice powder infant formula
FUF = follow-up formula
FOS = fructo-oligosaccharide

B.3.3 Safety Assessment Reports Prepared by International Agencies or other National Government Agencies

U.S.: An expert panel was convened to evaluate the safety of Yixi's 2'-FL and concluded that Yixi's 2'-FL is self-determined to be GRAS under the conditions of intended use (in non-exempt infant formula for term infants at a maximum use level of 2.4 g/L as consumed). The complete safety dossier was submitted to the U.S. FDA in February 2025 and currently awaiting FDA's response.

B.4 Information on Dietary Intake of the Nutritive Substance

FSANZ has previously assessed the safety of 2'-FL under its currently permitted use conditions (specifically, Application A1155, A1190, A1233, A1277, and A1283).

In this application, Yixi's 2'-FL is intended to replace other 2'-FL ingredients currently available on the market in Australia and New Zealand, with no changes requested to the currently permitted uses or usage levels of 2'-FL.

Consequently, the safety conclusions regarding 2'-FL as established in previous applications remain applicable to Yixi's 2'-FL, and no change to the current dietary intake of 2'-FL as a component of infant formula products are anticipated.

B.5 Information related to the Nutritional Impact of a Nutritive Substance other than Vitamins and Minerals

The purpose for the addition of Yixi's 2'-FL to infant formula products is the same as those described in previous FSANZ approvals (Application A1155, A1190, A1233, A1277, and A1283), i.e., to more closely mimic the composition of human milk, to contribute to a healthy intestinal microbiota (bifidogenic effect), and to reduce the binding of pathogens (C. jejuni) to intestinal epithelial cells.

B.6 Information related to potential impact on consumer understanding and behaviour

Yixi's 2'-FL is intended to substitute to other 2'-FL ingredients which have already been evaluated by FSANZ and are already on the market in Australia and New Zealand. Thus, no changes in consumer perception or behavioral responses to infant formula products containing Yixi's 2'-FL are expected. Likewise, the addition of Yixi's 2'-FL to infant formula products, in accordance with uses currently permitted in the Code, is not expected to impose any adverse effect to population groups.

C SPECIAL PURPOSE FOOD - INFANT FORMULA PRODUCTS

FSANZ has previously assessed the safety of 2'-FL for use as an ingredient in infant formula products (specifically, Application A1155, A1190, A1233, A1277, and A1283).

Yixi's 2'-FL is chemically identical to 2'-FL naturally occurring in human milk, and the 2'-FL ingredients previously approved by FSANZ. Yixi is not seeking to change the uses or use levels of 2'-FL currently permitted in the Code. Therefore, there are no public health and safety concerns associated with Yixi's proposed use of 2'-FL in infant formula products.

Yixi is not seeking changes to the composition of infant formula products as currently specified in Standard 2.9.1 of the Code. The safety, nutritive purpose, and physiological benefits of the addition of 2'-FL to infant formula products has been assessed by FSANZ previously. The addition of Yixi's 2'-FL to infant formula products is not expected to result in changes to the dietary intake of infant formula products, or to the labelling requirements of these products.

D SUMMARY

The information presented above supports the conclusion that Yixi's 2'-FL produced by fermentation using recombinant strain of *E. coli* EC102 is safe and suitable for use in accordance with uses currently permitted (i.e., as a nutritive substance in infant formula products under *Schedule 29*), and thus, *Schedules 3*, *26*, and *29* of the Code is expected to be amended accordingly.

This conclusion is based on the following:

• The Code already permits the use of 2'-FL as a nutritive substance in infant formula products, and Yixi is not seeking to alter the permitted use levels for 2'-FL.

• Yixi's 2'-FL has been demonstrated to be chemically and structurally identical to 2'-FL isolated from human milk.

• Yixi's specifications are comparable to other 2'-FL ingredients currently permitted for use in Australia and New Zealand, the EU, the U.S. and China.

• Yixi's 2'-FL consistently meets the proposed specifications.

• The stability of Yixi's 2'-FL has been verified by 6-month accelerated stability studies.

• Toxicity studies of Yixi's 2'-FL demonstrate that the ingredient is not genotoxic and does not result in compound-related adverse effects upon 90-day administration to rats at doses up to 8.00 g/kg bw/day.

• The host organism, *E. coli* BL21(DE3), is well-characterized, has a long history of use in the manufacture of food ingredients, and is considered to be a safe strain for such uses.

• The modifications made to the host organism have been demonstrated to result in a production organism that is functionally and genotypically stable; evaluation of the production strain indicates no safety concerns resulting from the genetic modifications.

• Protein, DNA and viable cells derived from Yixi's production strain, recombinant *E. coli* EC102, are absent in the final 2'-FL ingredient; the ingredient is therefore considered unlikely to pose safety concerns.

• In previous assessments, FSANZ concluded that there is no evidence of a nutritional concern regarding the use of 2'-FL in infant formula products at concentrations occurring naturally in human milk (i.e., up to 2.4 g/L). Yixi has not identified information in a recent comprehensive search of the published literature that would contradict FSANZ's previous conclusions.

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