Application to amend the Australia New Zealand Food Standards Code Schedule 26 Schedule 26 – Food produced using gene technology to permit "2'-fucosyllactose from the source organism, *Corynebacterium glutamicum* containing the gene for alpha-1,2-fucosyltransferase from *Corynebacterium urelyticum*"

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3.1.1 GENERAL REQUIREMENTS

B Applicant details

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C Purpose of the application

The purpose is to permit 2'-fucosyllactose (2'-FL) produced from a new genetically modified source organism to be used as a nutritive substance in infant formula products.
To amend Schedule 26 to add "*2'-fucosyllactose from the source organism Corynebacterium glutamicum* containing the gene for alpha-1,2-fucosyltransferase from *Corynebacterium urelyticum*".

There is no change to:

- "S3—51 Specification 2'-fucosyllactose sourced from *Corynebacterium glutamicum*", since the strain is not currently specified in this Schedule.
- the uses and levels of 2'-FL in "Standard 2.9.1 Infant Formula Products". The intended use levels are consistent with the permitted levels for 2'-FL in Schedule 29 (96 mg/100 kJ, equivalent to 2.4 g/L) (Schedule S29-54, Infant formula products – substances permitted as nutritive substances)

D Justification for the application

To provide an additional/alternative source of the Human Milk-identical Oligosaccharide 2'-Fucosyllactose (2'-FL) for use in infant formula products, under the controls already laid down in Standard 2.9.1 Infants Formula Products.

This source will enable alternative suppliers and therefore increase competition to keep raw materials costs as low as possible to provide economic benefits to the consumer.
The applicant's 2'-FL is chemically and structurally identical to the 2'-FL ingredients previously assessed and found safe by FSANZ (applications A1155, A1190, A1233, A1251, A1277, A1283)
In the European Union (EU) Cataya's 2'-FL is in the process of submission for the Amendment of the Union List of Novel Food[1] Specification for 2'-Fucosyllactose (2'-FL) (microbial source) to include 2'-FL produced by a derivative strain (CGMCC 7.559) of *Corynebacterium glutamicum* ATCC 13032. Its Novel Food Application Number is EFSA-Q-2025-00169. The dossier is currently at the validation stage with the European Food Safety Authority.

[1] 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. Available at: EUR-Lex - 02017R2470-20250220 - EN - EUR-Lex

In the United States (US) self-GRAS (Generally Recognized as Safe) was achieved 16 July 2024 and initially notified to the FDA on July 19th 2024. The FDA acknowledged the notification with GRN No 1238 on Feb 28th 2025. The notification review is currently in progress.
In China our partner is also in progress with an application for its approval as a new food additive/nutrient fortifier submitted on Dec 4th 2024.

D.1 Regulatory impact information

D.1.1 Costs and benefits of the application

As stated above, an additional source of 2'-FL will provide more competition between suppliers to keep the cost to infant formula manufacturers and therefore the consumers down.
There are no additional costs to governments from this application, beyond the normal costs of ensuring compliance with food laws.

D.1.2 Impact on international trade

There is no significant change to international trade, beyond increasing cost-competitiveness, which may have the potential to enhance international trade in respect of both the import and export of infant formula products containing added 2'-FL.

E Information to support the application

The application has addressed the FSANZ Application Handbook requirements. In particular, after pre-application discussions with FSANZ:

- sections 3.3.2.D and 3.3.2.E of the Handbook have been addressed to enable FSANZ to adequately assess Cataya's production organism, and
- sections 3.3.3.A and 3.3.3.B of the Handbook have been addressed to enable FSANZ to adequately assess technical aspects of Cataya's 2'-FL.

F Assessment procedure

2'-FL is already approved in the Code. This application is limited to the approval a new source organism for its production, consequently, General Procedure, level 1 or 2, is the appropriate procedure to be adopted in assessing this application.

G Confidential Commercial Information (CCI)

This application contains confidential commercial information (CCI) for which confidential treatment is requested. The CCI has been developed with significant financial resources and investment of time, and is commercially sensitive information that would incur significant negative commercial impact if competitors acquired access to the information.
CCI is framed in red boxes in this application and non-confidential versions are also provided in which the red boxes have been redacted. Please refer to Table G-1 below which summarises the CCI.

Table G-1 Confidentiality (CCI) Requests for Dossier Data	
Dossier Section/Annex	**Study Title**
Section B.4.1.	Detailed Description of the production process
Section B.4.2	Information on input material used in the production process of the novel food
Annex 1	Production Strain Deposition Certificate
Annex 2	Whole Genome Sequence Data
Annex 3	EFSA GMO Panel Category 1 Requirements
Annex 4	Structural Analysis: Analytical Reports of NMR and LCMS/MS
Annex 5	Analysis of Residual DNA from the Corynebacterium glutamicum CgBud-584 production strain in 2'-FL
Annex 6	Analysis of the Absence of Corynebacterium glutamicum CgBud-584 Production Strain in 2'-FL. Absence of viable cells in 2'- FL human milk oligosaccharide
Annex 7	2'-Fucosyllactose Acute Oral Toxicity Test Report (JC20240001 A1)
Annex 8	90-day Oral Toxicity Test Report of 2'-Fucosyllactose in Rats (JC20240001 M4)
Annex 9	2'-Fucosyllactose Teratogenicity Test Report (JC20240001 R1)
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Annex 11	2'-Fucosyllactose Chromosome Aberration Test Report (JC20240001 G3)
Annex 12	2'-Fucosyllactose In-vivo Mammalian Erythrocyte Micronucleus Test Report (JC20240001 G2)

H Other confidential information

I Exclusive capturable commercial benefit (ECCB)

This application seeks exclusive permission for the use of the 2'-FL from "*Corynebacterium glutamicum* containing the gene for alpha-1,2-fucosyltransferase from *Corynebacterium urelyticum*. Cataya requests a period of exclusivity of 15 months from the time of gazettal of the permission in Schedule 26. The brand name of the applicant's 2'-FL product is currently under discussion and to be decided internally.

In addition, the following questions from the Application Handbook have been addressed:

- Why are you making this application? What are you hoping to get out its approval?
    - As indicated in Section 3.1.1-D, there is a profitable/viable market in Australia and New Zealand for the manufacture of infant formula products both for use in Australia and New Zealand, as well as for export to other countries, specifically in South East Asia and China. The conferring of an exclusive permission will allow Cataya to market to manufacturers of infant formula in Australia and New Zealand and to establish early access to these markets relative to other competitors.
- How will you benefit from the approval of your application?
    - Sales and revenue in Australia and New Zealand and indirect sales in other markets which regard their respect for the FSANZ approval process.
- Who besides you, will benefit from the approval of your application? How and why will they benefit?
    - Infant formula companies will benefit from greater competition in prices for 2'-FL and this will be passed on to the consumer, keeping inflationary pressure down on infant formula products.
- If your application is approved, whose permission will be required before anyone can

derive a benefit from that approval?
    - Only Cataya's permission will be required.
- Who holds the intellectual property in the subject matter of your application?
    - Cataya

J International and other national standards

J.1 International standards

There is no Codex Alimentarius Standard that specifically includes 2'-FL

J.2 Other national standards or regulations

J.2.1 European Union

Current approvals for 2'-FL, from all sources are summarised in 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[2]. The specific sources approved are:

- Synthetic
- Genetically modified strain of *Escherichia coli* K-12
- Genetically modified strain of *Escherichia coli* BL-21
- Genetically modified strain of *Corynebacterium glutamicum* ATCC 13032

J.2.2 United States

Successful Generally Recognized as Safe (GRAS) Notifications for 2'-FL to date are summarised in Table J.2.2-1 below.

J.2.2-1 Successful Generally Recognized as Safe

GRN No.	Substance	Date of closure	FDA's Letter
1091	2'-fucosyllactose	Dec 1, 2023	FDA has no questions (in PDF) (272 kB)
1060	2'-fucosyllactose	Apr 4, 2023	FDA has no questions (in PDF) (333 kB)
1051	2'-fucosyllactose	Nov 21, 2023	FDA has no questions (in PDF) (298 kB)
1034	2'-fucosyllactose	Oct 21, 2022	FDA has no questions (in PDF) (258 kB)
1014	2-fucosyllactose	Jul 15, 2022	FDA has no questions (in PDF) (295 kB)
932	2'-fucosyllactose	Feb 18, 2021	FDA has no questions (in PDF) (223 kB)
929	2'-fucosyllactose	Feb 26, 2021	FDA has no questions (267 kB)
897	2'-*O*-fucosyllactose	Jun 12, 2020	FDA has no questions (in PDF) (715 kB)
852	2'-fucosyllactose	Nov 15, 2019	FDA has no questions (in PDF) (108 kB)
749	2'-O-fucosyllactose	Apr 23, 2018	FDA has no questions (in PDF) (95 kB)
735	2'-Fucosyllactose	Apr 6, 2018	FDA has no questions (in PDF) (103 kB)
650	2'-*O*-fucosyllactose	Nov 23, 2016	FDA has no questions
571	2'-Fucosyllactose	Nov 6, 2015	FDA has no questions
546	2'-O-fucosyllactose	Sep 16, 2015	FDA has no questions

[2] 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. Latest consolidated version available online at: EUR-Lex - 02017R2470-20241107 - EN - EUR-Lex

J.2.3 Canada

Table J.2.-1 Completed Novel Food Safety Assessments for 2'-FL by Health Canada		
Decision date	**Product**	**Proponent**
2022/07/22	2'-fucosyllactose (2'-FL) from *Escherichia coli* K-12 (DH1) MDO MAP1001d strain	Glycom A/S
2021/11/03	2'-Fucosyllactose from genetically engineered E. coli K12 MG1655 strain (sINB000846)	DuPont Nutrition & Biosciences
2021/06/09	2'-Fucosyllactose (2'-FL) in toddler formulas	Abbott Nutrition
2018/12/05	2'-fucosyllactose (2'-FL) from Escherichia coli BL21 (DE3) Strain #1540	Jennewein Biotechnologie, GmbH

J.2.4 Brazil

Table J.2.4-1 Approvals for 2'-FL in Brazil			
Name of Ingredient	**Requesting Company**	**Manufacturer**	**Approval**
2'-Fucosil-lactose (2'-FL) obtido a partir de processo fermentativo da linhagem Escherichia coli BL21 (DE3) #1540	Danone Ltda.	Jennewein Biotechnologie GmbH	RESOLUÇÃO RE Nº 1.351, de 31/03/2021
2'-O-Fucosillactose (2'-FL) obtido por processo fermentativo de Escherichia coli K-12 MG1655 INB000846	Danisco Brasil Ltda	Dupont Nutrition & Health	RESOLUÇÃO RE Nº 1.547 DE 14 DE ABRIL DE 2021
2-Fucosillactose (2-FL) obtido por processo fermentativo de Escherichia coli K-12 SCR6 e Lacto-N-neotetraose (LNnT) obtido por processo fermentativo de Escherichia coli K-12 MP572	Foodstaff S/C Ltda	Glycom de Alimentos Ltda - Dinamarca	Publicado deferimento RE 1020 de 17/04/2019
2-Fucosillatcose (2'-FL) obtido por processo fermentativo de Escherichia coli BL21 (DE3) 1540	Mead Johnson do Brasil Comércio e Importação de Produtos de Nutrição Ltda	Jennewein Biotechnologie GmbH - Alemanha	Publicado deferimento RE 3427 de 03/09/2020
2-Fucosillatcose (2'-FL) obtido por processo fermentativo de Escherichia coli K-12 (DH1) MAP1001d	Foodstaff S/C Ltda	Glycom Manufacturing A/S - Dinamarca	Publicado deferimento RE nº 4409 de 29/10/2020
2-O-Fucosil-lactose obtida por fermentação microbiana por meio da Escherichia coli K-12 SCR6 ou Escherichia coli K-12 (DH1) MAP1001d	DSM Produtos Nutricionais Brasil S.A	Glycom A/S, Dinamarca	Resolução RE nº 4802 de 23/12/2021
Approvals available online at : Microsoft Power BI			

K Statutory declaration

Please refer to separate Statutory Declaration signed and dated May 15, 2025.

L Checklist

See separate attached checklist

Structure of the application

FSANZ has advised Cataya that given the nature of previous assessments of 2'-FL and that this application is only requesting approval of a new source of 2'-FL, that the following Sections of the Applications Handbook apply:

3.1.1 – General Requirements

3.3.2 - Processing aids (only parts C2, C3, D1, D2, D3, and E1) – this covers the production methods for nutritive substances which make use of genetically modified organisms

3.3.3 - Substances used for a nutritive purpose

Pre-application discussions also noted that the Application Handbook's section 3.6.2 – Special Purpose Foods – Infant Formula Products – does not need to be addressed as 2'-FL is already permitted for use in infant formula products and we do not propose any changes to the current permitted conditions of use for 2'-FL *per se*.

3.3.2 PROCESSING AIDS

D Information related to safety of ~~enzyme~~ processing aid derived from a microorganism

D.1 Information on the source microorganism

D.1.1 Taxonomic information on the microorganism

The taxonomic information for the parental and production microorganisms *Corynebacterium glutamicum (C. glutamicum*) is provided in Table D.1.1-1.

Table D.1.1-1 Taxonomic Identification	
Phylum	*Actinobacteria*
Order	*Actinomycetales*
Family	*Corynebacteriaceae*
Genus	*Corynebacterium*
Species	*Corynebacterium glutamicum*
Strain – Host	*Corynebacterium glutamicum* ATCC 13032/ DSM 20300
Strain - Production	*Corynebacterium glutamicum* CGMCC 7.559

The production strain has been deposited at the China General Microbiological Culture Collection Centre (CGMCC) as *Corynebacterium glutamicum* strain number 7.599. The deposition certificate is provided as Annex 1. Within the deposition certificate the "strain reference given by depositor" is "Cgbud-584".

D.1.2 Information on the source organism used to manufacture 2'-FL

The production source organism is:
Corynebacterium glutamicum containing the gene for alpha-1,2-fucosyltransferase from *Corynebacterium urelyticum*

Please find attached as Annex 2 the whole genome sequence data, prepared in accordance with EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain (EFSA, 2021). In addition, Whole Genome Sequencing (WGS) Data Study Report in Annex 2 includes methodology and quality control measures that confirm the taxonomic identity of the host organism.

Host

A summary of the publicly available data about the genome sequence of *C. glutamicum* ATCC 13032 together with data from the applicant's assembly of the parental strain *C. glutamicum* ATCC 13032 WGS sequence, is provided in Table D.1.2-1. FSANZ has previously evaluated and approved 2'-Fucosyllactose (2'-FL) from the *C. glutamicum* APC199 production strain under application number A1283 (FSANZ, 2024). This production strain is a genetically modified derivative of the same host strain *C. glutamicum* ATCC 13032. EFSA has also issued a positive scientific opinion, resulting in approval, of 2'-FL from the production strain *C. glutamicum* APC199 (EFSA NDA Panel, 2022).

Table D.1.2-1– Whole Genome Sequence Data (Annex 2)		
	Applicant's Corynebacterium glutamicum ATCC 13032 (host strain)	*Corynebacterium glutamicum* ATCC 13032 *(*Public Data Source*)*
Main genome scaffold total	1	1
Main genome contig total	1	1
Main genome scaffold sequence total (Mbp)	3.3	3.3
Main genome contig sequence total (Mbp)	3.3	3.3
% gap	0	0
Scaffold L50	1	1
Scaffold N50 (Kbp)	3312.563	3309.401
Number of scaffolds >50 Kbp	1	1

D.1.3 Method used to manufacture 2'-FL

Please refer to Section 1.3 of Annex 3 - EFSA GMO Panel Category 1 Requirements.

The *C. glutamicum* parental strain was engineered in a targeted manner to over-express the 2'-FL synthesis pathway and to produce higher yields of 2'-FL. General strain engineering methods were used to introduce genetic modifications like gene deletions and insertions into the production strain genome. Gene deletions and insertions were verified by Polymerase Chain Reaction (PCR), Sanger sequencing, and Whole Genome Sequencing (WGS). WGS analysis confirmed that there are no unintended modifications in the final production strain. In addition, the final production strain is plasmid-free and antibiotic marker free, as validated by WGS analysis.

All heterologous genes inserted into the modified strain were produced by DNA synthesis. As such, this sourcing method ensures no risk of undesirable or unintended genes from the source organism being introduced to the production host. The source organism from which each heterologous gene was derived is specified in Table D.1.3-1.

Table D.1.3-1 Source Organisms for Heterologous Genes Inserted into The Production Strain	
Source Organism	**Inserted Heterologous Gene**
Escherichia coli	*Gmd*
Campylobacter jejuni	*DdahC*
Oryza sativa	*GME*
Escherichia coli	*lacy*
Corynebacterium urealyticum	*CuFucT*

The production strain was genetically engineered to produce 2'-FL from glucose or sucrose and lactose through fermentation. *C. glutamicum* has an endogenous metabolic pathway to biosynthesize GDP-D-mannose for producing glycoproteins and glycolipids in cell wall (Jackson and Brennan, 2009; Mishra *et al.*, 2011). Three heterologous enzymes (GDP-D-mannose-4,6-dehydratase, GDP-4-keto-6-deoxymannose reductase and GDP-D-rhamnose epimerase) were introduced to convert GDP-D-mannose to GDP-L-fucose. Lactose permease (LacY) was introduced into the strain to transport lactose across the cell membrane. Finally, α-1,2-fucosyltransferase (CufucT) was introduced for fucosylation of lactose to produce 2'-FL identical to that in human milk. Table D.1.3-2 provides a summary listing of all heterologous enzymes inserted into the engineered production strain and their respective functions leading to the production of 2'-FL. A schematic diagram of this biosynthetic pathway is provided below in Figure D.1.3-1.

Table D.1.3-2 Summary of Heterologous Enzymes and Their Respective Functions in The Production Strain	
Enzyme	**Function**
Gmd	GDP-D-mannose-4,6-dehydratase. Dehydrates GDP-D-mannose to GDP-4-keto-6-deoxymannose
DdahC	GDP-4-keto-6-deoxymannose reductase. Reduces GDP-4-keto-6-deoxymannose to GDP-D-rhamnose
GME	GDP-D-rhamnose epimerase. Converts GDP-D-rhamnose to GDP-L-fucose
LacY	Lactose permease. Transports lactose across cell membrane to the cytoplasm
CuFucT	α -1,2-fucosyltransferase. Fucosylates lactose to produce the product 2'-FL

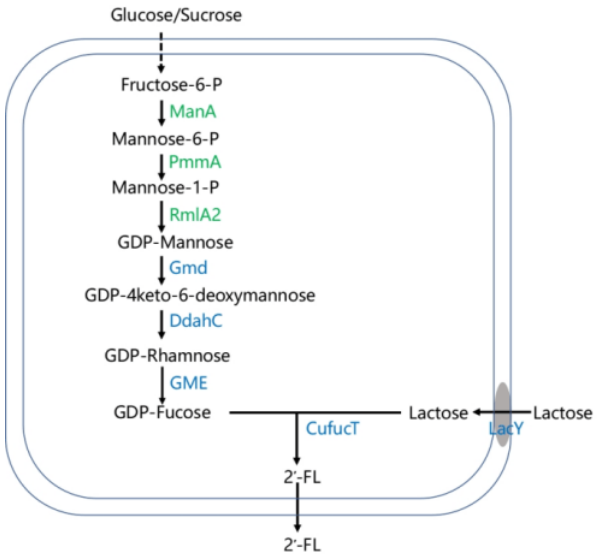


Figure D.1.3-1 Biosynthesis Pathway for Generation of 2'-FL by a Genetically Modified *C. glutamicum* Strain.

Abbreviations:

ManA = mannose-6-phosphate isomerase; PmmA = phosphomannomutase; RmlA2 = mannose-1-phosphate guanylyltransferase; Gmd = GDP-mannose 4,6-dehydratase; DdahC = GDP-4-keto-6-deoxymannose reductase; GME = GDP-D-rhamnose epimerase; CufucT = a1,2-fucosyltransferase; LacY = lactose permease.

Notes: Green font represents the endogenous enzymes in *C.glutamicum*. Blue font represents the heterologous enzymes that were introduced in production strain.

D.2 Information on the pathogenicity and toxicity of the source microorganism

D.2.1 Source microorganism

Corynebacterium glutamicum is a non-spore-forming, non-motile, Gram-positive bacterium isolated from soil. It has a short rod to club-shaped morphology and belongs to the genus *Corynebacterium* in the family *Corynebacteriaceae*. It has been recognized by the U.S. Food and Drug Administration (FDA) as "Generally Recognized as Safe" (GRAS) (Woo & Park, 2014; Lee *et al.*, 2016). *C. glutamicum* was first reported for use in L-glutamate fermentation production in 1957 (Kinoshita *et al.*, 1957) and has since been widely employed in the industrial production of amino acids such as L-lysine, L-isoleucine, L-valine, and L-arginine (Wendisch *et al.*, 2016). In recent years, *C. glutamicum* has developed into an important industrial chassis organism, capable of producing various natural and non-natural products from diverse renewable raw materials (Becker *et al.*, 2018; Wendisch, 2020). *C. glutamicum* is widely distributed in nature, found on surfaces of soil, water, and plant rhizospheres.

Regulatory authorities including the European Food Safety Authority (EFSA) and Japan's Ministry of Health, Labour and Welfare (MHLW) have acknowledged *C. glutamicum* as a safe production host for enzymes, amino acids, and other metabolites, due to its non-pathogenic nature and long history of safe industrial use (EFSA BIOHAZ, 2025, Ministry of Health, Labour and Welfare (MHLW), Japan. 2003). *Corynebacterium glutamicum* has been granted Qualified Presumption of Safety (QPS) status in the European Union (EU) by EFSA (EFSA BIOHAZ, 2025) and is considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes. The specific current entry in the current QPS is reproduced in Table D.2.1-1 below.

Table D.2.1-1 QPS Listing for *C. glutamicum*							
Microbiological Group	**Microbiological Subgroup**	**Family**	**Genus**	**Species**	**Synonyms commonly used in the feed and food industry/ anamorph (for yeasts)/ previous name**	**Qualification 1**	**Qualification 2**
Bacteria	Gram-positive non-sporulating bacteria		*Corynebacterium*	*Corynebacterium glutamicum*	*Brevibacterium lactofermentum*	The strains should not harbour any acquired antimicrobial resistance genes to clinically relevant antimicrobials.	QPS applies for 'production purposes only' *(the qualification 'for production purpose only' implies the absence of viable cells of the production organism in the final product and can also be applied for food and feed products based on microbial biomass*.

In addition, the heterologous genes integrated into the genetically modified CGMCC 7.559 2'-FL production strain were produced by DNA synthesis, ensuring no risk of undesirable or unintended genes from the source organism being introduced to the production host. Furthermore, the potential toxigenicity and pathogenicity of the introduced heterologous genes were assessed, and it was concluded that these genes present an insignificant risk of pathogenicity or toxicity in the genetically modified CGMCC 7.559 2'-FL production strain. (Annex 3).

Please refer to Annex 3 for the EFSA GMO Panel Category 1 Requirements of the production organism (EFSA GMO Panel, 2011).
Full sequencing of the parent strain did not reveal any previously undiscovered antibiotic resistance markers in the original *C. glutamicum* ATCC 13032 strain. The final production strain does not contain any antibiotic resistance markers or pathogenicity traits.
The production strain CGMCC 7.559 is plasmid-free and antibiotic marker free.

D.3 Information on the genetic stability of the source organism

D.3.1 Stability of phenotype of the production strain

To evaluate the genetic stability of the production strain *C. glutamicum* CGMCC 7.559, the strain was cultured over five consecutive subculturing passages, and cell density, growth rate and 2'-FL synthesis were analysed to assess the genetic stability of the strain over the five consecutive passages. Briefly, the production strain CGMCC 7.559 was inoculated at a 1:100 ratio into 20 mL of modified TSBG liquid medium (5 g/L glucose, 9 g/L soya peptone, 5 g/L yeast extract, 1 g/L $K_2HPO4{\cdot}3H_2O$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L urea, 0.5 g/L succinic acid, 400 µg/L biotin, 100 µg/L vitamin B1, and 20 g/L MOPS (pH 7.2)) in a flask and cultured overnight. After overnight cultivation, the OD_{562} of the bacterial culture was measured and the culture was inoculated into 20 mL of modified TSBG liquid medium in a flask at an initial OD_{562} of 0.1. This flask (designated F1) was incubated at 30°C with shaking at 200 rpm, and OD measurements were taken at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 23 h until the strain reached the stationary growth phase. The next day, sample from F1 was inoculated into a new flask (F2) with 20 mL of modified TSBG liquid medium at an initial OD_{562} of 0.1. F2 was grown and OD measurements were done similarly to F1. The process was repeated for a total of 5 passages (5 flasks). Culture samples collected at the end of each passage (from F1 to F5) were inoculated to 96-well plates and cultured for 3 days to measure 2'-FL production. To measure 2'-FL concentration, samples were centrifuged at 4,000 rpm for 10 minutes to separate the upper liquids from the pellet, and the supernatant was filtered using a 0.2-µm filter before being analysed on HPLC analysis. The 2'-FL concentration was determined on HPLC-1260 (Agilent) with RID detector using YMC-Triart-Diol-HILIC, 250 x 4.6 mm 5 µm column at 35 °C. The eluent solution consisted of ultrapure water mixed with acetonitrile at 250.0:589.5 (w/w). An isocratic flow was used at 0.8 ml/min. Data analysis was conducted using Agilent CDS2.7 software and ultrapure standard of 2'-FL was used to quantify 2'-FL concentration.

OD measurements showed that the five passages of CGMCC 7.559 had no significant differences in growth characteristic, and growth pattern was consistent with what is expected for *C. glutamicum* strains. Grow rate (µ) was calculated for cells in the F1-F5 passages, and there is no statistically significant difference in growth rate (Table D.3.1-1, $p>0.05$).

Table D.3.1-1 *C. glutamicum* CGMCC 7.559 Growth Rate Across 5 Consecutive Subculturing Passages					
Measured Parameter	F1	F2	F3	F4	F5
Growth Rate (μ)	0.49±0.017	0.49±0.007	0.50±0.012	0.49±0.004	0.49±0.002
Mean+ standard deviation					

Additionally, the final 2'-FL product yields after 72 hours of cultivation under the same conditions were analysed for five consecutive passages, and results showed that 2'-FL yield remained stable. 2'-FL product yield in F2 to F5 has no statistical significance, compared to F1 (Table D.3.1-2, $p>0.05$).

Table D.3.1-2 *C. glutamicum* CGMCC 7.559 Production of 2'-FL Across 5 Consecutive Subculturing Passages					
Measured Parameter	F1	F2	F3	F4	F5
2'-FL (g/gDCW)	0.59±0.004	0.58±0.017	0.60±0.022	0.59±0.027	0.59±0.015
Mean+ standard deviation DCW: dry cell weight					

D.3.2 Stability of the genotype of production strain

Genetic modifications on the production strain *Corynebacterium glutamicum* CGMCC 7.559 are all chromosomal integration and the production strain does not contain any plasmid-based systems. Therefore, the production source microorganism is plasmid-free and antibiotics marker-free, and the genotype of the productions strain is stable over extended fermentation cycles.

E Additional information related to the safety of a processing aid derived from a genetically-modified microorganism

E.1 Information on the methods used in the genetic modification of the source microorganism

E.1.1 Method for gene deletions (knock-out) and insertions (knock-in) in the genome

CGMCC 7.559 strain was genetically engineered using site-specific genomic integrations of DNA constructs via homologous recombination at stable, non-essential regions of the genome. The general method to introduce genetic modifications like gene deletions and insertions in the genome is based on widely used engineering methods for *C. glutamicum*, and described in detail in Section 1.3 of Annex 3- EFSA GMO Panel Category 1 Requirements.

E.2 Genetic information regarding plasmid

The production strain CGMCC 7.559 strain does not contain plasmids.

E.3 Donor Organisms for the Introduced Genes

Please refer to Section 1.2.2 of Annex 3 - EFSA GMO Panel Category 1 Requirements. All incorporated DNA is produced via DNA synthesis.

E.4 Stability of the genotype of the plasmid

The production strain CGMCC 7.559 strain does not contain plasmids.

3.3.3 SUBSTANCES USED FOR A NUTRITIVE PURPOSE

A Information on the use of the nutritive substance

A.1 Information on the purpose of the use of a nutritive substance

There is no proposed change to the conditions of use of 2'-FL as laid down within Schedule 29-7 (Infant formula and special medical purpose for infants – Standard 1.5.2)) and 29-8 (Follow-on formula Standard 1.5.2) of the Food Standards Code. I.e. Maximum amount per 100 kJ = 96 mg.

A.2 General data requirements for supporting evidence

All studies presented below have been conducted on test article which is representative of the commercial product on which approval is sought.

B Technical information on the use of the nutritive substance

B.1 Information to enable identification of the nutritive substance

B.1.1Identification of the Nutritive Substance

B.1.1.1 Chemical name, when appropriate, according to IUPAC nomenclature rules

There is no change from the existing approval:

2'-fucosyllactose

(2R,3R,4R,5R)-4-[(2S,3R,4S,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3S,4R,5S,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-2,3,5,6-tetrahydroxyhexanal

B.1.1.2 CAS number (if this has been attributed) and other identification numbers

There is no change from the existing approval

41263–94–9

B.1.1.3 Synonyms, trade names, abbreviations

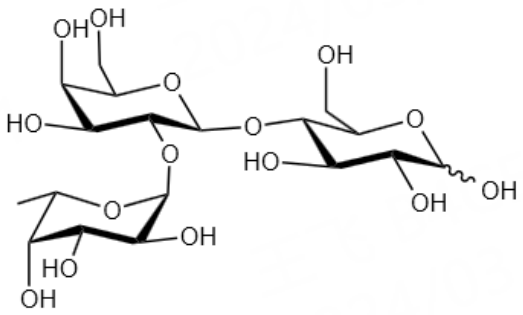
There is no change from the existing approval

- α-L-Fucopyranosyl-(1 →2)-β-D-galactopyranosyl-(1 →4)-D –glucopyranose
- 2'-Fucosyl-D-lactose
- 2'-O-fucosyllactose

B.1.1.4 Molecular and structural formulae; stereochemistry

There is no change from the existing approval

$C_{18}H_{32}O_{15}$



B.1.1.5 Molecular mass (Da)

There is no change from the existing approval

488.44

B.1.1.6 InChI and InChIkey

There is no change from the existing approval

HWHQUWQCBPAQQH-BWRPKUOHSA-N

B.1.1.7 SMILES Canonical and SMILES Isometric

There is no change from the existing approval

C[C@H]1[C@H]([C@H]([C@@H]([C@@H](O1)O[C@@H]2[C@H]([C@H]([C@H](O[C@H]2O[C@H]([C@@H](CO)O)[C@@H]([C@H](C=O)O)O)CO)O)O)O)O)O

B.1.1.8 Identity tests of the relevant constituents with the most relevant analytical techniques

The 2'-FL produced by the applicant using microbial fermentation has been demonstrated using nuclear magnetic resonance (1 H-NMR, 13C-NMR and 2D NMR) spectroscopy to be chemically and structurally equivalent to 2'-FL in human milk. Additionally, each batch of the applicant's 2'-FL was compared to the 2'-FL standard using liquid chromatography with mass spectrometry (LC-MS/MS) to confirm the identity. The NMR and LC-MS/MS analytical reports are provided in Annex 4 – Structural Analysis: Analytical Reports of NMR and LC-MS/MS.

B.1.1.9 Particle size, shape and distribution if particles are present in the final product

In accordance with the decision tree laid down in EFSA Scientific Committee Guidance on technical requirements (Section 2.3.1) for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA Scientific Committee, 2021), the solubility was determined to be in excess of 419 ± 14 g/L by a contract laboratory accredited to ISO 17025. This exceeds the threshold criteria of 33.3 g/L and thus further assessment with respect to nanoparticles is not required.

B.1.1.10 Comparison with chemical standards, certified reference material, authentic biological specimens, naturally occurring compound or other relevant material

Please refer to section B.1.1.8

B.1.1.11 In case of simple chemical mixtures, description of potential chemical or physical interactions altering the properties of the single component or their behaviour in the body

2'-FL is already approved as a novel food ingredient. There is no change to the structure or chemical properties for 2'-FL from *Corynebacterium glutamicum (C.glutamicum)* CGMCC 7.559.

B.2 Information on the chemical and physical properties of the nutritive substance

B.2.1Incorporation into Food Matrices

There are no proposed changes to existing approved use conditions for 2'-FL under Standard 2.9.1 Infant Formula Products and specifically Schedule 29-7.

B.2.2 Product Stability

The stability of 2'-FL has been well-characterized in stability studies summarized in previous applications (A1155, A1190, A1233, A1251, A1277, A1283). The compositional similarities between Cataya's 2'-FL and other 2'-FL preparations indicate that the stability of the ingredients will be similar. Stability tests using Cataya's 2'-FL have been carried out on 5 representative, independently produced batches.

B.2.2.1Real-time stability

The stability of five representative production lots of 2'-FL was investigated under real-time storage conditions (25°C ± 2°C/60% RH ± 5% RH) performed in an ISO 17025/CNAS (China National Accreditation Service for Conformity Assessment) qualified laboratory facility. The study is ongoing and currently demonstrates at least 12 months stability. The results are presented in Table B.2.2.1-1 below.

Table B.2.2.1-1 Stability Results of 2'-FL Produced by Fermentation Under Real-time Storage Conditions (25°C, 60% Relative Humidity)				
Test Item	**Specification**	**0**	**6-month**	**12-month**
Lot No.: 23100101				
Assay	%dwb ≥94.0%	97.4%	98.0%	97.5%
D-lactose, %w/w	≤ 3.0% dwb	1.4%	1.5	1.4%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.1%	1.3%	1.2%
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	ND	NA	NA
D-Glucose (% w/w dry matter)	≤ 3.0	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	ND	ND	ND
Water content	≤ 9.0%	3.34%	3.68%	3.29%
Lot No.: 23102101				
Assay	%dwb ≥94.0%	98.1%	98.2%	98.2%
D-lactose, %w/w	≤ 3.0% dwb	0.5%	0.5%	<0.5%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.2%	1.4%	1.3%
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	ND	NA	NA
D-Glucose (% w/w dry matter)	≤ 3.0	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	ND	ND	ND
Water content	≤ 9.0%	2.91%	3.07%	2.98%
Lot No.: 23102202				
Assay	%dwb ≥94.0%	96.6%	97.7%	97.8%
D-lactose, %w/w	≤ 3.0% dwb	1.1%	1.2%	1.1%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.0%	1.2%	1.1%
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	ND	NA	NA
D-Glucose (% w/w dry matter)	≤ 3.0	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	ND	ND	ND
Water content	≤ 9.0%	2.47%	2.54%	2.49%
Lot.: 23101302				
Assay	%dwb ≥94.0%	97.5%	97.4%	97.5%
D-lactose, %w/w	≤ 3.0% dwb	1.0%	1.1%	1.0%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.3%	1.5%	1.4%
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	ND	NA	NA
D-Glucose (% w/w dry matter)	≤ 3.0	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	ND	ND	ND

Water content	≤ 9.0%	1.69%	1.82%	1.64%
Lot.: 23101401				
Assay	%dwb ≥94.0%	97.3%	98.2%	98.1%
D-lactose, %w/w	≤ 3.0% dwb	0.9%	1.0%	0.8%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.2%	1.4%	1.3%
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	ND	NA	NA
D-Glucose (% w/w dry matter)	≤ 3.0	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	ND	ND	ND
Water content	≤ 9.0%	2.72%	3.62%	3.11%
Abbreviations: ND = Not detected, dwb = dry weight basis, NA=Not Analysed				

B.2.2.2. Accelerated Stability

The stability of five representative production lots of 2'-FL was investigated under accelerated storage conditions (40°C, 75% relative humidity) performed in an ISO 17025/CNAS qualified laboratory facility. Accelerated storage data for carbohydrate and water content are summarised in Tables B.2.2.2-1 below, demonstrating 2'-FL to be stable for up to 26 weeks under accelerated conditions (equivalent to 2 years real-time basis). No appreciable changes in 2'-FL assay, impurities and water content were observed.

Table B.2.2.2-1 Stability Results of 2'-FL Produced by Fermentation Under Accelerated Storage Conditions (40°C, 75% Relative Humidity)							
Test Item	**Specification**	**0**	**Week 1**	**Week 4**	**Week 8**	**Week 13**	**Week 26**
Lot No.: 23100101							
Assay	%dwb ≥94.0%	97.4%	96.6%	97.9%	96.4%	98.1%	98.0%
D-lactose, %w/w	≤ 3.0% dwb	1.4%	1.3%	1.4%	1.4%	1.4%	1.5%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.1%	1.1%	1.1%	1.1%	1.1%	1.3%
3-Fucosyllactose (% w/w)	≤ 3.0 dwb	ND	NA	NA	NA	NA	NA
D-Glucose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
Water content	≤ 9.0%	3.34%	3.16%	3.47%	3.53%	3.34%	3.54%
Lot No.: 23102101							
Assay	%dwb ≥94.0%	98.1%	97.7%	98.9%	97.6%	98.4%	98.6%
D-lactose, %w/w	≤ 3.0% dwb	0.5%	0.5%	0.5%	< 0.5%	0.5%	0.5%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.2%	1.2%	1.2%	1.2%	1.2%	1.4%
3-Fucosyllactose (% w/w)	≤ 3.0 dwb	ND	NA	NA	NA	NA	NA
D-Glucose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
Water content	≤ 9.0%	2.91%	2.65%	2.93%	2.75%	3.07%	3.13%
Lot No.: 23102202							
Assay	%dwb ≥94.0%	96.6%	96.7%	97.8%	97.6%	97.4%	97.7%
D-lactose, %w/w	≤ 3.0% dwb	1.1%	1.1%	1.1%	1.1%	1.1%	1.3%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.0%	1.0%	1.0%	1.1%	1.1%	1.2%
3-Fucosyllactose (% w/w)	≤ 3.0 dwb	ND	NA	NA	NA	NA	NA
D-Glucose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
Water content	≤ 9.0%	2.47%	2.38%	2.45%	2.33%	2.57%	2.64%

Lot.: 23101302							
Assay	%dwb ≥94.0%	97.5%	97.3%	97.6%	96.9%	97.5%	97.6%
D-lactose, %w/w	≤ 3.0% dwb	1.0%	0.9%	1.0%	1.0%	1.0%	1.1%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND	ND	ND	ND
L-Fucose, %w/w	≤ 3.0% dwb	1.3%	1.3%	1.3%	1.3%	1.3%	1.5%
3-Fucosyllactose (% w/w)	≤ 3.0 dwb	ND	NA	NA	NA	NA	NA
D-Glucose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
Water content	≤ 9.0%	1.69%	1.50%	1.59%	1.64%	1.70%	1.96%
Lot.: 23101401							
Assay	%dwb ≥94.0%	97.3%	96.7%	98.4%	97.3%	97.0%	97.2%
D-lactose, %w/w	≤ 3.0% dwb	0.9%	0.8%	0.9%	0.9%	0.9%	1.0%
Difucosyl-D-lactose, %w/w	≤ 2.0% dwb	ND	ND	ND	ND	ND	ND
L-Fucose, %w/w	≤ 2.0% dwb	1.2%	1.2%	1.2%	1.2%	1.3%	1.5%
3-Fucosyllactose (% w/w)	≤ 3.0 dwb	ND	NA	NA	NA	NA	NA
D-Glucose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w)	≤ 3.0 dwb	ND	ND	ND	ND	ND	ND
Water content	≤ 9.0%	2.72%	2.90%	2.74%	2.58%	3.11%	2.81%
Abbreviations: ND = Not detected, dwb = dry weight basis, NA=Not Analysed							

The microbiological stability of the 5 representative production lots of 2'-FL was investigated under accelerated storage conditions (40°C, 75% relative humidity). Data summarised in Table B.2.2.2-2 below demonstrates 2'-FL to be microbiologically stable for up to 26 weeks under accelerated conditions (equivalent to 2 years real-time).

Table B.2.2.2-2 Microbiological Stability Results of 2'-FL Produced by Fermentation under Accelerated Storage Conditions (40°C, 75% Relative Humidity - RH)			
Test Items	**Specification**	**Week 0**	**Week 26 (40°C, 75%RH)**
Lot No.: 23100101			
Standard Plate Count	≤ 500 CFU/g	200 CFU/g	<100 CFU/g
Yeast and Mould	≤ 100 CFU/g	<10 CFU/g	<10 CFU/g
Aflatoxin M1 (µg/kg)	≤ 0.025	ND	ND
Coliforms (CFU/g)	≤ 10	<10	<10
Endotoxins (EU/mg)	≤ 10	0.005	0.0086
Lot No.: 23102101			
Standard Plate Count	≤ 500 CFU/g	300 CFU/g	< 100 CFU/g
Yeast and Mould	≤ 100 CFU/g	<10 CFU/g	<10 CFU/g
Aflatoxin M1 (µg/kg)	≤ 0.025	ND	ND
Coliforms (CFU/g)	≤ 10	<10	<10
Endotoxins (EU/mg)	≤ 10	0.002	0.004
Lot No.: 23102202			
Standard Plate Count	≤ 500 CFU/g	< 100 CFU/g	< 100 CFU/g
Yeast and Mould	≤ 100 CFU/g	<10 CFU/g	<10 CFU/g
Aflatoxin M1 (µg/kg)	≤ 0.025	ND	ND
Coliforms (CFU/g)	≤ 10	<10	<10
Endotoxins (EU/mg)	≤ 10	0.0018	0.0017
Lot No.: 23101302			
Standard Plate Count	≤ 500 CFU/g	< 100 CFU/g	< 100 CFU/g
Yeast and Mould	≤ 100 CFU/g	<10 CFU/g	<10 CFU/g
Aflatoxin M1 (µg/kg)	≤ 0.025	ND	ND
Coliforms (CFU/g)	≤ 10	<10	<10
Endotoxins (EU/mg)	≤ 10	0.004	0.004
Lot No.: 23101401			
Standard Plate Count	≤ 500 CFU/g	100 CFU/g	< 100 CFU/g
Yeast and Mould	≤ 100 CFU/g	<10 CFU/g	<10 CFU/g
Aflatoxin M1 (µg/kg)	≤ 0.025	ND	ND
Coliforms (CFU/g)	≤ 10	<10	<10
Endotoxins (EU/mg)	≤ 10	0.0014	0.0096
Abbreviations: ND = Not detected, CFU: colony forming units			

B.3 Information on the impurity profile

We propose no change to the current Specification 2'-fucosyllactose sourced from *Corynebacterium glutamicum* defined under Schedule 3 (S3-51) of the Food Standards Code (Section B.5). Therefore, the product specifications for other carbohydrate components of the 2'-fucosyllactose ingredient, such as lactose, fucose, 3-Fucosyllactose, Difucosyl-D-lactose, glucose, and galactose are the same as those defined in S3-51 (section B.5). Analysis of the 5 representative production lots demonstrates the 2'-fucosyllactose product is compliant with specifications in S3-51 (section B.5.2)

In addition, 3 representative production lots were thoroughly analysed and confirmed to be absent of viable cells or residual DNA from the *C. glutamicum* production strain (section B.7)

B.4 Manufacturing process

B.4.1 Detailed Description of the Production Process

The production process consists of two stages: the upstream fermentation (first) stage and the downstream purification (second) stage.

B.4.2 Non-confidential Summary of the Production Process

The first stage, upstream processing (USP), is fermentation for the production of 2'-FL in a stirred type reactor (STR). Fermentation of the production organism is performed in a complex medium including yeast extract, soy peptone and trace elements with well controlled process parameters (e.g., temperature, pH, dissolved oxygen (DO), aeration). Glucose or sucrose is fed continuously as a carbon source and lactose is also fed as substrate for cells to synthesize 2'-FL. The 2'-FL ingredient is excreted into the media. At the end of the fermentation step, the production organism is inactivated by thermal treatment and removed by microfiltration.

After biomass removal, the fermentation broth undergoes a series of downstream purification processes (DSP) to purify 2'-FL by removing impurities sequentially and followed by drying steps. First, large molecules, mainly proteins, are removed by ultrafiltration, followed by a further concentration step (nanofiltration, evaporation) to reduce its volume. Next, active charcoal is applied to remove colour and organic matter, followed by electrodialysis and ion exchange to remove charged inorganic salts. Next, saccharide impurities are removed by chromatography, followed by spray drying to obtain the final 2'-FL ingredient.

B.4.3 Information on input material used in the production process of the novel food

All raw materials and processing aids used in the production of 2'-FL are food-grade and none are major allergens or are derived from major allergens.

B.4.4 Detailed description of the measures implemented for production control and quality and safety assurance (e.g. HACCP, GMP, ISO)

The manufacturing process for Cataya's 2'-FL will be controlled by current Good Manufacturing Practices (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP) plan. Critical Control Points (CCPs) are identified, and in-process controls are implemented throughout the process based on the HACCP plan. Raw materials and processing aids are food-grade, and packaging materials are permitted for food contact use. Master operating instructions are followed, all records are kept, and the final 2'-FL product is controlled and documented by production of a Certificate of Analysis and lot release procedures.

Cataya's 2'-FL product will be manufactured at a site where the food safety management system will comply with Food Safety Systems Certification (FSSC) 22000 or equivalent, International Organization for Standardization (ISO) 9001 and be conducted in accordance with cGMP as established by 21 CFR §117 (U.S. FDA, 2024) and the Food Safety Modernization Act (FSMA).

B.5 Specification for identity and purity

B.5.1 Product Specifications for 2'-FL

We propose no change to the current Specification 2'-fucosyllactose sourced from *Corynebacterium glutamicum* defined under Schedule 3 (S3-51) of the Food Standards Code, as reproduced below.

S3—51 **Specification 2′-fucosyllactose sourced from *Corynebacterium glutamicum***

For 2′-fucosyllactose (2′-FL) sourced from *Corynebacterium glutamicum*, the specifications are the following:

(a) chemical name—α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose;
(b) chemical formula—$C_{18}H_{32}O_{15}$;
(c) molecular weight—488.44 g/mol;
(d) CAS number—41263-94-9;
(e) description—white to off-white/ivory powder;
(f) 2′-FL—not less than 94% (water free);
(g) D-lactose—not more than 3.0% (water free);
(h) L-fucose—not more than 3.0% (water free);
(i) 3-fucosyllactose—not more than 3.0% (water free);
(j) difucosyl-D-lactose—not more than 2.0% (water free);
(k) glucose—not more than 3.0% (water free);
(l) galactose—not more than 3.0% (water free);
(m) water—not more than 9.0%;
(n) ash, sulphated—not more than 0.5%;
(o) ethanol—not more than 1,000 mg/kg (for crystallised product from solvent only);
(p) residual proteins—not more than 0.005%;
(q) lead—not more than 0.02 mg/kg;
(r) arsenic—not more than 0.03 mg/kg;
(s) cadmium—not more than 0.01 mg/kg;
(t) mercury—not more than 0.05 mg/kg;
(u) microbiological:
  (i) total plate count—not more than 500 cfu/g;
  (ii) coliforms—not more than 10 cfu/g;
  (iii) yeasts and moulds—not more than 100 cfu/g;
  (iv) aflatoxin M1—not more than 0.025 µg/kg;
  (v) residual endotoxins—not more than 10 EU/mg

B.5.2 Batch Analysis

B.5.2.1 Analytical methods

Please refer to Table B.5.2.1-1 Compliance Details for Analytical Methods for all methods used. Concerning the measurement of 3-fucosyllactose (3FL), the production strain does not produce 3-FL. Representative production batches have been analysed both in-house at Cataya and by an external laboratory, with results confirming that 3-FL is not detected in the final product. Compliance with the specification is further confirmed by calculation.

Similarly, Cataya does not utilize ethanol at any stage of the production process, nor is it produced by the production organism. Consequently, ethanol analysis not conducted and compliance with the current specification outlined in Schedule 3 (S3-51) of the Food Standards Code is ensured by default.

Table B.5.2.1-1 Compliance Details for Analytical Methods		
Parameter *(e.g. purity, heavy metals, residual solvents, etc.)*	**Analytical method used** *(indicate the name of the method and the relevant file e.g. xxxx.pdf)*	**Limit of Detection (LOD)/ Limit of Quantification (LOQ)**
Appearance, Colour	Visual, ISO6658	N/A
Appearance, Form	Visual, ISO6658	N/A
pH (20°C, 5% solution)	AOAC 981.12	N/A
Assay (w/w)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	N/A
Water content (%)	Karl Fischer titration Current USP/NF	N/A
Ash, (% w/w)	AOAC 923.03	LOQ: 0.01%
Protein content (%)	Bradford assay: SOP_Bud_006, validated by ISO17025 lab	LOQ: 21.7 mg/kg LOD: 6.5 mg/kg
D-lactose (% w/w)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	LOQ: 0.5% LOD: 0.2%
3,2'-Difucosyl-D-lactose (% w/w)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	LOQ: 0.5% LOD: 0.2%
L-Fucose (% w/w)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	LOQ: 0.5% LOD: 0.2%
D-Glucose (% w/w dry matter)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	LOQ: 0.5% LOD: 0.2%
D-Galactose (% w/w dry matter)	HPLC-RID internal method：SOP_Bud_004, validated by ISO17025 lab	LOQ: 0.5% LOD: 0.2%
3-fucosyllactose (% w/w dry matter)	HPAEC-PAD internal research method	LOQ: 0.5% LOD: 0.2%
Arsenic (As) (mg/kg)	EN 15763:2009	LOQ: 0.005 mg/kg
Lead (Pb) (mg/kg)	EN 15763:2009	LOQ: 0.02 mg/kg
Cadmium (Cd) (mg/kg)	EN 15763:2009	LOQ: 0.005 mg/kg
Mercury (Hg) (mg/kg)	EN 15763:2009	LOQ: 0.003 mg/kg
Aflatoxin M1 (µg/kg)	ISO 14501:2021	LOQ 0.01 µg/kg LOD 0.005 µg/kg
Standard Plate Count (CFU/g)	ISO 4833-2	N/A
Yeast and Mould (CFU/g)	ISO21527-2:2008	N/A
Coliforms (CFU/g)	ISO 4832:2006	N/A
Endotoxins (EU/g)	Ph. Eur. 2.6.14	LOQ: 0.05 EU/g
N/A: Not available.		

B.5.2.2 Batch analysis data

The analytical results for five representative production lots of 2'-FL are summarised in Table B.5.2.2.-1 below. These analyses demonstrate that 2'-FL consistently meets the specifications provided.

Table B.5.2.2-1 Batch Analysis Data								
Specification Parameter	**Specification （same as S3-51）**	**Method**	**Lot No.: 23100101 “Batch 1”**	**Lot No.: 23102101 “Batch 2”**	**Lot No.: 23102202 “Batch 3”**	**Lot No.: 23101302 “Batch 4”**	**Lot No.: 23101401 “Batch 5”**	**Mean**
Appearance	White to off-white/ivory powder	Visual, ISO6658	Complies	Complies	Complies	Complies	Complies	Complies
Assay (% w/w dry matter)	≥ 94.0	HPLC-RID	97.4	98.1	96.6	97.5	97.3	97.4
D-Lactose (% w/w dry matter)	≤ 3.0	HPLC-RID[c]	1.4	0.5	1.1	1.0	0.9	1
L-Fucose (% w/w dry matter)	≤ 3.0	HPLC-RID[c]	1.1	1.2	1.0	1.3	1.2	1.2
3-Fucosyllactose (% w/w dry matter)	≤ 3.0	HPAEC-PAD[c]	ND	ND	ND	ND	ND	ND
Difucosyl-D-lactose (% w/w dry matter)	≤ 2.0	HPLC-RID[c]	ND	ND	ND	ND	ND	ND
D-Glucose (% w/w dry matter)	≤ 3.0	HPLC-RID[c]	ND	ND	ND	ND	ND	ND
D-Galactose (% w/w dry matter)	≤ 3.0	HPLC-RID[c]	ND	ND	ND	ND	ND	ND
Water content (%)	≤ 9.0	Karl Fischer USP/NF	3.34	2.91	2.47	1.69	2.72	2.6
Ash (% w/w)	≤ 0.5	AOAC 923.03[a]	<0.01	<0.01	0.01	0.01	0.02	0.01
Protein content (%)	≤ 0.005	Bradford assay[b]	<0.0016	<0.0016	<0.0016	<0.0016	<0.0016	<0.0016
Arsenic (As) (mg/kg)	≤ 0.03	EN 15763:2009[d]	0.00696	<0.005	<0.005	<0.005	<0.005	<0.005
Lead (Pb) (mg/kg)	≤ 0.02		<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Cadmium (Cd) (mg/kg)	≤ 0.01		<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Mercury (Hg) (mg/kg)	≤ 0.05		0.00851	<0.003	<0.003	<0.003	<0.003	<0.005
Aflatoxin M1 (µg/kg)	≤ 0.025	ISO 14501:2021	ND	ND	ND	ND	ND	ND

Total Plate Count (CFU/g)	≤ 500	ISO 4833-2	200	300	<100	<100	100	<500
Yeast and Mould (CFU/g)	≤ 100	ISO 21527-2:2008	<10	<10	<10	<10	<10	<10
Coliforms (CFU/g)	≤ 10	ISO 4832:2006	<10	<10	<10	<10	<10	<10
Endotoxins (EU/mg)	≤ 10	Ph. Eur. 2.6.14[e]	0.005	0.023	0.018	0.004	0.014	0.013

Abbreviations: 2'-FL: 2'-fucosyllactose; CFU: colony forming units; AOAC: Association of Official Analytical Collaboration; dwb: dry weight basis; EU: Endotoxin unit.
HPLC-RID: High Performance Liquid Chromatography - Differential Refractive Index Detector (Internal Method); ISO: International Organization for Standardization; LOQ: Limit of quantitation; LOD: Limit of detection; ND: Not Detected; Ph. Eur.: European Pharmacopeia; USP: United States Pharmacopeia; EN: European Standard. The above methods have been validated or tested in ISO 17025/CNAS condition.
HPAEC-PAD: High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection, research method.
[a] LOQ = 0.01%.
[b] LOD = 16 mg/kg
[c] LOQ = 0.5%; LOD=0.2%
[d] LOQ: Arsenic (As) = 0.005 mg/kg; Lead (Pb)=0.02 mg/kg; Cadmium (Cd)=0.005 mg/kg; Mercury (Hg)=0.003 mg/kg
[e] LOQ = 0.05 EU/g

B.6 Analytical method for detection

B.6.1 Analysis for the absence of 2'-Fucosyllactose

The High Performance Liquid Chromatography coupled with Refractive Index Detector (HPLC-RID) was developed internally to analyse Cataya's 2'-FL product. This analytical method has been validated by an ISO 17025 lab. This method was employed in the batch analysis in Section B.5.2.2 and also stability tests as discussed in Section B.2.2.

An ion chromatography-based method (AOAC 2022.04) will be used to measure the presence of the 2'-FL in nutritive substance in the foods in which it is proposed to be used (Haselberger, P. 2023).

B.6.2 Analysis for the Absence of residual DNA

The 2'-FL is produced with a genetically modified microorganism (GMM) and falls under Category 1 according to the decision tree of EFSA's Guidance on the risk assessment of GMMs and their products intended for food and feed use (EFSA Panel on Genetically Modified Organisms Panel, 2011). For Category 1 GMMs and their products, information to demonstrate the absence of the GMM in the final product and information on the possible presence of recombinant DNA in the final product is required. The absence of GMM DNA was analysed using a validated culture-based method following the "Guidance on the characterisation of microorganisms used as feed additives or as production organisms", Section 3.2, 'Presence of DNA from the production strain' following EFSA guidance (EFSA FEEDAP Panel, 2018). The full report is provided as Annex 5 – "Analysis of Residual DNA from the *Corynebacterium glutamicum* CgBud-584 Production Strain in 2'-FL". As indicated in Annex 1, CgBud-584 = Strain Deposition Certificate CGMCC 7.559.

The residual DNA from the production strain was analysed using a polymerase chain reaction (PCR)-based method. Genomic DNA was isolated in triplicate from three product batches. Positive controls with samples spiked with known quantity and a dilution series of total DNA from the production strain, added to the sample before DNA extraction, were included.

DNA from the production strain was not detected in any of the three batches. The limit of detection (LoD) of the production strain DNA in the product analysed was 1 ng DNA in 1 g of the fermentation product. No PCR inhibition was observed. Please refer to Table B.6.2-1 below.

Table B.6.2-1 Analysis of DNA of the Production Strain in Test Material by PCR

Sample	No-template Control	Positive Control	DNA from Lot 23100101	DNA from Lot 23101302	DNA from Lot 23102202
PCR Analysis	ND	676 bp[a]	ND[b]	ND[b]	ND[b]

ND = Not detected

[a] The limit of detection (LoD) of DNA of the production strain in 2'-FL product analysed by PCR was 1 ng DNA in 1 g of the product. No PCR inhibition was observed.

[b] Three replicate samples from each batch were analysed.

B.6.3 Analyses of presence of viable cells in the novel food

In addition to the absence of DNA, the absence of the production strain was analysed using a validated culture-based method following the "Guidance on the characterisation of microorganisms used as feed additives or as production organisms", Section 3.1, (Absence of the production strain) (EFSA FEEDAP Panel, 2018). According to the Guidance, the absence of the production strain should be investigated by means of a culture-based method targeted to the detection of viable cells. Please refer to Annex 6 for the full study report "Analysis of the

Absence of *Corynebacterium glutamicum* CgBud-584 Production Strain in 2'-FL". As indicated in Annex 1, CgBud-584 = Strain Deposition Certificate CGMCC 7.559.

The need for and type of selective pressure against possible contaminating microbiota in the samples were tested. The culturing time of the production strain under the selected conditions in the absence of the product was determined. The absence of viable cells of the production strain was analysed from a total of 9 g of three independent production batches, each sampled in triplicate. Controls with samples from each of the three test material batches spiked with low counts of viable production strain cells, were included to confirm that the test material did not inhibit the growth of the production strain, if present, in the samples. Extended culturing time was applied to enable recovery of possible stressed cells in the samples.

The culturing time of the production strain at 30 °C on agar was two days. Test material samples spiked with the production strain verified that the medium and culturing conditions enabled the growth of possible production strain cells remaining in the product, and that the product itself did not inhibit the growth of the production strain cells. One colony was detected from 9 g of the product. The colony was analysed using molecular methods. This confirmed that the colony did not represent the production strain. Positive and negative controls were used to verify that the molecular methods enabled the detection of the production strain.

The absence of viable cells of the production strain was demonstrated in nine samples, three from each of the three batches. Each of the nine samples of 1 g was taken from 10-g subsamples. Altogether, 9 g of test material were analysed. No production strain was detected. Please refer to Table B.6.3-1 below.

Table B.6.3-1 Microbial Count in 9 Samples of Test Material

Batch Number	Sample A CFU/g of test material	Sample B CFU/g of test material	Sample C CFU/g of test material	Total CFU/g of test material
23100101	0	0	0	0
23101302	0	0	1[a]	1[a]
23102202	0	0	0	0

[a] Analysis of the detected colony confirmed that the colony does not represent the production strain.

B.7 Proposed food label

The label will remain the same on the final food, i.e. "2'-fucosyllactose"

C Information related to the safety of the nutritive substance

C.1 Information on the toxicokinetics and metabolism of the nutritive substance and, if necessary, its degradation products and major metabolites

There is no change to the toxicokinetics of the purified 2'-FL that is the subject of the application compared to that of existing approved sources.

C.2 Information from studies in animals or humans that is relevant to the toxicity of the nutritive substance and, if necessary, its degradation products and major metabolites

C.2.1 Toxicology Testing Strategy

This application is to amend the specification for 2'-Fucosyllactose from Genetically modified strain of *Corynebacterium glutamicum* ATCC 13032, which is currently already approved in Schedule 26 of the Code. Consequentially the key data provided above demonstrate that:

1. the 2'-FL is structurally identical to 2'-FL from human milk
2. the production organism has a QPS host and a full in-silico assessment, based on whole genome sequence, demonstrate the absence of external genes with toxigenic potential, allergenic potential or antimicrobial resistance potential
3. The purity matches that for the currently approved 2'-FL from the same host organism
4. There are no changes to the proposed uses or exposure of the 2'-FL

As further supporting data, which has been generated to support mandatory requirements for the Peoples Republic of China (PRC), the following studies have been provided, and are discussed in detail in the sections below:

- Acute oral toxicity test report
- Short-term - Repeated dose 90-day oral toxicity test in the rat
- Developmental - Teratogenicity test in the rat
- *Genotoxicity*
    - *In vitro* Bacterial reverse mutation test
    - *In vitro* mammalian chromosome aberration test
    - *In vivo* mammalian erythrocyte micronucleus test

The studies were sponsored by our commercial partner.

Please refer to Table C.2.1-1 for the details of the study protocols and comparison of GB standards to OECD Guidelines.

Table C.2.1-1 Toxicology Study Guidelines Followed		
Study Title	**Chinese Standard**	**Equivalent OECD Standard**
Acute oral toxicity test report	GB 15193.3-2014	OECD Test No. 420 (OECD, 2002)
Repeated dose 90-day oral toxicity test in the rat	GB 15193.13-2015	OECD Test No. 408 (OECD, 2018)
Teratogenicity test in the rat	GB 15193.14-2015	OECD Test No. 414 (OECD, 2018)
In vitro Bacterial reverse mutation test	GB 15193.4-2014	OECD Test No. 471 (OECD, 2020)
In vitro mammalian chromosome aberration test	GB 15193.23-2014	OECD Test No. 473 (OECD, 2014)
In vivo mammalian erythrocyte micronucleus test	GB 15193.5-2014	OECD Test No. 474 (OECD, 1997)

All tests were conducted in CMA (China Metrology Accreditation) accredited laboratories, in Hunan Institute for Occupational Disease Prevention Institute, following the evaluation procedures and methods of the National Food Safety Standard GB 15193 (GB 15193. *National Food Safety Standard – General Rules for Toxicological Assessment of Food*. Standardization Administration of the People's Republic of China (SAC), Beijing.).

C.2.2 Acute oral toxicity test

GB 15193.3-2014. OECD Test No. 401 (OECD, 1987)
Annex 7 Hunan Occupational Disease Prevention and Treatment Institute Inspection Report acceptance number: JC20240001-A1. 30 January 2024. Proprietary and Confidential.

Whilst the EFSA Scientific Guidance does not mandate or specifically include acute study data, it is mandated by the PRC guidance and so is included here for the sake of transparency and completeness.

10 male and 10 female SD rats of SPF grade were selected and, based on the limit test, dosed orally at 10.0 g/kg bodyweight in a single administration. The rats were monitored continuously for 14 days and any manifestations of toxicity and mortality recorded.

No apparent signs of toxicity or mortality were observed during the 14-day observation period. The animals were sacrificed at the end of the test, and no abnormalities were found in gross pathology. The result of acute oral toxicity test was a lethal dose $(LD)_{50}$ value of at least 10.0 g/kg bodyweight, suggesting the test sample 2'-FL belonged to the actual non-toxic grade according to the acute toxicity classification in GB 15193.3-2014.

C.2.3 Repeated dose 90-day oral (dietary administration) toxicity test in the rat

GB 15193.13-2015. OECD Test No. 408 (OECD, 2018)
Annex 8 Hunan Occupational Disease Prevention and Treatment Institute Inspection Report acceptance number: JC20240001-M4. 30 January 2024. Proprietary and Confidential.

A total of 60 female and 60 male of SD rats of SPF grade (4 weeks old) were used in the experiment. Forty female and forty male rats were selected and randomised into three dose groups (low, medium, high) and a control group (20 rats per group, half male and half female). Additionally, a mid-term control satellite group, a mid-term high-dose satellite group, a recovery control satellite group, and a recovery high-dose satellite group were established, with 10 rats in

each group, half male and half female. The test sample was incorporated directly into the feed. The concentrations of test sample in the feed for the high-dose group (including high-dose satellite groups), medium-dose group and low-dose group were set at 10%, 5% and 2.5% respectively. Control groups and their satellite groups were fed with basic feed without test sample.

Test animals were fed continuously for 90 days (mid-term satellite groups were fed for 45 days), while the recovery satellite groups were observed for an additional 28 days after stopping giving the test sample. General performance, behaviour, signs of toxicity and mortality of the animals were monitored daily. Weekly records of body weight, food consumption and food utilization rate were kept.

Ophthalmic examinations were conducted before and at the end of the test period. Haematology, blood biochemistry and urinalyses examinations were performed at mid-term (satellite groups), end of the test period and end of the recovery period (satellite groups). All animals underwent gross pathology at end of the test and end of the recovery period (satellite groups).

Bodyweight and food consumption

During the test period, comparisons between the body weight at different time points, total weight gain, food consumption at different time points, total food consumption, food utilization rate at different time points and overall food utilization rate in the various dose groups showed no statistically significant differences (P>0.05) or biological significance compared to the control group. The body weight changes during the test period are illustrated in Figure C.2.3-1 and Figure C.2.3-2. During the recovery observation period, there were no statistically significant differences (P>0.05) in body weight at different time points, total weight gain, food consumption at different time points, total food consumption, food utilization rate at different time points and overall food utilization rate between the high-dose satellite group and control satellite group. Based on body weight and food consumption, the actual intake doses of female and male in the low, medium and high dose groups were calculated to be 2.28, 4.51, 9.16 g/kg bodyweight/day and 1.89, 3.67, 7.43 g/kg bodyweight/day, respectively.

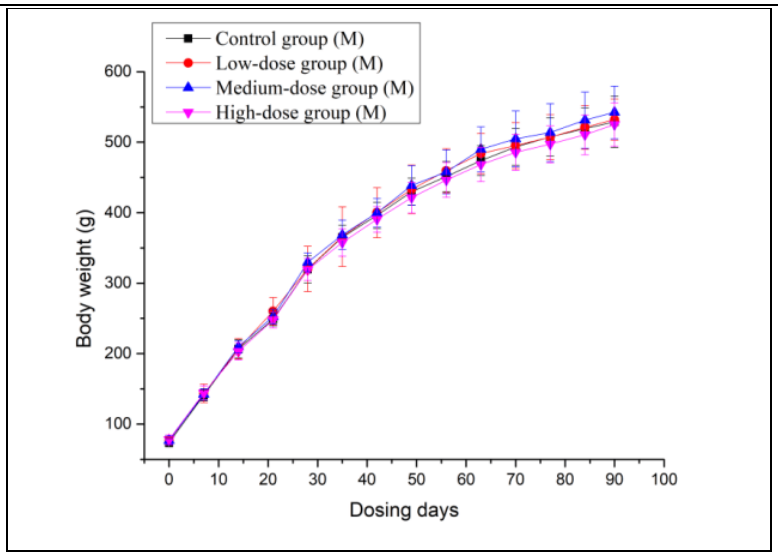


Figure C.2.3-1 Effects of 2'-FL on body weight of male rats

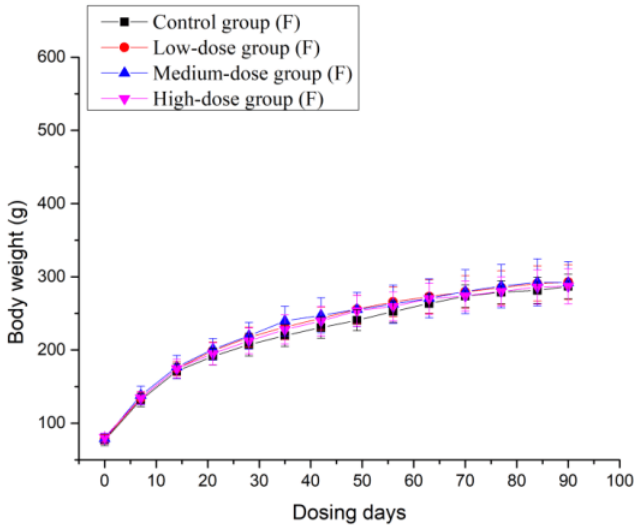


Figure C.2.3-2 Effects of 2'-FL on body weight of female rats

Examination of eyes

Eye examinations (cornea, lens, bulbar conjunctiva, iris) were performed before and at the end of the test, and no obvious pathological changes were observed in the eyes of rats. All examination results were normal.

Haematology

At the end of the test, comparisons of haematological indicators between the various dose groups and the control group showed no statistically significant differences (P>0.05) as indicated in Table C.2.3-1 As shown in Table C.2.3-2, during the mid-term of the test, in the high-dose satellite group, male rats had a statistically significant decrease in activated partial thromboplastin time (APTT) values compared to the control satellite group after 45 days of giving test sample (P<0.05). However, this difference was within the historical control range of the laboratory, indicating no biological significance. Other haematological indicators in both male and female of the high-dose satellite group did not show statistically significant differences compared to the control satellite group (P>0.05). At the end of the recovery period, comparisons of haematological indicators between the high-dose satellite group and control satellite group in both male and female showed no statistically significant differences (P>0.05).

Table C.2.3-1 Haematology Summary Data – Main Dose Groups					
Sex	**Test item**	**Group (N=10)**			
		Control group	**Low-dose group**	**Medium-dose group**	**High-dose group**
Female	WBC ($\times10^9$/L)	2.79±0.60	2.72±0.72	3.17±0.71	2.37±0.73
	NEU (%)	19.01±5.20	18.88±6.68	18.43±5.64	17.83±4.72
	LYM (%)	73.14±5.98	70.01±12.82	74.27±7.63	75.01±5.45
	MON (%)	4.67±1.11	3.80±2.01	3.65±1.81	3.71±1.77
	EOS (%)	3.18±0.97	3.30±1.32	3.61±1.44	3.45±1.08
	BAS (%)	0.00±0.00	0.01±0.03	0.04±0.13	0.00±0.00
	RBC ($\times10^{12}$/L)	8.10±0.44	7.93±0.54	7.89±0.38	7.87±0.41
	HGB (g/L)	162.8±9.2	160.5±6.3	157.4±5.9	157.5±9.5
	HCT (%)	45.7±2.4	45.0±2.1	44.1±1.6	44.0±2.7
	PLT ($\times10^9$/L)	901±83	909±81	910±107	924±105
	PT (sec)	14.94±0.60	14.76±0.43	14.83±0.70	14.41±0.45
	APTT (sec)	13.87±2.90	14.15±2.36	13.70±1.99	14.61±2.32
Male	WBC ($\times10^9$/L)	4.44±1.65	4.71±1.46	4.73±1.32	3.83±1.04
	NEU (%)	24.84±8.06	30.01±4.34	29.23±8.21	29.56±7.24
	LYM (%)	67.87±10.07	61.71±5.31	62.25±10.45	61.62±7.75
	MON (%)	4.38±1.75	4.61±1.18	4.69±2.21	5.19±1.47
	EOS (%)	2.91±1.46	3.65±179	3.83±1.29	3.63±1.17
	BAS (%)	0.00±0.00	0.02±0.04	0.00±0.00	0.00±0.00
	RBC ($\times10^{12}$/L)	9.16±0.71	9.77±0.83	9.26±0.50	9.31±0.44
	HGB (g/L)	172.5±11.2	181.0±13.1	177.6±6.2	173.6±8.1
	HCT (%)	50.6±3.2	53.1±3.6	52.4±2.7	50.4±2.7
	PLT ($\times10^9$/L)	971±102	873±155	924±122	1003±157
	PT (sec)	15.92±0.97	16.56±0.97	16.37±1.12	16.65±0.95
	APTT (sec)	18.16±1.82	17.84±0.93	18.21±1.51	17.56±1.26

Note: N indicates the number of animals in each group.
Abbreviations: WBC, white blood cell count; NEU, neutrophils; LYM, Lymphocytes; MON, Monocytes; EOS, Eosinophils; BAS, Basophils; RBC, red blood cell count; HGB, haemoglobin; HCT, haematocrit; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time.

Table C.2.3-2 Haematology Summary Data – Satellite Dose Groups					
Sex	**Test item**	**Group (N=5)**			
		Mid-term of the test (45 d)		**End of the recovery period**	
		Control satellite group	**High-dose satellite group**	**Control satellite group**	**High-dose satellite group**
Female	WBC ($\times10^9$/L)	4.27±0.71	6.87±3.48	4.06±0.65	3.53±0.38
	NEU (%)	14.92±2.79	17.48±1.33	13.00±3.43	17.02±2.07
	LYM (%)	79.22±3.19	76.26±1.56	79.30±5.63	74.80±2.29
	MON (%)	4.80±1.98	4.84±0.77	6.24±2.48	6.74±1.29
	EOS (%)	1.06±0.34	1.38±0.61	1.46±0.44	1.44±0.60
	BAS (%)	0.00±0.00	0.04±0.09	0.00±0.00	0.00±0.00
	RBC ($\times10^{12}$/L)	7.86±0.42	8.23±0.36	7.63±0.49	7.32±0.32
	HGB (g/L)	159.2±11.3	165.2±8.9	149.0±13.3	144.2±5.4
	HCT (%)	46.2±3.4	48.5±1.9	42.3±3.6	41.4±1.5
	PLT ($\times10^9$/L)	780±130	823±112	1077±309	875±132
	PT (sec)	17.80±0.51	18.04±1.14	16.58±0.58	15.61±0.47
	APTT (sec)	16.06±0.61	16.90±0.80	14.26±1.33	14.86±1.65
Male	WBC ($\times10^9$/L)	2.48±0.50	2.94±1.57	5.13±1.86	3.75±0.58
	NEU (%)	14.22±8.71	16.18±8.42	21.10±6.06	18.70±3.01
	LYM (%)	81.68±9.55	79.60±10.65	72.52±8.09	73.90±4.57
	MON (%)	2.78±1.00	4.38±2.47	4.46±2.79	5.94±1.98
	EOS (%)	1.32±0.90	1.84±1.54	1.92±0.50	1.46±0.81
	BAS (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	RBC ($\times10^{12}$/L)	7.53±0.55	8.27±0.52	7.70±0.77	7.12±0.41
	HGB (g/L)	153.0±7.5	163.8±10.5	148.4±12.5	139.0±5.0
	HCT (%)	44.2±2.2	47.7±3.4	42.6±4.6	39.6±2.1
	PLT ($\times10^9$/L)	756±131	680±76	860±105	916±174
	PT (sec)	18.25±0.32	18.65±0.56	17.59±0.72	18.17±1.36
	APTT (sec)	16.46±0.84	15.06±1.02*	15.82±1.61	14.74±1.86

Note: N indicates the number of animals in each group. *Compared with the respective control group, the difference is statistically significant, P<0.05.
Abbreviations: WBC, white blood cell count; NEU, neutrophils; LYM, Lymphocytes; MON, Monocytes; EOS, Eosinophils; BAS, Basophils; RBC, red blood cell count; HGB, haemoglobin; HCT, haematocrit; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time.

Blood biochemistry

As demonstrated by the data provided in Table C.2.3-3, comparisons of blood biochemistry indicators between the various dose groups and the control group showed no statistically significant differences (P>0.05). According to Table C.2.3-4, after 45 days of giving the test sample, during the mid-term of the test, comparisons of blood biochemistry indicators between the high-dose satellite group and their respective control groups in both male and female did not show any statistically significant differences (P>0.05). At the end of the recovery period, in the high-dose satellite group, the Albumin (Alb) value in female rats was higher than that in the control satellite group, and this difference was statistically significant (P<0.01). However, this difference was within the historical control range of the laboratory, indicating no biological significance. Other blood biochemistry indicators in both male and female rats did not show statistically significant differences compared to the control group (P>0.05)(Table 3.2.3-4).

Table C.2.3-3 Blood Biochemistry Summary Data – Main Dose Groups					
Sex	**Test item**	**Group (N=10)**			
		Control group	**Low-dose group**	**Medium-dose group**	**High-dose group**
Female	TP (g/L)	63.2±4.5	60.7±2.8	64.5±4.4	65.8±3.1
	Alb (g/L)	36.6±3.4	35.6±1.9	37.9±2.5	38.6±1.9
	ALT (U/L)	33.7±12.4	36.1±12.3	46.5±19.5	41.0±21.0
	AST (U/L)	113.1±25.5	115.0±19.8	138.6±59.9	117.9±36.7
	ALP (U/L)	62.3±16.8	61.8±16.3	56.7±20.1	47.7±11.3
	Cr (μmol/L)	60.5±11.3	66.6±5.0	56.7±11.1	63.2±11.1
	Urea (mmol/L)	8.86±1.10	8.58±0.90	8.70±1.30	9.18±0.68
	TC (mmol/L)	2.60±0.44	2.50±0.43	2.69±0.41	2.62±0.22
	TG (mmol/L)	0.40±0.07	0.40±0.06	0.38±0.06	0.37±0.07
	Glu (mmol/L)	7.99±1.40	9.04±1.38	8.37±1.11	8.56±0.83
	GGT (U/L)	0.34±0.42	0.29±0.34	0.25±0.28	0.18±0.31
	Na^+ (mmol/L)	140.9±2.0	140.5±1.3	141.2±0.9	141.0±0.8
	K^+ (mmol/L)	3.73±0.29	3.66±0.25	3.80±0.31	3.67±0.19
	Cl^- (mmol/L)	109.8±2.0	108.5±1.4	110.2±1.4	108.0±4.1
Male	TP (g/L)	57.3±3.5	56.5±4.4	55.0±2.5	55.2±2.5
	Alb (g/L)	32.5±1.2	32.3±1.0	31.5±0.8	31.1±1.5
	ALT (U/L)	26.5±5.1	27.5±3.1	34.2±9.7	26.3±4.7
	AST (U/L)	114.4±31.4	111.6±21.4	128.7±45.4	111.4±29.0
	ALP (U/L)	95.2±20.7	104.7±30.3	105.2±15.7	103.9±14.3
	Cr (μmol/L)	56.6±7.9	57.8±8.6	58.0±7.4	55.7±10.1
	Urea (mmol/L)	7.39±1.12	7.05±0.81	7.01±0.70	6.86±0.85
	TC (mmol/L)	1.76±0.30	1.76±0.44	2.08±0.64	1.83±0.26
	TG (mmol/L)	0.24±0.06	0.24±0.07	0.30±0.08	0.27±0.04
	Glu (mmol/L)	8.41±0.96	7.55±1.52	7.52±0.79	6.97±0.98
	GGT (U/L)	0.29±0.31	0.22±0.39	0.24±0.61	0.28±0.22
	Na^+ (mmol/L)	139.6±1.8	140.3±1.7	141.3±0.7	141.7±1.8
	K^+ (mmol/L)	4.37±0.32	4.84±1.79	4.19±0.22	4.33±0.25
	Cl^- (mmol/L)	108.9±1.8	109.4±1.2	109.5±1.9	110.1±2.0

Note: N indicates the number of animals in each group.
Abbreviations: TP, Total Protein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Cr, creatinine; TC, total cholesterol; TG, triglyceride; Glu, glucose; GGT, Gamma-glutamyl transpeptidase; Na+, sodium; K+, potassium; Cl-, chlorine.

Table C.2.3-4 Blood Biochemistry Summary Data – Satellite Dose Groups					
Sex	**Test item**	**Group (N=5)**			
		Mid-term of the test (45 d)		**End of the recovery period**	
		Control satellite group	**High-dose satellite group**	**Control satellite group**	**High-dose satellite group**
Female	TP (g/L)	54.1±2.9	53.4±1.2	66.0±3.7	70.9±0.9
	Alb (g/L)	32.8±1.1	32.2±0.7	39.0±1.6	42.8±0.8**
	ALT (U/L)	37.7±11.0	30.0±4.8	36.6±15.5	49.7±6.8
	AST (U/L)	138.7±49.3	102.1±17.5	128.8±49.4	153.0±44.1
	ALP (U/L)	184.0±35.5	235.1±40.5	48.5±10.2	49.7±12.6
	Cr (μmol/L)	49.4±8.2	47.2±5.3	48.9±3.7	51.6±5.2
	Urea (mmol/L)	7.40±0.88	7.59±0.82	7.87±1.18	7.41±0.57
	TC (mmol/L)	1.56±0.32	1.70±0.33	2.83±0.70	2.89±0.85
	TG (mmol/L)	0.26±0.09	0.37±0.17	0.43±0.07	0.40±0.07
	Glu (mmol/L)	9.30±2.71	8.36±2.34	8.70±1.71	9.41±1.70
	GGT (U/L)	0.90±0.23	0.88±0.23	0.20±0.27	0.34±0.34
	Na^+ (mmol/L)	137.6±1.3	139.0±0.4	138.3±1.1	139.2±0.8
	K^+ (mmol/L)	4.51±0.47	4.58±0.37	3.91±0.36	3.56±0.19
	Cl^- (mmol/L)	106.4±1.9	108.2±1.0	108.2±1.7	109.1±0.6
Male	TP (g/L)	58.4±2.1	57.6±2.6	56.3±1.3	56.3±3.5
	Alb (g/L)	35.3±0.7	34.8±1.3	31.6±1.3	33.2±0.6
	ALT (U/L)	36.4±25.5	33.3±14.4	49.0±22.7	40.1±22.2
	AST (U/L)	108.6±34.2	117.4±47.3	142.8±61.8	100.2±28.9
	ALP (U/L)	99.8±20.0	104.6±35.1	90.2±14.8	100.6±6.8
	Cr (μmol/L)	55.0±7.6	50.4±6.9	63.3±14.8	73.6±24.2
	Urea (mmol/L)	10.3±0.9	9.1±1.4	7.51±0.82	7.46±1.99
	TC (mmol/L)	2.02±0.41	2.02±0.24	2.22±0.60	1.67±0.31
	TG (mmol/L)	0.34±0.08	0.27±0.04	0.44±0.30	0.36±0.18
	Glu (mmol/L)	7.82±1.41	7.21±1.19	10.3±2.9	10.1±2.9
	GGT (U/L)	0.72±0.31	0.56±0.38	0.36±0.34	0.32±0.27
	Na^+ (mmol/L)	139.2±1.0	139.5±1.9	139.1±1.7	137.3±2.4
	K^+ (mmol/L)	4.35±0.57	4.40±0.22	4.25±0.38	4.29±0.55
	Cl^- (mmol/L)	110.1±1.9	110.7±1.8	108.5±0.8	107.5±1.3

Note: N indicates the number of animals in each group. **Compared with respective control group, the difference is statistically significant, P<0.01.
Abbreviations: TP, Total Protein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Cr, creatinine; TC, total cholesterol; TG, triglyceride; Glu, glucose; GGT, Gamma-glutamyl transpeptidase; Na+, sodium; K+, potassium; Cl-, chlorine.

Urinalyses

The results showed that the urine in both female and male rats was normal in appearance. As depicted in Table C.2.3-5, the comparison of urine indicators between the various dose groups and the control group at the end of the test showed no statistically significant differences (P>0.05). Similarly, as shown in Table C.2.3-6, at the mid-term of the test and at the end of the recovery period, the comparison of various urine indicators between the high-dose satellite group and the control satellite group indicated no statistical significance (P>0.05).

Table C.2.3-5 Urinalysis Summary Data - Main Dose Groups					
Sex	**Test item**	**Group (N=10)**			
		Control group	**Low-dose group**	**Medium-dose group**	**High-dose group**
Female	Appearance	faint yellow	faint yellow	faint yellow	faint yellow
	SG	1.000~1.005	1.005	1.000~1.005	1.000~1.005
	PH	8~9	7~8	8~9	7~9
	ERY	0/10	0/10	0/10	0/10
	PRO	0/10	0/10	0/10	0/10
	GLU	0/10	0/10	0/10	0/10
Male	Appearance	faint yellow	faint yellow	faint yellow	faint yellow
	SG	1.000~1.015	1.000~1.010	1.000~1.020	1.000~1.010
	PH	7~9	8~9	7~9	8~9
	ERY	0/10	0/10	0/10	0/10
	PRO	0/10	0/10	0/10	0/10
	GLU	0/10	0/10	0/10	0/10
Note: The data shown in GLU, PRO, ERY and other columns of the test items represent the number of detected animals/the number of inspected animals; N indicates the number of animals in each group. Abbreviations: SG, Specific gravity; PH, potential of hydrogen; ERY, erythrocytes; PRO, protein; GLU, glucose.					

Table C.2.3-6 Urinalysis Summary Data - Satellite Dose Groups					
Sex	**Test item**	**Group (N=5)**			
		Mid-term of the test (45 d)		**End of the recovery period**	
		Control satellite group	**High-dose satellite group**	**Control satellite group**	**High-dose satellite group**
Female	Appearance	faint yellow	faint yellow	faint yellow	faint yellow
	SG	1.000~1.020	1.000~1.025	1.000~1.005	1.000~1.005
	PH	7~9	7~9	9	9
	ERY	0/5	0/5	0/5	0/5
	PRO	0/5	0/5	0/5	0/5
	GLU	0/5	0/5	0/5	0/5
Male	Appearance	faint yellow	faint yellow	faint yellow	faint yellow
	SG	1.000~1.005	1.000~1.005	1.000~1.020	1.000
	PH	8~9	8~9	9	8~9
	ERY	0/5	0/5	0/5	0/5
	PRO	0/5	0/5	0/5	0/5
	GLU	0/5	0/5	0/5	0/5
Note: The data shown in GLU, PRO, ERY and other columns of the test items represent the number of detected animals/the number of inspected animals; N indicates the number of animals in each group. Abbreviations: SG, Specific gravity; PH, potential of hydrogen; ERY, erythrocytes; PRO, protein; GLU, glucose.					

Organ weight and organ/body ratio

From Table C.2.3-7, it can be seen that at the end of the test the thymus weight of male rats in the low-dose group was lower than that of the control group. However, due to the absence of a dose-response relationship, it holds no biological significance. When comparing the weights of various organs of male and female in each dose group with their respective control groups, no statistically significant differences were observed ($P>0.05$). As per Table C.2.3-8, at the end of the recovery period, the brain weight of male rats in the high-dose satellite group was lower than that of the control satellite group, showing statistical significance ($P<0.05$). Nevertheless, the brain-to-body weight ratio did not exhibit any statistically significant difference, thus lacking biological significance (data not shown). When comparing the weights of other organs of male and female rats in the high-dose satellite group with their control satellite group, no statistically significant differences were found ($P>0.05$). Furthermore, the organ-to-body weight ratios of

various organs in each dose group at the end of the test and in the high-dose satellite group at the end of the recovery period, when compared with their respective control groups, showed no statistically significant differences (P>0.05) (data not shown).

Table C.2.3-7 Organ Weight Summary Data – Main Dose Groups					
Sex	**Test item**	**Group (N=10)**			
		Control group	**Low-dose group**	**Medium-dose group**	**High-dose group**
Female	Body weight after fasting	269.0±14.8	274.2±23.7	271.2±29.6	269.7±24.8
	Heart	0.859±0.082	0.889±0.118	0.924±0.148	0.901±0.103
	Thymus	0.327±0.049	0.368±0.120	0.290±0.039	0.315±0.076
	Liver	7.536±1.325	7.338±0.843	7.509±0.619	7.342±0.800
	Spleen	0.521±0.061	0.501±0.075	0.538±0.069	0.471±0.057
	Kidney	1.706±0.122	1.742±0.110	1.745±0.223	1.715±0.147
	Adrenal gland	0.085±0.009	0.084±0.017	0.082±0.014	0.079±0.013
	Brain	1.850±0.122	1.851±0.119	1.798±0.130	1.787±0.101
	Ovary	0.157±0.042	0.165±0.038	0.137±0.039	0.144±0.047
	Uterus	0.821±0.234	0.734±0.190	0.801±0.174	0.939±0.195
Male	Body weight after fasting	499.2±31.9	504.2±27.7	504.8±37.1	478.7±44.8
	Heart	1.432±0.161	1.445±0.134	1.401±0.138	1.323±0.086
	Thymus	0.535±0.123	0.414±0.074*	0.491±0.102	0.435±0.102
	Liver	12.375±1.465	12.133±0.801	12.166±1.114	11.453±1.063
	Spleen	0.831±0.164	0.874±0.154	0.935±0.101	0.844±0.152
	Kidney	3.067±0.315	2.964±0.248	2.933±0.224	2.910±0.298
	Adrenal gland	0.074±0.021	0.080±0.018	0.074±0.016	0.061±0.007
	Brain	2.032±0.132	2.071±0.104	1.974±0.178	2.049±0.113
	Testis	3.596±0.312	3.669±0.436	3.836±0.407	3.735±0.463
	Epididymis	1.639±0.110	1.548±0.195	1.646±0.140	1.582±0.248
Note: * Compared with control group, the difference is statistically significant, P<0.05; N indicates the number of animals in each group.					

Table C.2.3-8 Organ Weight Summary Data – Main Dose Groups			
Sex	**Test item**	**Group (N=5)**	
		Control satellite group (recovery period)	**High-dose satellite group (recovery period)**
Female	Body weight after fasting	278.0±28.5	290.0±14.8
	Heart	0.896±0.053	0.933±0.048
	Thymus	0.322±0.055	0.340±0.035
	Liver	7.772±0.796	8.472±0.516
	Spleen	0.548±0.103	0.524±0.048
	Kidney	1.675±0.155	1.717±0.093
	Adrenal gland	0.067±0.011	0.072±0.008
	Brain	1.804±0.095	1.835±0.082
	Ovary	0.113±0.024	0.147±0.056
	Uterus	0.981±0.146	1.153±0.155
Male	Body weight after fasting	528.4±57.1	496.4±30.5
	Heart	1.356±0.157	1.257±0.111
	Thymus	0.459±0.131	0.393±0.029
	Liver	12.828±1.848	12.343±0.930
	Spleen	0.822±0.079	0.879±0.034
	Kidney	3.094±0.346	2.529±0.520
	Adrenal gland	0.057±0.010	0.058±0.008
	Brain	2.123±0.102	1.989±0.055*
	Testis	3.553±0.251	3.906±0.328
	Epididymis	1.523±0.137	1.552±0.184
Note: * Compared with control group, the difference is statistically significant, P<0.05; N indicates the number of animals in each group.			

Gross anatomy and histopathology

At the end of the test and at the end of the recovery period, gross examinations of the body surface, skull, thoracic and abdominal cavities, as well as their respective organs in all groups of rats revealed no abnormal changes. Histopathology examinations indicated that some rats in the high-dose group exhibited scattered histopathology alterations; however, these changes were sporadic, showed no inter-group variance, and were considered to be related to spontaneous animal pathology rather than the test sample. Therefore, it can be concluded that under the conditions of this test, apart from spontaneous animal pathologies, no histopathological changes related to 2'-FL were observed.

Conclusions

Under the conditions of this test, the no observed adverse effect level (NOAEL) for the repeated dose 90-day oral toxicity test of 2'-FL in female and male rats were determined to be 9.16 and 7.43 g/kg·bw/day, respectively.

C.2.4 Developmental toxicity Teratogenicity test in the rat

GB 15193.14-2015 . OECD 414 (OECD, 2018)

Annex 9 Hunan Occupational Disease Prevention and Treatment Institute Inspection Report acceptance number: JC20240001-R1. 10 July 2024. Proprietary and Confidential.

85 male and 85 non-mated female SD rats of SPF grade (11-12 weeks old) were used in the experiment. After 5 days of acclimation, male and non-mated female rats were paired at a 1:1 ratio, and females were checked for successful mating every day. Each day, mated females were housed in specialized rat breeding boxes (2-4 per box). The day of confirmed mating was designated as day 0 of gestation. Mated females were then randomly assigned to each

experimental group based on body weight using a randomized block design. Each group included 20 successfully mated females. Three dose groups (2500, 5000, and 10000 mg/kg bodyweight/day), along with a solvent control group (purified water) were established. From days 6 to 15 of pregnancy, either the test sample or purified water were administered orally via gavage to the pregnant rats each morning. Daily observations were conducted on the pregnant rats (including skin, fur, eyes, etc) to detect any signs of miscarriage or premature birth. On day 20 of pregnancy, the pregnant rats were sacrificed and necropsy performed to assess parental pregnancy status and foetal development and mortality, preparation and examination of foetal skeletal specimens, and internal organ inspections of the foetuses.

In-life observations

During the test, pregnant rats in all dose groups displayed normal activity and feeding habits, showing no overt signs of toxicity, premonitory miscarriage, or mortality. As gestation progressed, the body weight of pregnant rats increased accordingly. When compared with the solvent control group on days 0, 6, 9, 12, 15, 18 and 20 of pregnancy, there were no statistically significant differences ($P>0.05$). At the end of the test, uterine weight with foetal, total weight gain during the test period, and net weight gain of pregnant rats across all dose groups revealed no statistically significant difference compared to the solvent control group ($P>0.05$).

Effect on foetal development

As indicated in Table C.2.4-1, comparisons of average number of corpus luteum, average number of implantation, average number of live foetus, live foetus rate, resorbed foetus rate, stillborn foetus rate and sex ratio across all dose groups with the solvent control group revealed no statistically significant difference ($P>0.05$). This suggests that 2'-FL has no significant impact on the reproductive capacity of pregnant rats.

Table C.2.4-1. Summary Data on Pregnancy Outcomes														
Group	**Dose (mg/kg bw)**	**Number of pregnant rats**	**Number of corpus luteum**		**Number of implantation**		**Number of live foetus**		**Live foetus rate**		**Stillborn foetus rate**		**Resorbed foetus rate**	
			Total	**Average ($\overline{X}$ ±s)**	**Total**	**Average ($\overline{X}$ ±s)**	**Total**	**Average ($\overline{X}$ ±s)**	**Live foetus / foetal rats**	**Percentage (%)**	**Stillborn foetus/ foetal rats**	**Percentage (%)**	**Resorbed foetus/ implantation**	**Percentage (%)**
Solvent control	0	16	239	14.9±3.8	204	12.8±3.6	196	12.3±3.8	196/196	100.0	0/196	0	8/204	3.9
Low-dose	2500	18	269	14.9±4.6	242	13.1±4.2	235	13.4±4.4	235/235	100.0	0/235	0	7/242	2.9
Medium-dose	5000	16	218	13.6±4.5	184	11.9±4.8	173	10.8±5.1	173/173	100.0	0/173	0	11/184	6.0
High-dose	10000	18	254	14.1±3.5	221	12.8±3.8	208	11.6±4.6	208/208	100.0	0/208	0	13/221	5.9
Abbreviations: bw = bodyweight														

Effects on growth and development of foetal rats

As shown in Table C.2.4-2, comparisons of body length, tail length and body weight of foetal rats, and placental weight among different dose groups with the solvent control group showed no statistically significant difference (P>0.05), indicating that 2'-FL has no significant impact on the growth and development of foetal rats.

Table C.2.4-2 Summary Data on Growth and Development result of live foetal rats in teratogenicity test								
Group	**Number of pregnant rats with live foetal**	**Number of live foetus**	**Sex**		**Body weight of foetal rats (g)**	**Placental weight (g)**	**Body length of foetal rats (mm)**	**Tail length of foetal rats (mm)**
			Female/male	**Ratio**				
Solvent control	16	196	104/92	1.13	4.84±0.88	0.67±0.13	41.81±2.47	15.65±0.75
Low-dose	18	235	120/115	1.04	4.50±0.75	0.67±0.11	40.37±2.32	15.16±0.92
Medium-dose	16	173	88/85	1.04	4.82±0.87	0.72±0.12	41.00±2.63	15.78±0.99
High-dose	18	208	115/93	1.24	4.91±0.70	0.74±0.12	41.36±1.78	15.81±0.67

Effects on appearance, internal organs and bones of foetal rats

The appearance examination of the head, trunk and limbs of foetal rats in each dose group did not reveal any deformities, and the internal organ examinations of the head, thoracic cavity and abdominal cavity of foetal rats also showed no abnormalities. The occurrences of skeletal malformation in the solvent control group, low-dose group, medium-dose group and high-dose group were 1 (1 litter), 2 (2 litters), 2 (2 litters) and 0 (0 litter) respectively. Based on the results of these examinations, the comparisons of the number of external malformation, internal organ malformation, skeletal malformation and total malformation rates of foetuses of foetal rats among the dose groups with the solvent control group showed no statistically significant difference ($P>0.05$), indicating that 2'-FL has no teratogenic effect on foetal rats. For detailed information on the total malformation rate of foetuses in each group, please refer to Table C.2.4-3.

Table C.2.4-3 Effect of 2'-FL on total malformation rate of foetuses								
Malformation type	**Solvent control group**		**Low-dose group**		**Medium-dose group**		**High-dose group**	
	Malformed litters/ litters examined	**Malformed foetal rats/foetal rats**	**Malformed litters/ litters examined**	**Malformed foetal rats/foetal rats**	**Malformed litters/ litters examined**	**Malformed foetal rats/foetal rats**	**Malformed litters/ litters examined**	**Malformed foetal rats/foetal rats**
External malformation	0/16	0/196	0/18	0/235	0/16	0/173	0/18	0/208
Internal organ malformation	0/16	0/94	0/18	0/115	0/16	0/84	0/18	0/99
Skeletal malformation	1/16	1/102	2/18	2/120	2/16	2/89	0/18	0/109
Total malformation rate (%)	0.51(1/196)		0.85(2/235)		1.16(2/173)		0(0/208)	

Conclusions on the teratology test

The above results indicate that, under the conditions of this test, the NOAEL of 2'-FL on pregnant rats and foetal rats is ≥10.0 g/kg bodyweight/day.

C.2.5 Genotoxicity Testing

C.2.5.1 Bacterial reverse mutation test

GB 15193.4-2014. OECD Test No. 71 (OECD, 2020)
Annex 10 - Hunan Prevention and Treatment Institute for Occupational Disease. Inspection acceptance number: JC20240001-G1. 3 March 2024. Proprietary and Confidential.

The strains used were *Salmonella typhimurium* TA97a, TA98, TA100, TA102 and TA1535. The 10% S9 mixture was prepared using auxiliary factors and rat liver microsomal enzymes (S9) (which was induced by the combination of phenobarbital sodium and β-naphthoflavone) as an *in vitro* metabolic activation system. Based on the result of preliminary test, the formal test included five dose groups: 5000, 1582, 500, 158 and 50 μg/plate, while the validation test included five dose groups: 5000, 1000, 200, 40 and 8 μg/plate. Untreated control group, solvent control group (purified water and DMSO) and positive control groups were set up simultaneously in the above two tests. The test was conducted using the plate incorporation method, and the treated plates were cultured at 37℃ for 48 h, and the results were observed.

Both the formal and validation tests revealed that, under the condition with/without S9 mixture, the revertant colony numbers in each dose group did not exceed twice the revertant colony numbers in the untreated control group, and no dose-response relationship was observed. In contrast, the revertant colony numbers in the positive control group exceeded twice the revertant colony numbers in the untreated control group. These findings indicate that the bacterial reverse mutation test for 2'-FL yielded negative results, suggesting that under the conditions of this test, 2'-FL does not possess mutagenic properties. Please refer to Table C.2.5.1-1 for the results of the formal test.

Table C.2.5.1-1. Results of bacterial reverse mutation test exposed to 2'-FL (formal test)											
Group		**Dose level (/plate)**	**TA97a**		**TA98**		**TA100**		**TA102**		**TA1535**	
			-S9	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
2'-FL		50 μg	114±5	110±3	36±5	41±6	122±13	151±3	271±11	285±16	11±3	10±3
		158 μg	110±13	98±6	35±8	43±7	140±39	134±30	270±24	256±10	12±3	14±3
		500 μg	112±8	97±5	37±2	40±3	157±33	129±18	268±15	275±22	11±1	12±1
		1582 μg	98±12	104±8	25±1	38±4	148±47	135±25	270±21	253±19	9±0	12±1
		5000 μg	107±16	98±8	34±7	41±4	155±46	125±25	264±10	277±19	13±2	12±1
Untreated control		—	108±7	106±12	39±2	38±2	161±13	119±1	268±16	259±16	14±1	13±4
Solvent control	H_2O	100 μL	105±14	100±12	31±4	43±6	145±26	143±27	265±8	253±9	11±1	12±3
	DMSO	100 μL	107±2	98±9	38±3	27±1	—	153±4	267±15	259±6	—	13±2
Positive control		—	2148±168	1192±144	923±102	1292±406	1500±241	1688±142	1124±367	1279±126	887±215	944±42
Note: Positive control and dose level: -S9-TA97a, TA98, TA102: dexon (50 μg/plate); TA100, TA1535: sodium azide (1.5 μg/plate); +S9-TA97a, TA98, TA100: 2-aminofluorene (10 μg/plate); TA102, TA1535: 1,8-dihydroxyanthraquinone (50 μg/plate).												

C.2.5.2 *In vitro* Mammalian chromosome aberration test

GB 15193.23-2014. OECD Test No. 473 (OECD, 2014)
Annex 11 Hunan Occupational Disease Prevention and Treatment Institute Inspection Report acceptance number: JC20240001-G3. 9 May 2024. Proprietary and Confidential.

Chinese Hamster Lung (CHL) cell line was used. The 10% S9 mixture was prepared using auxiliary factors and rat liver microsomal enzymes (S9) (which was induced by the combination of phenobarbital sodium and β-naphthoflavone) as an *in vitro* metabolic activation system.

Three dose groups were established at 1250, 2500 and 5000 μg/mL, alongside solvent control (DMEM culture solution) and positive controls (without S9 mixture: methyl methanesulfonate (MMS, 20 μg/mL), with S9 mixture: cyclophosphamide (CP, 10 μg/mL)).

The day before the test, CHL cells were seeded in culture bottles and cultured in a CO_2 incubator. During the test, the culture solution was removed from the bottles, and 100 μL of test sample or control solution, 0.5 mL of 10% S9 mixture (or serum-free culture solution when S9 mixture is not added) and 4.4 mL of serum-free culture solution was added, and the bottles were cultured for 4 hours in the incubator at 37°C. After the treatment, the culture solution containing the test sample was removed, cells were washed three times with PBS solution, and 5 mL of complete culture solution was added for further incubation at 37°C for 24 hours. Four hours before the end of the incubation, 50 μL colchicine at 100 μg/mL was added to the culture bottles.

Afterwards, cells underwent digestion, hypotonic treatment and fixation were added to slides and stained before microscopic analysis. Under the microscope, 100 well-dispersed metaphase cells were analysed in each group to record the coordinates and types of aberrant cells. Additionally, a validation test without the S9 mixture was conducted, extending the exposure time of test sample to 24 hours, using the same doses, with the final concentration of methyl methane sulfonate (positive control) adjusted to 10 μg/mL.

As shown in Table C.2.5.2-1, under the conditions of exposure to the test sample for 4 hours with or without S9 mixture, the chromosome aberration rates in the solvent control group were 1.0% and 2.0%. The chromosome aberration rates in the dose groups ranged from 0% to 2.0%, with no statistical significance compared with the corresponding solvent control groups ($P>0.05$). The chromosome aberration rates in the positive control group (MMS and CP) were 10.0% and 10.0%, showing statistically significant differences compared to the corresponding solvent control groups ($P<0.05$ and $P<0.01$, respectively).

The results presented in Table C.2.5.2-2 demonstrate that under the conditions of exposure to the test sample for 24 hours, without S9 mixture, the chromosome aberration rates in the dose groups ranged from 1.0% to 3.0%. These differences were not statistically significant when compared to the solvent control group (chromosome aberration rate was 1.0%) ($P>0.05$). However, the chromosome aberration rate in the positive control group (MMS) was 9%, showing statistically significant differences compared to the solvent control group ($P<0.01$).

The above results indicated that under the conditions of this test, 2'-FL did not induce chromosomal aberrations in CHL cells, and the result of in vitro mammalian chromosome aberration test was negative for both aneurgenicity and clastogenicity.

Table C.2.5.2-1 Effects of 2'-FL on chromosome aberration rate of CHL cells (4 h)

Group		Final concentration (/mL)	S9mix	Number of metaphase cells	Chromosome number change			Chromosome structure change					Number of chromosome aberration	Number of aberrant cells	Chromosome aberration rate (%)
					Aneuploid /polyploid	Endoreduplication		Gap	Breakage	Fragment	Microbody	Ring	Others			
Solvent control		—	-	100	0	0		0	1	0	0	0	1	2	2	2.0
			+	100	0	0		0	1	0	0	0	0	1	1	1.0
2'-FL		1250 μg	-	100	0	0		0	0	1	1	0	0	2	2	2.0
			+	100	0	0		0	0	0	0	0	0	0	0	0
		2500 μg	-	100	0	0		0	0	1	0	0	0	1	1	1.0
			+	100	0	0		0	2	0	0	0	0	2	2	2.0
		5000 μg	-	100	0	0		0	1	0	0	0	0	1	1	1.0
			+	100	0	0		0	0	0	1	0	0	1	1	1.0
Positive control	MMS	20 μg	-	100	0	0		2	4	3	1	0	4	14	10	10.0*
	CP	10 μg	+	100	1	0		1	3	3	0	0	3	11	10	10.0**

Note: Other changes in chromosome structure in the table include double centromere, multiple centromere, chromatid exchange, comminution, multiple aberration, etc. Chromosome gap is not included in the number of aberrant cells.

* Compared with solvent control group, the difference is statistically significant, $P<0.05$; ** compared with solvent control group, the difference is statistically significant, $P<0.01$.

Table C.2.5.2-2 Effects of 2'-FL on chromosome aberration rate of CHL cells (24 h)

Group	Final concentration (/mL)	S9 mix	Number of metaphase cells	Chromosome number change			Chromosome structure change					Number of chromosome aberration	Number of aberrant cells	Chromosome aberration rate (%)
				Aneuploid /polyploid	Endoreduplication	Gap	Breakage	Fragment	Microbody	Ring	Others			
Solvent control	—	-	100	0	0	0	1	0	0	0	0	1	1	1.0
2'-FL	1250 μg	-	100	0	0	0	0	1	0	0	0	1	1	1.0
	2500 μg	-	100	0	0	0	1	0	0	0	1	2	2	2.0
	5000 μg	-	100	0	0	0	1	1	0	0	1	3	3	3.0
Positive control (MMS)	10 μg	-	100	0	0	1	3	4	1	0	2	11	9	9.0**

Note: Other changes in chromosome structure in the table include double centromere, multiple centromere, chromatid exchange, comminution, multiple aberration, etc. Chromosome gap is not included in the number of aberrant cells.

** compared with solvent control group, the difference is statistically significant, $P<0.01$.

C.2.5.3 *In vivo* mammalian erythrocyte micronucleus test

GB 15193.5-2014. OECD Test No. 474 (OECD, 1997)
Annex 12 Hunan Occupational Disease Prevention and Treatment Institute Inspection Report acceptance number: JC20240001-G2. 14 June 2024. Proprietary and Confidential.

Select specific pathogen free (SPF)-grade healthy adult Kunming (KM) mice (25 males and 25 females) were divided into 5 groups (10 mice per group, with an equal distribution of males and females). The test included three dose groups: 10000, 5000 and 2500 mg/kg bodyweight, along with the solvent control group (purified water) and positive control group (cyclophosphamide). The method of giving test sample twice in 30 h period was adopted, and the interval between administrating the two samples was 24 h. The sternal bone marrow samples were collected 6 hours after the final dose for slide preparation. Under the microscope, 2000 polychromatic erythrocytes (PCE) per mouse were examined to calculate the micronucleus incidence, and 200 total red blood cells (RBC) were examined to calculate the proportion of PCE in the total red blood cells.

As shown in Table C.2.5.3-1, the micronucleus incidences in the positive control groups of female and male were 27.8‰ and 25.3‰, respectively, which were significantly higher than that in the solvent control group ($P<0.01$). In contrast, the micronucleus incidences in the dose groups ranged from 1.7‰ to 2.2‰, and did not differ significantly from that in the solvent control group ($P >0.05$). The micronucleus incidence in solvent control group, dose groups and positive control group were generally within respective historical control data (HCD) ranges. These results indicate that under the conditions of this test, 2'-FL did not have a significant impact on the micronucleus incidence in the polychromatic erythrocytes of mice.

Table C.2.5.3-1. Results of *in vivo* mammalian erythrocyte micronucleus test exposed to 2'-FL								
Group	**Dose level (/kg·bw)**	**Sex**	**Number of animals**	**Number of RBC**	**Number of PCE containing micronucleus**	**Micronucleus incidences (‰, $\overline{X}$ ±s)**	**PCE/RBC**	**PCE/RBC ($\overline{X}$ ±s)**
Purified water	20 mL	Female	5	10000	20	2.0±0.5	549/1000	0.55±0.03
		Male	5	10000	19	1.9±0.2	553/1000	0.55±0.05
Test samples	2500 mg	Female	5	10000	22	2.2±0.3	530/1000	0.53±0.03
		Male	5	10000	17	1.7±0.3	541/1000	0.54±0.04
	5000 mg	Female	5	10000	17	1.7±0.3	533/1000	0.53±0.07
		Male	5	10000	19	1.9±0.2	614/1000	0.61±0.04
	10000 mg	Female	5	10000	18	1.8±0.3	501/1000	0.50±0.04
		Male	5	10000	19	1.9±0.4	596/1000	0.60±0.06
Cyclophosphamide	40 mg	Female	5	10000	278	27.8±1.4**	536/1000	0.54±0.03
		Male	5	10000	253	25.3±1.4**	626/1000	0.63±0.06
Note: **Compared with solvent control group, P<0.01.								

C.3 International safety assessments

See Section 3.1.1 D and J.2 under General Requirements

D.1 List of food groups or foods likely to contain the nutritive substance

See Section 3.1.1 C. No change to currently approved 2'-FL from other sources.

D.2 Proposed maximum levels in food groups or foods

See Section 3.1.1 C. No change to currently approved 2'-FL from other sources.

D.3 Likely level of consumption

See Section 3.1.1 C. No change to currently approved 2'-FL from other sources.

D.4 Percentage of food group to use nutritive substance

Please refer to Section 3.1.1 D. No change to currently approved 2'-FL from other sources.

D.5 Use in other countries (if available)

Please refer to Section 3.1.1 D.

D.6 Where consumption has changed, information on likely consumption

No change to currently approved 2'-FL from other sources.

E.1 Need to permit addition of vitamin or mineral

Not applicable

E.2 Demonstrated potential to address deficit or health benefit

No change to currently approved 2'-FL from other sources.

F.1 Nutritional purpose (other than vitamins and minerals)

Non-digestible oligosaccharide

G.1 Consumer awareness and understanding

No change to currently approved 2'-FL from other sources.

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