SYNAURA 虹摹生物

May 30, 2023

To
Paulette Gaynor, PhD
GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway College Park, MD 20740-3835
USA

Subject: GRAS Notification for the intended use of 2'-Fucosyllactose as a Food Ingredient

Dear Dr. Gaynor:

In accordance with 21 CFR § 170 Subpart E consisting of sections § 170.203 through § 170.285, Synaura Biotechnology (Shanghai) Co., Ltd. (Synaura) hereby submits, the enclosed notice of a claim that 2'-Fucosyllactose (2'-FL) produced by fermentation with a recombinant *Escherichia coli* EB011065 strain, as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

As required, please find enclosed three copies of the GRAS notification. If you have any questions or require additional information, please feel free to contact me by phone at: +86-0571-89716570 or by email at < wing.yu@cirs-group.com >. Thank you

Sincerely,

[redacted]

Application R&D Director
Synaura Biotechnology (Shanghai) Co., Ltd.

Enclosure: Three copies of GRAS notification

虹摹生物科技（上海）有限公司
Synaura Biotechnology (Shanghai) Co., Ltd.
上海市浦东新区芙蓉花路 500 弄 2 号楼 1-2 层
Floor 1-2, Building 2, Lane 500, Furong Hua Road, Pudong New Area, Shanghai, CHINA

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF

2'-FUCOSYLLACTOSE (2'-FL)

AS A FOOD INGREDIENT

Submitted To:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway College Park, MD 20740-3835
USA

Submitted By:
Synaura Biotechnology (Shanghai) Co., Ltd.
Floor 1-2, Building 2, Lane 500,
Furong Hua Road, Pudong New Area,
Shanghai, China

May, 2023

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF 2'FUCOSYLLACTOSE (2'-FL) AS A FOOD INGREDIENT

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1. PART I- SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR § 170 Subpart E consisting of sections § 170.203 through § 170.285, Synaura Biotechnology (Shanghai) Co., Ltd. (Synaura) hereby informs the FDA that 2'-Fucosyllactose (2'-FL) produced by fermentation with a recombinant *Escherichia coli* EB011065 strain, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Synaura's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described below.

Synaura Biotechnology (Shanghai) Co., Ltd. [a holding subsidiary of China Mengniu Dairy Company Limited, registered in China as Hongmo Shengwu Keji（Shanghai）Youxian Gongsi] has developed 2'-Fucosyllactose, subject of present GRAS.

1.1. Basis of Conclusion:

This GRAS conclusion for the use of 2'-Fucosyllactose (2'-FL) has been reached in accordance with the requirements in 21 CFR 170.220.

1.2. Name and address of organization:

Synaura Biotechnology (Shanghai) Co., Ltd.
Floor 1-2, Building 2, Lane 500,
Furong Hua Road, Pudong New Area,
Shanghai, CHINA

1.3. Name of substance:

The name of the substance of this GRAS assessment is 2'-Fucosyllactose. The substance is also known as 2'-FL; 2'-Fucosyl-D-lactose; 2'-O-fucosyllactose.

1.4. Intended conditions of use:

2'-Fucosyllactose (2'-FL) is intended to be used as a food ingredient in milk and soy-based, non-exempt infant formula for term infants at a maximum level of 2.4 g/L of formula as consumed; in toddler formulas (intended for children > 12 months of age) and meal replacement drinks for children ages 1-3 years at a maximum level of 2.4 g/L, as consumed; in infant and toddler foods at maximum levels of 10.0 g/L in drinks, 10.9 g/kg in cereals and desserts, 57 g/kg in dry snacks; in beverages (sports and "energy" drinks, flavored waters, fruit juices and drinks, milk drinks, dairy analogs, milk-based meal replacements) at maximum levels ranging from 0.8-6 g/L; and in the following foods, at maximum levels ranging from 4.8-80 g/kg: breakfast cereals; frozen dairy desserts; puddings, fillings, mousses; yogurt; meal replacement and snack bars; syrups; and jams and jellies. It is recognized that there are Standard of Identity requirements for some of these specified foods and these foods will not be referred by their commonly recognized names.

1.5. Statutory Basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.6. Exemption from Premarket approval requirements:

Synaura has concluded that 2'-FL is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion that 2'-FL, meeting the specifications cited herein, and when used as a food ingredient in selected conventional food products and infant formula, is GRAS and is therefore exempt from the premarket approval requirements.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that 2'-FL, when used as described in this dossier, is GRAS based on scientific procedures.

1.7. Availability of data and information:

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting

Synaura Biotechnology (Shanghai) Co., Ltd.
Floor 1-2, Building 2, Lane 500,
Furong Hua Road, Pudong New Area,
Shanghai, CHINA

Phone: +86-13811045993
Email: yaofei@mengniu.cn

The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.8. Data exempt from Disclosure:

Parts II through VII of this GRAS notification does not contain any data or information that is exempt from disclosure under the Freedom of Information Act. There is no privileged or confidential information such as trade secrets and/or commercial or financial information in this document. Therefore, the information contained in this dossier can be made publicly available.

1.9. Certification:

Synaura certifies that, to the best of its knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by Synaura, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of 2'-FL. Synaura accepts responsibility for the GRAS determination that has been made for 2'-FL as described in this dossier.

1.10. Name, position/title of responsible person who signs dossier and signature:

Fei Yao
Application R&D Director
Synaura Biotechnology (Shanghai) Co., Ltd.
Floor 1-2, Building 2, Lane 500,
Furong Hua Road, Pudong New Area,
Shanghai, CHINA

Phone: +86-13811045993
Email: yaofei@mengniu.cn

Signature: 

1.11. FSIS/USDA – Use in Meat and/or Poultry:

Synaura does not intend to add 2'-FL to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

2. PART II- IDENTITY AND TECHNICAL INFORMATION

2.1. Description and Characterization

The subject of this GRAS assessment, 2'-fucosyllactose (2'-FL), is a standardized preparation produced by fermentation using a genetically engineered *Escherichia coli* EB011065 strain, and contains not less than 94% 2'- FL. It is a white powder that primarily consist of 2'-FL and minor quantities of other chemically-related sugars or by-products. General descriptive characteristics of 2'-FL are summarized in Table 1. 2'-FL is a fucosylated, neutral trisaccharide composed of L-fucose, D-galactose, and D-glucose units. The molecular constitution of 2'-FL can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by α-(1→2) bond to form the trisaccharide. It is a trisaccharide that occurs only as one specific constitutional isomer. The chemical structure of 2'-FL is provided in Figure 1.

Table 1. General Descriptive Characteristics of 2'-Fucosyllactose (2'-FL)

Parameter	Description*
Source	By fermentation of *Escherichia coli* EB011065 strain
Common name	2'-Fucosyllactose; 2'-*O*-fucosyllactose
Common Abbreviation	2'-FL; 2-FL; 2FL
Alternative names	2'-O-Fucosyllactose; 2'-Fucosidolactose; 2'-O-L-Fucosyl-D-lactose; Fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc-α-(1→2)-Gal-β-(1→4)-Glc
IUPAC name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucose
CAS No.	41263-94-9
Chemical formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol
Appearance	Powder
Color	White to almost white
Odor	Neutral
Taste	Lactose like
Storage	Store sealed in a cool, dry place
Shelf life	2 years
Accelerated Stability	6 months

*Based on information provided by Synaura and other publicly available

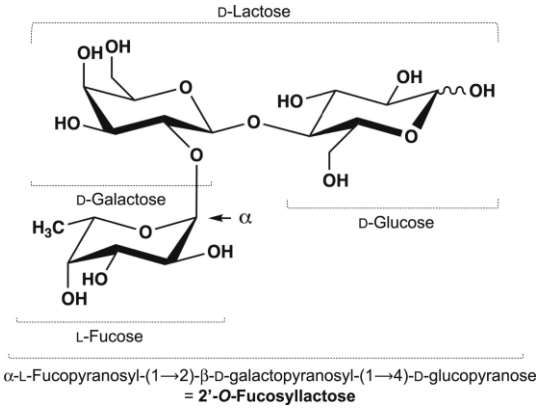


Figure 1. Chemical Structure of 2'-Fucosyllactose (2'-FL).

2’-FL is one of the most prevalent oligosaccharides present in human milk (Urashima et al., 2012). Additionally, it is also found in the milk of goats, pigs, chimpanzees, bonobos, and orangutans (Castanys-Muñoz et al., 2013; Chaturvedi et al., 2001). Given the significant levels of 2'-FL in human milk, it is often categorized as a human milk oligosaccharide (HMO). 2'-FL, the subject of present GRAS manufactured by Synaura, has been fully characterized and identity is established by HPAEC-PAD and other identification methods as described below. It is demonstrated to be qualitatively identical to 2’-FL that is present in human milk from lactating women.

2.2. Manufacturing Process

2’-Fucosyllactose (2’-FL) from Synaura is manufactured according to current good manufacturing practices (cGMP) for food ingredients. As described below, 2’-FL is produced by fermentation using a genetically engineered *E. coli* EB011065 strain.

Production Organism:

To improve the GDP-Fucose pool, several specific genetic manipulations were performed in the genome of recipient *E. coli* BL21 (DE3) strain by Synaura Biotechnology (Shanghai) Co., Ltd., which included knocking out of *lac*Z, *fuc*Ik, *ara*A, *rha*A, *wca*J, and *pfk*A genes, respectively, and knocking in of *lac*Y gene.

Additionally, heterologous genes necessary to biosynthesize 2’-FL in the above engineered *E.coli* BL21 (DE3) were inserted into the pACYCduet-1 and pET28a plasmids, respectively, resulting in the construction of the pBCGW plasmid (Appendix D Figure D1) and pGT065 plasmid (Appendix D Figure D2). The pBCGW plasmid contains four heterologous genes and pGT065 plasmid contains α-1,2-fucosyl-transferase gene to enable 2’-FL biosynthesis in the engineered *E.coli* BL21 (DE3). The heterologous genes are *man*B (*E.coli* Phospho-mannomutase), *man*C (*E.coli* mannose-1-phosphate guanylyltransferase), *gmd* (*E.coli* GDP-mannose-4,6- dehydratase) and *wca*G (*E.coli* GDP-fucose synthase) and *gt065* gene (*Neisseria* α-1,2-fucosyltransferase). Synaura’s 2’-FL producing strain, designated as *E.coli* EB011065, was obtained after transforming the pBCGW and pGT065 into the engineered *E.coli* BL21 (DE3). Overexpression of the above-mentioned genes were achieved by using a strong bacteriophage T7 promoter, and The T7 terminator was used to terminate the transcription. Additional details of genetic manipulations and genetic stability testing are described in Appendix D and Appendix E, respectively.

The available information shows that genetically modified *E. coli* has been used in the production of human-identical milk oligosaccharides (HMOs) including several GRAS ingredients such as 2’-FL; lacto-N-neotetraose (LNnT); 2’-fucosyllactose/difucosyllactose (2’-FL/DFL); lacto-N-tetraose (LNT); 6’-sialyllactose (6’-SL) sodium salt; 3’-sialyllactose (3’-SL) sodium salt; 3-fucosyllactose (3-FL); and lacto-N-fucopentaose I/2’-fucosyllactose (LNFP-I/2’-FL).

Raw Materials and Processing Aids:

The raw materials and processing aids used in the manufacture of 2’-FL are all typically used in the microbial fermentation carried out for food ingredient production. These include: Glucose; Lactose; Peptone; Yeast extract; Sodium hydroxide (NaOH; pH control); Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$); Potassium dihydrogen phosphate

(KH_2PO_4); Sodium Sulfate (Na_2SO_4); Magnesium sulfate ($MgSO_4$); Sodium chloride (NaCl); Ammonium chloride (NH_4Cl); Calcium chloride ($CaCl_2$); Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$); Ferric Chloride ($FeCl_3$); Ammonia water/Liquid ammonia (NH_3) and IPTG (Isopropyl ß-D-1-thiogalactopyranoside; small amount to induce 2'-FL biosynthesis). The majority of these ingredients are fermentation nutrients, while others are used for foam control; pH control; or as an inducer in fermentation. In addition to the processing aids in the manufacturing of 2'-FL, the following purification aids are used: Cation exchange resin; Anion exchange resin; Diatomite Activated carbon; Ethanol; and Flocculant. All raw materials and processing aids are food-grade quality and are safe and suitable for use in the manufacture of food ingredients consistent with appropriate U.S. federal regulations, or have previously been determined to be GRAS.

Process Description:

The production of 2'-fucosyllactose (2'-FL) by *E. coli* EB011065 complies with the current Good Manufacturing Practice (cGMP) requirements, and the raw materials and processing aids used meet the quality requirements for food additive production. The purity of the culture strains is checked during the fermentation process, including seed culture, by microscopic observation and/or agar plating. The production process of 2'-FL includes two steps: fermentation and purification.

A. Fermentation:

The fermentation process is carried out as the extracellular expression by production strain of *E. coli* converting the glucose and lactose in the medium into 2'-FL. The entire fermentation process is controlled in a closed fermenter under sterile conditions, and the temperature and pH are precisely controlled. The schematic diagram of the fermentation process is shown in Figure 2, and the specific operation steps are as follows:

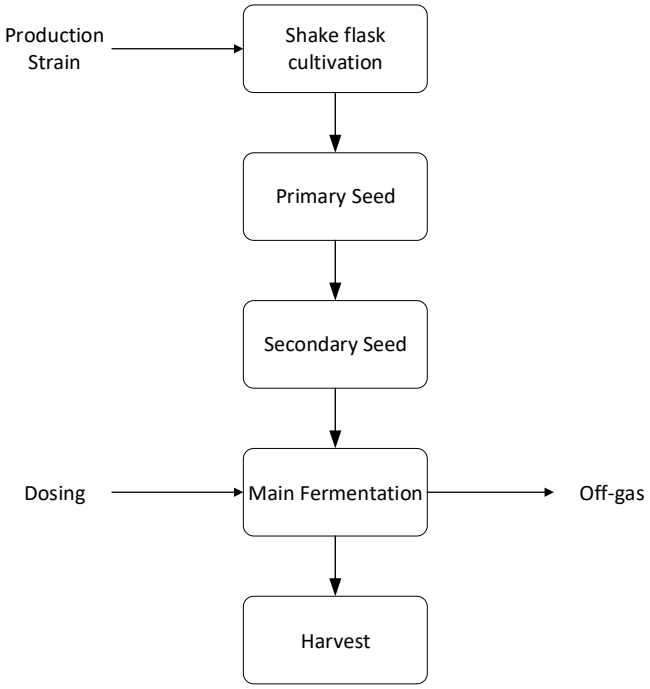


Figure 2. 2'-Fucosyllactose (2'-FL) Fermentation process flow

1) Preparation of culture medium

Add a prescribed amount of water into the mixing tank; weigh raw materials according to the recipe of the culture medium; and, add the raw materials to the mixing tank to dissolve.

2) Calibration of DO electrode and pH electrode

Calibrate the dissolved oxygen (DO) electrode and pH electrode and install them on the seed tank and main fermenter, transfer the prepared medium to the seed tank, main fermenter and dosing tank, respectively. This is followed by adjusting the volume according to the process requirements and afterwards performing Medium sterilization.

3) Seed cultivation

Take the production strains out from the cell bank; then inoculate and cultivate in shake flasks. Transfer the shake flask broth into the primary seed tank by a certain ratio; cultivate at above 30°C for about several hours until OD_{600} reach certain value; and, then inoculate the secondary seeds at a predefined ratio. In the secondary seed tank, cultivate for several hours to reach predefined OD_{600} value to enrich the biomass.

4) Actual Fermentation

Inoculate the secondary seeds into the main fermenter by a certain ratio, and adjust the temperature, DO, pH and other parameters according to the process control requirements. Add inducer-IPTG and lactose when biomass concentration reaches a certain level. During the fermentation process, the growth and metabolism of production strain, lactose consumption and product accumulation were all monitored.

5) Harvest

When the speed of 2'-FL synthesis slows down the fermentation is stopped (normally in several days), and the fermentation broth was harvested. (Heat treatment can be applied to be fermentation broth.)

B. Purification:

The purification of 2'-FL is comprised of several steps which are shown in Figure 3. In order to satisfy different customer requirements, the sequence and selection of purification steps can be adjusted to ensure that the final product is in compliance with physical, chemical and microbial specifications. The processing aids used in the purification process are all suitable for food processing.

Detailed purification process steps are described below:

1) Production strain removal

The purpose of this step is to remove production strain from fermentation broth. For this, suitable solid-liquid separation method can be used, such as centrifugation, flocculation, and membrane filtration, and the filtrate is subjected for subsequent purification operations. The isolated production strains are subjected to heating or other inactivation treatment.

After this process, the production strains are removed and the proteins will be denatured. As a result, the final product doesn't contain viable producer strain.

2) Ultra-Filtration

The molecular weight of 2'-FL is 488.4. Based on this property, the permeate can be obtained by ultrafiltration using a suitable ultrafiltration membrane. In this way, most of the macromolecules like protein, DNA, endotoxin and other substances, can be removed, and the product can also be clarified during this process.

In addition, based on product quality requirements or customer needs, other types of membranes (different molecular weight cut-offs) can be selected for purification or concentration purposes.

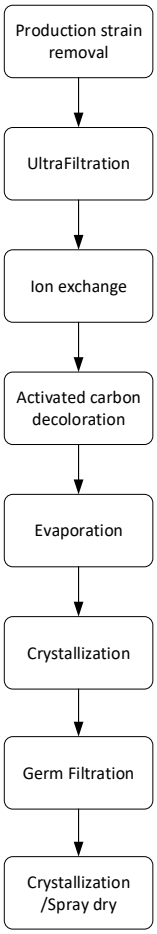


Figure 3. 2'-FL Purification process flow

3) Ion exchange

To remove anions and cations contained, the product will pass through an activated cation exchange resin and an anion exchange resin, respectively; first the cation column and then anion column. After this treatment, rinsing of the column with purified water is performed.

Impurities such as salts, amino acids, and pigments can be removed in this step.

4) Decolorization

To take advantage of the strong adsorption capacity of activated carbon, the product is also treated with activated carbon for decolorization at room temperature.

This step can remove the pigment and other impurities in the product.

5) Germ filtration

In this step, the product is filtered through a sterilizing filter. It can remove foreign particles and reduce the bioburden as well. Normally, this step will be carried out before the final drying or concentration operation.

6) Crystallization

In this step, the product is concentrated to above 40% (w/v) at certain temperature, then ethanol is added dropwise with stirring until a white powder precipitates. The product is then cooled down to ambient temperature, and the product is filtered and dried. By doing this, the product purity is improved simultaneously.

7) Drying

Dry the material at about suitable temperature to produce powders with uniform particle size.

Sampling and testing should be finished before the final product is released by the quality assurance department to ensure full specifications compliance.

As described above, the fermentation process is strictly controlled under sterile conditions. After the fermentation, the high heat treatment will greatly reduce the number of the viable strain, which also denatures most proteins. Through ultrafiltration, endotoxin, residual protein and DNA molecules are removed; then salts, amino acids and pigments in the product are removed through ion exchange treatment; pigments are removed by a decolorization step; and, in the last step, water is removed through crystallization, fine drying or spray drying.

As a product of genetically engineered microorganisms, 2'-FL is of high purity and does not contain viable production strains, DNA or protein fragments. This is demonstrated by the absence of the modified strain, DNA or protein fragments in the final product, thus supporting the safety of the final product. The *E. coli* strain used in the production of 2'-FL is considered as non-pathogenic and non-toxigenic.

None of the raw materials used in the fermentation are major allergens or are derived from major allergens. Although lactose used in the fermentation is derived from milk, a concentration of residual lactose in the finished ingredient is not significant.

2.3. Specifications and Identity

In order to ensure a consistent and safe product, Synaura has established food grade specifications for 2'-FL (Table 2). The analytical methods used for the qualitative and quantitative analysis of the individual specification parameters are validated for their intended use. Analytical results from five lots of 2'-FL demonstrate that it is consistently manufactured and meets the standard specifications. The purity of 2'-FL is at least 94% of the product and is based on High-Performance Liquid Chromatography – Refractive Index Detector (HPLC-RID) analysis (area normalization method) method. HPLC chromatograms of monosaccharides for L-Fucose, D-Glucose, 2'-fucosyllactulose, D-Lactose, 2'-FL, and Difucosyllactose are provided in Appendix C-1.

To ensure the purity of the final product, upper limits have been established for the raw

materials and processing aids used in the manufacturing (e.g., D-lactose, acetic acid), the carbohydrates formed during the fermentation (e.g., L-fucose, difucosyllactose, 2'-fucosyl-D-lactulose), residual solvent, heavy metals, and microbiological parameters. Analytical data from three non-consecutive representative batches of 2'-FL (Table 3) demonstrate compliance with the product's physical, chemical and microbiological specifications and the ability of the method of manufacture to produce a consistent product.

2.3.1. Chemical Identity of 2'-Fucosyllactose

By employing HPAEC-PAD, Synaura has demonstrated that its 2'-FL is chemically and structurally identical to those of the reference materials (Dalian Institute of Chemical Physics, Chinese Academy of Sciences; Batch number is 20220727, Purity greater than 98%). This analysis confirmed the chemical equivalence of Synaura 2'-FL to reference 2'-FL. A brief summary of these analyses is as follows:

In the HPAEC-PAD analysis, similar retention times were observed for the main component of Synaura 2'-FL compared with the reference 2'-FL (the retention time of reference 2'-FL is 9.91 min, the retention time for three batches of Synaura 's 2'-FL is 9.92 min, 9.91 min, 9.91 min). This information suggest that Synaura 2'-FL is substantially equivalent or is identical to the 2'-FL reference. The HPAEC-PAD analysis of Synaura produced 2'-FL along with the reference standard is presented in Appendix C-2.

In addition to HPAEC-PAD, Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) and Infrared Spectroscopy was used to confirm identity of 2'-FL. This information revealed identical positive fragmentation patterns between the main component of the Synaura's 2'-FL and reference 2'-FL. Furthermore, identity of the product was also confirmed by comparison with the ^{1}H and ^{13}C NMR spectra of Synaura's 2'-FL to reference samples. The results of other spectrogram such as Dept135, COSY, HSQC, HMBC, and HSQC-TOCSY spectra of reference and three batches are also basically consistent. All this information further confirms the identity of the subject of the present GRAS.

Table 2. Food Grade Specifications of 2'-Fucosyllactose (2'-FL)

Parameters	Specification	Analytical Methods
Color	White to off-white	In-house: Transfer a suitable amount of sample into a clean and dry white porcelain dish or beaker, and observe its color and form under natural light.
Form	Powder	
2'-Fucosyllactose (calculated on the dried basis), *w*/%	≥ 94%	HPLC-RID (Area normalization method) See A.1 of Appendix A
pH (5% in water)	3.2–7.0	General Chapter <0631>, Volume IV, ChP 2020
Sulfated ash, *w*/%	≤ 0.2%	GB 5009.4-2016
Water content, *w*/%	≤ 9.0%	Karl Fischer Titration, GB 5009.3-2016
Content of residual proteins	≤ 100 mg/kg	Spectrophotometry (See Appendix B)
Residual DNA	Negative	General Chapter <3407>, Volume Ⅳ, ChP 2020
Residual ethanol	≤1000 ppm	General Chapter <0861>, Volume Ⅳ, ChP 2020
Related substances		

D-Lactose	≤ 3.0%	HPLC-RID (Area normalization method) (See A.2 of Appendix A)
2'-Fucosyl-D-lactulose	≤ 1.0%	
Difucosyllactose	≤ 1.0%	
L-Fucose	≤ 1.0%	
D-Glucose	≤ 1.0%	
Total impurities	≤ 5%	
Heavy metals		
Arsenic (calculated as As)	≤ 0.1 mg/kg	ICP-MS, GB 5009.11-2014
Cadmium (Cd)	≤ 0.1 mg/kg	Atomic Absorption Spectroscopy, GB5009.15-2014
Mercury (Hg)	≤ 0.05 mg/kg	ICP-MS, GB 5009.17-2014
Lead (Pb)	≤ 0.02 mg/kg	ICP-MS, GB 5009.12-2017
Aflatoxins		
Aflatoxin B_1	⩽ 0.1 g/kg	Method I, GB 5009.22-2016
Aflatoxin B_2	⩽ 0.1 g/kg	Method I, GB 5009.22-2016
Aflatoxin G_1	⩽ 0.1 g/kg	Method I, GB 5009.22-2016
Aflatoxin G_2	⩽ 0.1 g/kg	Method I, GB 5009.22-2016
Aflatoxin M_1	⩽ 0.05 g/kg	Method I, GB 5009.24-2016
Microbial limits		
Aerobic plate count	⩽ 500 CFU/g	GB 4789.2-2016
Molds and yeasts count	⩽ 100 CFU/g	Method I, GB 4789.15-2016
Coliforms count	< 3 MPN/g	Method I, GB 4789.3-2016
Enterobacteriaceae	< 10 CFU/g	Method I, GB 4789.41-2016
Salmonella/(25 g)	Absent	GB 4789.4-2016
Cronobacter (*Enterobacter sakazakii*)/(100 g)	Absent	Method I, GB 4789.40-2016
Shigella/(25 g)	Absent	GB 4789.5-2016
Staphylococcus aureus/(25 g)	Absent	Method I, GB 4789.10-2016
Bacterial endotoxins	⩽ 300EU/g	Gel-Clot Method, General Chapter <1143>, Volume IV, ChP 2020

ChP = Chinese Pharmacopoeia; CFU = colony forming unit; MPN = most probable number; GB = Chinese Nations standard (Guojia Biaozhun).

Table 3. Batch Analysis Data of 2'-Fucosyllactose (2'-FL)

Tests	Test results		
	2102-0215-P1	**2102-0218-P1**	**2102-0220-P1**
Color	Off-white	Off-white	Off-white
Form	Powder	Powder	Powder
pH (5% in water)	5.7	5.8	5.7
Sulfated ash	0.051%	0.043%	0.043%
Water content	0.25%	0.21%	0.25%
Content of residual proteins	Not detected	Not detected	Not detected
Residual DNA	Negative	Negative	Negative
Residual solvent (ethanol)	26.4 ppm	25.6 ppm	24.2 ppm
2'-Fucosyllactose (calculated on the dried basis)	99.5%	99.4%	99.3%
D-Lactose	Not detected	0.02%	0.07%
2'-Fucosyl-D-lactulose	0.34%	0.42%	0.44%
Difucosyllactose	Not detected	Not detected	Not detected
L-Fucose	0.05%	0.06%	0.06%
D-Glucose	Not detected	Not detected	Not detected
Total impurities	0.5%	0.6%	0.7%
Total Arsenic (calculated as As)	Not detected	Not detected	Not detected
Cadmium (Cd)	Not detected	Not detected	Not detected
Mercury (Hg)	Not detected	Not detected	Not detected
Lead (Pb)	Not detected	Not detected	Not detected
Aflatoxin B_1	Not detected	Not detected	Not detected
Aflatoxin B_2	Not detected	Not detected	Not detected
Aflatoxin G_1	Not detected	Not detected	Not detected
Aflatoxin G_2	Not detected	Not detected	Not detected
Aflatoxin M_1	Not detected	Not detected	Not detected
Aerobic plate count	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Molds and yeasts count	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Coliforms count	Absent	Absent	Absent
Enterobacteriaceae	Absent	Absent	Absent
Salmonella/(25 g)	Absent	Absent	Absent
Cronobacter (*Enterobacter sakazakii*)/(100 g)	Absent	Absent	Absent
Shigella/(25 g)	Absent	Absent	Absent
Staphylococcus aureus/(25 g)	Absent	Absent	Absent
Bacterial endotoxins	Not detected	Not detected	Not detected

Limit of detection (LOD) for 'not detected' tests are as follows: Residual protein 17 mg/kg. Total Arsenic (calculated as As) 0.01 mg/kg; Cadmium (Cd) 0.003 mg/kg; Mercury (Hg) 0.003 mg/kg; Lead (Pb) 0.05 mg/kg; Aflatoxin B_1 0.1 µg /kg; Aflatoxin B_2 0.1 µg /kg; Aflatoxin G_1 0.1 µg /kg; Aflatoxin G_2 0.1 µg /kg; Aflatoxin M_1 0.05 µg /kg.

2.3.2. Similarity with Other GRAS Products

As described in details later in Part VI Narrative section, 2'-FL has been the subject of 12 GRAS notices submitted to FDA. A comparison of the specifications of 2'-FL, subject of this present GRAS with 2'-FL from other GRAS notices that received "no question" letters from FDA is provided in Table 4. The comparative information on specifications presented in Table 4 suggest that 2'-FL from Synaura is comparable with the information from other GRAS notices, such as GRN 932 by APTech, GRN 735 by Glycosyn, GRN 650 by Glycom, GRN 571 by Jennewein and GRN 546 by Glycom (these GRAS notices are available at FDA GRAS Inventory website and the web links to these notices, including amendments, are also provided in Table 6). This comparison suggest that the subject of this present GRAS is substantially similar to other GRAS notices. As described later, the safety data described in these GRAS notices (Table 4) that received no question letter from FDA, is applicable to this present GRAS. Residual components present in Synaura's 2'-FL ingredient are also present in human milk or otherwise naturally present in the human body and were present in the material used in the Synaura's 2'-FL toxicological studies.

Table 4. Comparison of 2'-Fucosyllactose from Different GRAS Notices with Present GRAS

Parameters	**2'-FL Derived by Fermentation**					**Synthetic 2'-FL**
	Current GRAS	APTech (GRN 932)	Glycosyn (GRN 735)	Glycom (GRN 650)	Jennewein (GRN 571)	Glycom (GRN 546)
Appearance, Form	Powder	Dry powder	Homogenous powder	Powder or agglomerates	Spray-dried powder	Powder
Appearance, Color	White to almost white	White to off-white/ivory	White	White to off white	White to ivory-colored	White to ivory-colored
Water, %	≤9.0%	≤ 9.0%	≤ 5	≤ 5.0%	≤ 9.0%	≤ 9.0%
Ash, %	≤ 0.2 (sulfated)	≤ 0.5%	≤ 0.2 (sulfated)	≤ 1.5% (sulfated)	≤ 0.5%	≤ 0.2% (Sulphated)
Acetic acid (as free acid and/or sodium acetate)	NS	NS	NS	≤ 1.0%	NS	≤ 0.3%
Residual proteins	≤ 100 mg/kg	≤ 100 µg/g	≤ 0.01%	≤ 0.01%	≤ 100 µg/g	0.1%
Purity	≥ 94%	≥ 94% (Area, dry wt basis)	≥ 90%	≥ 94.0% (water free)	≥ 90% (area)	≥ 95.0% (water free)
Lactose, %	≤3%	≤ 5 (Area)	≤ 3%	≤ 3%	≤ 5% (Area)	NS
Difucosyllactose, %	≤1%	≤ 5 (Area)	NS	≤ 1.0	≤ 5% (Area)	NS
3-FL, %	NS	≤ 5 (Area)	NS	NS	≤ 5% (Area)	NS
Fucosyl-galactose, %	NS	≤ 3 (Area)	NS	NS	≤ 3 (Area)	NS
2'-Fucosyl-D-lactulose, %	≤1%	NS	NS	≤ 1.0	NS	NS

Glucose, %	≤1%	≤ 3 (Area)	≤ 2%	NS	≤ 3% (Area)	NS
Galactose, %	NS	≤ 3 (Area)	≤ 2%	NS	≤ 3% (Area)	NS
Fucose, %	≤1%	≤ 3 (Area)	≤ 2%	≤ 1.0	≤ 3% (Area)	NS
Allo-lactose, %	NS	NS	≤ 2%	NS	NS	NS
Total HMOs, %	NS	NS	NS	≥96	NS	NS
Heavy metals						
Aluminum, ppm	NS	NS	≤ 4.8	NS	NS	NS
Lead, ppm	≤0.02	≤ 0.02	≤ 0.05	≤ 0.1	≤ 0.02	≤ 0.8
Arsenic, ppm	≤0.1	≤ 0.1	≤ 0.1	NS	≤ 0.2	NS
Cadmium, ppm	≤0.1	≤ 0.01	≤ 0.01	NS	≤ 0.1	NS
Mercury, ppm	≤0.05	≤ 0.05	≤ 0.05	NS	≤ 0.5	NS
Microbial limits						
Total plate count or aerobic mesophilic total count, CFU/g	≤500 CFU/g	≤ 500	≤ 3,000	≤ 500	≤ 10,000	≤ 500
Yeast, CFU/g	≤100 CFU/g	≤ 100 (Yeast and Mold)	≤ 10	≤ 10	≤ 100 (Yeast and Mold)	≤ 10
Mold, CFU/g	≤100 CFU/g		≤ 10	≤ 10		≤ 10
Salmonella	Absent in 25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 100 g	ND in 25 g
Enterobacteriaceae	< 10 CFU/g	NS	ND in 10 g	ND in 10 g	ND in 11 g (w/ Coliform)	ND in 10 g
*Cronobacter sakazakii**	ND in 100g	ND in 10 g	ND in 25 g	ND in 10 g	ND in 100 g	ND in 10 g
Listeria monocytogenes	NS	NS	NS	ND in 25 g	NS	ND in 25 g
Bacillus cereus, CFU/g	NS	NS	≤ 100 (presumptive)	≤ 50	NS	≤ 50
Escherichia coli	NS	ND in 25 g	ND in 10 g	NS	NS	NS
Staphylococcus aureus, CFU/g	Absent in 25 g	ND in 1 g	ND in 1 g	NS	NS	NS
Sulphite reducing *clostridia* spores, CFU/g	NS	NS	≤ 30	NS	NS	NS
Clostridium perfringens, CFU/g	NS	NS	ND in 1 g	NS	NS	NS
Residual endotoxins, EU/g	≤ 300	≤ 100	≤ 10,000	NS	≤ 300	≤ 50,000
Aflatoxin M_1, µg/kg	ND	NS	≤ 0.2	NS	≤ 0.025	NS
Residual GMO detection	Negative	NS	Negative	NS	Negative	NS

Adapted from GRN 932, GRN 735. ND=not detected; NS=not specified; **Enterobacter sakazakii*/100 g

2.3.3. Potential Impurities

In order to control the levels of residual impurities and carbohydrate by-products, as well as heavy metals, microbes, and production organism-derived DNA and endotoxin, process controls and product specifications are in place to ensure a consistent, food-grade finished ingredient. The microbial residues generated during the production of 2'-FL were removed by flocculation and centrifugation and are strictly controlled in the final product through testing.

The test results showed that the level of *Enterobacteriaceae* was less than 10 CFU/g, and *Cronobacter* (*Enterobacter sakazakii*) was not detected, as shown in Table 3.

The total proteins generated during the production of 2'-FL were removed by ultrafiltration and ion exchange in process control and are strictly controlled by a modified Bradford method in the in final product testing. The test results showed that no residual proteins were detected, as shown in Table 3. See Appendix B for the analytical procedures.

The nucleic acids generated during the production of 2'-FL were removed by ultrafiltration and ion exchange in process control and are strictly controlled by PCR in the final product testing. The test results showed that no residual nucleic acids were detected, as shown in Table 3.

2.3.4. Microbial Endotoxins

Typical ranges of endotoxin load have been reported for cow's milk (Gehring et al., 2008) and infant formula powder (Townsend et al., 2007). The 2'-FL specification for endotoxin is established to ensure that exposures do not exceed the usual levels that are expected for infant formula powder that are currently on the market. Batch analyses of 2'-FL from three non-consecutive batches demonstrate compliance to the endotoxin specification.

3. PART III- DIETARY EXPOSURE

3.1. Intended Uses and Food Categories

Synaura intends to use 2'-Fucosyllactose (2'-FL) as a food ingredient in infant formula as well as in selected conventional foods. The food categories are as follows:

- In milk and soy-based, non-exempt infant formula for term infants at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder).
- In formulas for toddler and meal replacement drinks for children ages 1-3 years at a maximum level of 2.4 g/L of formula, as consumed (ready-to-drink or reconstituted formula prepared from powder). While FDA does not have a regulatory definition for "toddler formula," it should be noted that the use is as formula intended for children > 12 months of age. Formulas for older infants (e.g., 9-12 months of age) would be included in the category of infant formula and comply with the infant formula regulations under Section 412 of the Federal Food, Drug, and Cosmetic Act (FD&C Act).
- In foods for infants and toddlers at maximum levels of 10 g/L in drinks, 10.9 g/kg in cereals and desserts, 57 g/kg in dry snacks; and
- In selected conventional food categories such as beverages (sports and "energy" drinks, flavored waters, fruit juices and drinks, milk drinks, dairy analogs, milk-based meal replacements) at maximum levels ranging from 0.8-6 g/L; and
- In other conventional foods that include: breakfast cereals; frozen dairy desserts; puddings, fillings, mousses; yogurt; meal replacement and snack bars; syrups; and jams and jellies, at maximum levels ranging from 4.8-80 g/kg.

The details of food categories to which 2'-FL is proposed to be added are summarized in Table 5, along with descriptions of the types of foods within the category, the serving size associated with each food type, and the maximum use level of 2'-FL. The product, 2'-FL by Synaura, is intended for use in the same foods, and at identical use levels, mentioned in the GRN 932 and GRN 735. There are no new food uses proposed by Synaura for 2'-FL. The substance mentioned in GRN 932 and GRN 735 is substantially equivalent to the subject of this present GRAS assessment.

The subject of this GRAS notice, 2'-FL, will not be used in any foods for which food standards would preclude its use. Foods such as meat and poultry products that come under USDA jurisdiction are excluded from the list of intended food uses of 2'-FL.

Table 5. Summary of the Individual Proposed Food Uses and Use Levels for 2'-Fucosyllactose

Proposed Food Category	Food Uses	Use Level (g/serving)	Serving Size (g or mL)	Use Level (g/100 g)
Non-exempt infant and follow-on formula	Infant formula* (0 to 6 months), including ready-to drink formula or reconstituted formula prepared from powder		NA	2.4 g/L
	Follow-on formula* (6-12 months), including ready-to drink formula or formula prepared from powder		NA	2.4 g/L
	Infant meal replacement products	0.24	100	0.24 (400 mg/100 kcal)
Baby foods	Milk formula for toddlers and children aged 12-36 months*		NA	2.4 g/L
	Ready-to-eat, ready-to serve, hot cereals	1.20	15 (dry) 110 (ready-to serve)	1.09 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.20	120	1.00
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.20	110	1.09
	Baby crackers, pretzels, cookies, and snack items	0.40	7	5.70
Beverages and beverage bases	Energy drinks	0.28	360	0.08
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360	0.08
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1.20	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8.00 3.00 2.00
	Hot cereals for adults and children	1.20	40 (dry) ~250 prepared	0.48 (as consumed)
Dairy product analogs	Milk substitutes such as soy milk and imitation milks	0.28	240	0.12
Frozen dairy desserts and mixes	Frozen desserts including ice creams and frozen yogurts, frozen novelties	1.20	~70	1.70
Gelatins, puddings and fillings	Dairy-based puddings, custards and mousses	1.20	~70	1.70
	Fruit pie filling	1.20	85	1.41
	Fruit filling in bars, cookies, yogurt and cakes	1.20	~40	3.00
Grain products and pastas	Bar, including snack bars, meal-replacement bars, and breakfast bars	0.48	40	1.20
Jams and jellies, commercial	Jellies and jams, fruit preserves, and fruit butters	1.20	~20	6.00
Milk, whole and skim	All Acidophilus or fortified milks, non-fat and low-fat fluids, including fluid milk and reconstituted milk powder	0.28	240	0.12

Milk products	Flavored milks, including milk, coffee drinks, cocoa, smoothies (dairy and fruit based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir	0.28	240	0.12
	Milk-based meal replacement beverages or diet beverages	0.28	240	0.12
	Yogurt	1.20	225	0.53
	Formula intended for pregnant women (-9 to 0 months)	1.20	200	0.60
Processed fruits and fruit juices	Fruit drinks, including vitamin and mineral fortified products	0.28	240	0.12
	Fruit juices	0.28	240	0.12
Sweet sauces, toppings, and syrups	Syrups used to flavor milk beverages	0.28	40	0.70

Adopted from GRN 932 (pages 6-8) and GRN 735 (pages 30-31). *ready-to-drink or reconstituted formulas prepared from powder; NA=not applicable' ~=Approximately

3.2. Estimated Daily Intake from the Proposed Uses

As indicated above, 2'-FL is intended for use in the same foods, and at identical levels of addition, as notified in GRN 932 and GRN 735. In GRN 932, it is stated that the intended uses of 2′-FL are identical to those listed previously in GRN 735. The proposed uses and use levels of 2'-FL are presented in Table 5. 2'-FL is proposed for use as a food ingredient in non-exempt term infant formulas, in toddler formulas and meal replacement drinks for children ages 1-3 years, and selected conventional foods at the levels listed in Table 5. The intended use of 2'-FL in the same foods and at the same levels as those in GRN 932 and GRN 735 is not expected to noticeably affect the intake of 2'-FL in the overall diet of the public from introduction into the market by another supplier who will have to compete in essentially the same markets and foods. In GRN 932, estimates for the intake of 2'-FL were determined using the U.S. National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES) 2017-2018. Food codes were grouped in food-use categories according to 21 CFR 170.3.

As 2'-FL will be added to the same food categories and at the same use levels mentioned earlier, the estimated daily intakes (EDIs) are expected to be the same as or similar to those reported in GRN 932 and GRN 735. In GRN 932, the notifier also presented its own comprehensive dietary exposure estimates, including uses proposed in GRN 932 as well as background levels of 2′-FL from limited uses not included in GRN 932 but addressed in earlier GRAS notifications for 2′-FL, and, thus, potentially part of the background diet.

Using the NHANES food consumption data and assuming maximum intended use levels, the estimated dietary exposures (eaters-only) for different age groups determined was as follows:

For infants 0-5 months of age the mean and 90th percentile intake was estimated to be 2.06 g/person/day (0.33 g/kg bw/day) and 3.17 g/person/day (0.53 g/kg bw/day), respectively.

For infants 6-11 months of age the mean and 90th percentile intake was estimated to be 2.60 g/person/day (0.29 g/kg bw/day) and 4.95 g/person/day (0.53 g/kg bw/day), respectively.

For children 1-3 years of age, the estimated dietary exposures to 2′-FL at the mean and 90th percentile were estimated to be 1.45 g/person/day (0.12 g/kg bw/day) and 2.23 g/person/day (0.19 g/kg bw/day), respectively.

For the total population (all ages), estimates of dietary exposure at mean and 90th percentile were estimated to be 1.77 g/person/day (0.034 g/kg bw/day) and 3.59 g/person/day (0.074 g/kg bw/day), respectively.

In GRN 932, based on the similarity of cumulative dietary exposure estimates (GRN 932 and background uses) to the dietary exposure estimates from intended uses in GRN 932 only, it was concluded that inclusion of potential uses outside the scope of the notice does not impact the overall dietary exposure to 2'-FL. This is also applicable to the present GRAS assessment.

In addition to the intake of 2'-FL from its uses in infant formula, in toddler formulas and meal replacement drinks, and in selected conventional foods, in GRN 932 and GRN 735 cumulative total intake of 2'-FL from the proposed uses and background intake (other foods) was also estimated. In response to a FDA query regarding inclusion of 2'-FL uses in certain food categories (e.g., carbonated beverages, flavored waters, and vegetable juices) described in other GRNs for 2'-FL, additional intake analysis, based on the 2017-2018 NHANES dataset, was provided in the amendment to GRN 932. Inclusion of the certain food categories, did not significantly affect the overall estimates of exposure to 2'-FL in the total diet. The mean and 90th percentile cumulative EDIs of 2'-FL in all users in all ages were reported as 1.77 and 3.59 g/person/day from all intended uses, respectively.

The proposed use levels of the subject of this present GRAS are similar to those described in previous 2'-FL GRAS notices. The above described estimates of 2'-FL intake are based on the assumption that the subject of this present GRAS, 2'-FL by Synaura, will replace currently marketed 2'-FL. Thus, cumulative exposures are not expected to change.

4. PART IV- SELF LIMITING LEVELS OF USE

2'-Fucosyllactose (2'-FL) does not have any self-limiting intake levels of use under the conditions of use described in this GRAS notification.

5. PART V- EXPERIENCE BASED ON COMMON USE IN FOODS BEFORE 1958

The statutory basis for the conclusion of GRAS status of 2'-Fucosyllactose (2'-FL) in this document is not based on common use in food before 1958. However, 2'-FL is found in human milk, a natural source of 2'-FL that have been consumed prior to 1958. Notwithstanding this, present GRAS assessment for the use of 2'-FL as a food ingredient is based on scientific procedures.

6. PART VI- NARRATIVE

6.1. Current and Historical Uses

In the published literature, the history of consumption of 2'-fucosyllactose (2'-FL) has been extensively described. Human breast milk contains numerous biomolecules, including human milk oligosaccharides (HMOs) that are the third most abundant component of breast milk, after lactose and lipids. Amongst the synthesized HMOs, 2'-FL and lacto-N-neotetraose (LNnT) are widely studied and are considered safe for infant nutrition. As a naturally occurring oligosaccharide present in human milk, 2'-FL is synthesized from lactose in the mammary gland (Castanys-Mufioz et al., 2013). 2'-FL has been detected in amniotic fluid in maternal serum during pregnancy, and its levels vary according to gestational age and Secretor status (Jantscher-Krenn et al., 2019; 2022). It has also been detected in cord serum samples and demonstrated to cross the human placenta *ex vivo* (Hirschmugl et al., 2019). Several factors, such as stage of lactation (levels generally decrease as lactation progresses), ethnicity, geographical location, and genetic traits (Lewis blood groups and Secretor status) are known to influence the levels of 2'-FL in human milk (Erney et al., 2000; Gabrielli et al., 2011; Galeotti et al., 2012; McGuire et al., 2017). These, and other, studies are also discussed in several GRAS notices (particularly in GRNs 546 and 650).

As the most abundant HMO, 2'-FL constitutes approximately 30% of the total HMOs among secretor mothers (Hegar et al., 2019). Based on the findings from 17 studies, the mean 2'-FL content in mature human breast milk has been reported as 2.35 g 2'-FL/L in full-term, mature human breast milk (as reported in GRN 546). In another GRAS notice (GRN 571), this value is reported as approximately 2.6 g/l in mature milk. 2'-FL values as high as 8.4 g/L in breast milk have been reported (Gabrielli et al., 2011; Galeotti et al., 2012; Musumeci et al., 2006). In a systematic review article, Thurl et al. (2017) calculated a mean concentration and 95% confidence limit of 2'-FL in the milk of secretor mother as 2.74 g/L and 2.43 -3.04 g/L, respectively. McGuire et al. (2017) reported levels of 2'-FL in breast milk in 11 rural and urban populations. The values of 2'-FL in these populations ranged from 0.7±0.1 (SEM) g/l in a rural Ghanaian population to 3.44±0.29 g/L in a California population with a mean across all populations equal to 1.93±0.22 g/L. As described in GRN 1014 (pages 14-18), based on several studies, the human milk levels of 2'-FL are reported to range from 0 to 9.5 g/L and vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth. Though the 2'-FL content declines as lactation continues, the volume of breast milk increases throughout the nursing period. Given this, infants born to a Secretor mother may ingest 2-3 g of 2'-FL per day (Castanys-Munoz et al., 2013).

In a detailed structure analysis of bovine milk that is commonly used in the production of infant formula in the United States, Aldredge et al. (2013) reported the oligosaccharide content to range from 100 to 1000 times lower as compared to human milk and fucosylated oligosaccharides constitute less than 1% of the oligosaccharide fraction. The 2'-FL content of infant formulas is considered negligible (< 2.4 mg/L). Given low levels of 2'-FL in infant formula, in recent years, synthetic forms of 2'-FL, including those derived by fermentation, are used in the infant formulas at levels up to 2.4 g/L. Additionally, 2'-FL is used in selected conventional foods at levels up to 600 g/kg, and enteral tube feeding formulas up to 6 g/L (GRN 546; GRN 571; GRN 650; GRN 735; GRN 749; GRN 815; GRN 852; GRN 897, GRN 929, GRN 932, GRN 1014, GRN 1034; also mentioned in Table 6).

In summary, 2'-FL is a naturally occurring oligosaccharide in human milk, and is synthesized from lactose in the mammary gland. The available information suggest that humans, particularly infants, are exposed to 2'-FL either through the ingestion of human milk, cow's milk, and/or products containing synthetic forms of 2'-FL. This also suggest that at naturally found levels, 2'-FL is unlikely to cause any adverse effects.

Table 6. Summary of Available GRAS Notices on 2'-Fucosyllactose that Received FDA "No Question" Letter

GRN No.	Substance	Date of closure	FDA's Letter
1034	2'-fucosyllactose	Oct 21, 2022	FDA has no questions
1014	2'-fucosyllactose	Jul 15, 2022	FDA has no questions
932	2'-fucosyllactose	Feb 18, 2021	FDA has no questions
929	2'-fucosyllactose	Feb 26, 2021	FDA has no questions
897	2-*O*-fucosyllactose	Jun 12, 2020	FDA has no questions
852	2-fucosyllactose	Nov 15, 2019	FDA has no questions
815	2'-fucosyllactose and difucosyllactose	Aug 20, 2019	FDA has no questions
749	2'-O-fucosyllactose	Apr 23, 2018	FDA has no questions
735	2'-Fucosyllactose	Apr 6, 2018	FDA has no questions
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

6.2. Data Pertaining to Safety

Given the natural occurrence of 2'-FL in foods, such as human milk, and in the human body, the need for systematic toxicity studies of 2'-FL has been diminished. However, as the most abundant HMO (2'-FL) in breast milk and given its role in shaping the gut microbiome, there has been considerable efforts to elucidate its importance in infant nutrition. Published literature contains several studies on human milk oligosaccharides, including 2'-FL. The available information suggest that 2'-FL is synthesized from lactose in the mammary gland.

For the present GRAS assessment, the safety determination of 2'-FL is based on the totality of the available evidence, including human clinical observations/trials, animal experimental studies and *in vitro* studies. Efforts have been made to present both the data supporting 2'-FL safety as well as any data on potential adverse effects. In addition to its role in shaping the gut microbiota, 2'-FL plays multiple roles in the human body that have been investigated in recent years. An attempt has been made to interpret these findings from relevant studies as it relates to the present GRAS assessment. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. In the following sections, relevant biological and toxicological studies on 2'-FL and structurally related substances are described that provide support for the conclusions reached in this determination.

6.2.1. Metabolic Fate

The available absorption, distribution, metabolism and excretion studies of 2'-FL have been comprehensively reviewed in previous GRAS Notices (listed in Table 6) of 2'-FL submitted to the FDA, as well as in the European Food Safety Authority (EFSA) Panel reports (EFSA, 2015, 2019). The available information suggest that HMOs, including 2'-FL, are non-digestible carbohydrates, highly resistant to the digestive enzymes of the gastrointestinal (GI) tract and only small amounts of 2'-FL is absorbed intact. Findings from *in vitro* studies revealed that <5% of ingested HMOs is digested. *In vivo* studies in infants and in rats have reported that 1 to 2% of the total amount of ingested HMOs is excreted unchanged in the urine. The remaining unabsorbed oligosaccharides passes through the GI tract, where it is either fermented by the select microbiota or excreted unchanged in the feces (Rudloff et al., 1996; Brand-Miller et al., 1998; Obermeier et al., 1999; Gnoth et al., 2000; Engfer et al., 2000; Chaturvedi et al., 2001; Rudloff et al., 2006; Rudloff et al., 2012; Rudloff and Kunz, 2012; Goehring et al., 2014; Dotz et al., 2014; Kuntz et al., 2019).

The absorption profiles of 2'-FL were demonstrated to be similar in plasma samples collected from infants fed formulas supplemented with 2'-FL and infants fed human milk (Marriage et al., 2015). The exact mechanisms of absorption of HMOs has not been fully elucidated. However, data from *in vitro* studies using Caco-2 human intestinal epithelial cells suggest that neutral HMOs are transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMOs are absorbed via the non-specific paracellular transport only (Gnoth et al., 2001).

Vazquez et al. (2017) investigated the absorption and urinary elimination of HMOs in rats. In an initial study, a single oral dose of 2'-FL, 6'-sialyllactose and lacto-N-neotetraose at different concentrations was administered to adult rats. The findings revealed that rats, similar to human infants, are able to effectively absorb a portion of HMOs from the intestine into plasma and to excrete them in urine. In a specific kinetic absorption study with 2'-FL in 9-11 day-old rat pups, a significant amount of 2'-FL was absorbed into the systemic circulation and subsequently excreted in urine during lactation in rats in a dose-depended manner. Basal levels of these HMOs in plasma and urine of adult rats as well as rat pups was noted as a natural result of nursing. The findings from this study confirm the results of previous studies that small amounts of 2'-FL are absorbed and metabolized at least in part to lactose either prior to, and/or after, absorption.

Kuntz et al. (2019) reported absorption of 2'-FL fermentation products into the systemic circulation and distribution to organs (liver, heart, spleen, kidney, and brain) of wild-type but not germ-free mice 2 hours following the oral administration of ^{13}C-labelled 2'-FL at a dose of 1 g/kg bw/day, with highest levels observed 5 hours post-administration. In the wild-type mice, ^{13}C was detected in urine but at lower levels than in feces, indicating that feces was the primary route of elimination. In contrast, in germ free animals, no ^{13}C was detected in the urine, indicating that the intestinal microbiota were required for the fermentation of 2'-FL. The findings from this study suggest that 2'-FL is not absorbed from the upper gastrointestinal tract, is fermented by the intestinal microbiota, and primarily excreted in the feces.

The content of other carbohydrates in Synaura's 2'-FL produced by fermentation is comparable to those in other 2'-FL ingredients (synthetic and produced by microorganism) that have been concluded to be GRAS and notified to the FDA. The other carbohydrates, including

lactose, fucose, and difucosyllactose, are naturally found in human milk or are breakdown products of naturally-occurring fucosylated oligosaccharides (fucosylgalactose). Also the glucose and galactose are naturally-occurring breakdown products of lactose and are common dietary components. These other carbohydrates will follow endogenous routes of absorption, distribution, metabolism, and excretion (ADME). Given this, no safety concerns are expected from these carbohydrates at the intended use levels.

In summary, the available information from a large number of studies suggest that HMOs, including 2'-FL, are resistant to hydrolysis by digestive enzymes in the upper digestive tract and are either partially fermented by the intestinal microbiota or excreted unchanged in the feces. 2'-FL absorption profiles were reported to be similar in plasma samples collected from infants fed formulas supplemented with 2'-FL and infants fed human milk. The available metabolism related information suggest that 2'-FL is unlikely to cause any adverse effects at the proposed use levels.

6.2.2. Human Studies of 2'-FL

In multiple clinical studies in infants and adults, the safety and tolerability of 2'-FL supplementation, either alone or in combination with other oligosaccharides, has been extensively investigated (Marriage et al., 2015; Goehring et al., 2016; Kajzer et al., 2016; Elison et al., 2016; Puccio et al., 2017; Gurung et al., 2018; Nowak-Wegrzyn et al., 2019; Storm et al., 2019; Palsson et al., 2020; Iribarren et al., 2020; Vandenplas et al., 2020; Roman et al., 2020; Parschat et al., 2021; Fonvig et al., 2021; Ryan et al., 2021; Alliet et al., 2022; Wallingford et al., 2022; Hascoet et al., 2022). Some of these studies of 2'-FL ingredients in different population groups have been reviewed and described in previous GRAS notifications to FDA. The available studies in infants, children and adults, including ones described in previous GRAS notices, are summarized in Tables 7 and 8, while some of the more recent studies are further described below. In the available studies, healthy, term infants or toddlers were fed formula containing 0.25 to 3 g 2'-FL/L for 6 weeks to 6 months and the findings from these studies suggest that 2'-FL is safe and well-tolerated. In studies in adults, the daily supplementation of up to 20 g 2'-FL is found to be safe and well-tolerated in adults.

In addition to the above mentioned studies, support for the safe use of 2'-FL in toddler formulas, foods for infants and young children, as well as in beverages (sports and "energy" drinks, flavored waters, fruit juices and drinks, milk drinks, dairy analogs, milk-based meal replacements) and in breakfast cereals, frozen dairy desserts, puddings, fillings, mousses; yogurt; meal replacement and snack bars; syrups, and jams and jellies is based on results of numerous clinical studies that evaluated the safety and tolerance of HMOs, including 2'-FL, as well as other non-digestible carbohydrates in infants, adults, sensitive populations and oral electrolytes solutions. The available information suggest that HMOs are well tolerated in infants at levels up to 1 g/day, and in adults at levels up to 20 g/day.

6.2.2.1. Studies in Infants and Children

The available clinical trials of 2'-FL in infants and children are summarized in Table 7. In a recent study, Alliet et al. (2022) studied the safety of a starter infant formula containing *Limosilactobacillus reuteri* DSM 17938 and supplemented with 2'-FL. In this study, healthy infants <14 days old (n = 289) were randomly assigned to a bovine milk-based formula containing *L. reuteri* DSM 17938 at $1x10^7$ CFU/g (control group) or the same formula with added 1.0 g/L 2'-FL (experimental group) until 6 months of age. A non-randomized breastfed

group served as reference (n = 60). The primary endpoint was weight gain through 4 months of age in the formula-fed infants. Secondary endpoints included additional anthropometric measures, gastrointestinal tolerance, stooling characteristics, adverse events (AEs), fecal microbiota and metabolism, and gut immunity and health biomarkers in all feeding groups. Weight gain in the experimental group was non-inferior to the control group. Anthropometric Z-scores, parent-reported stooling characteristics, gastrointestinal symptoms and associated behaviors, and adverse events were comparable between formula groups. The investigators concluded that *L. reuteri*-containing infant formula with 2'-FL supports age-appropriate growth and is well-tolerated.

In a double-blind randomized controlled trial, Wallingford et al. (2022) investigated the effects of 2'-FL on growth of healthy term infants. In this study, infant formula with and without 2'-FL or human milk was fed to healthy term infants (37-42 weeks of gestation) for 16 weeks. There were no effects of addition of 2'-FL (1 g/L) on growth or AEs. The investigators concluded that the addition of a physiologic level of 2'-FL had no effect on growth or incidence of adverse effects of formula-fed infants, adding evidence of safe use of this HMO in the infant formula.

Hascoet et al. (2022) investigated the effects of HMO supplementation on feeding tolerance, growth, and safety in pre-term infants (27 – 33 weeks' gestation, birth weight <1,700 g). In this study, pre-term infants, early after birth, were randomized to receive HMO supplement (n = 43) 2'-FL and lacto-N-neotetraose (LNnT) in a 10:1 ratio (0.374 g/kg bw/day) or an isocaloric placebo (n = 43) consisting of only glucose (0.140 g/kg/day) until discharge from the neonatal unit. The primary outcome was feeding tolerance, measured by non-inferiority (NI) in days to reach full enteral feeding (FEF) from birth in HMO vs. placebo group. The mean number of days on intervention prior to FEF was 8.9 and 10.3 days in HMO and placebo, respectively. The adjusted mean time to reach FEF from birth was 2 days shorter in HMO (12.2) vs. placebo (14.3), although not statistically significant. There was no difference in weight-for-age z-scores between groups throughout the FEF period until discharge. Length-for-age z-scores were higher in HMO at FEF day 14 and 21. Head circumference-for-age z-score was higher in HMO vs. placebo at discharge. Occurrence of AEs was similar in both groups and relatively common in this population, whereas 2.3 and 14.3%, respectively, experienced investigator-confirmed, related AEs. The investigators stated that HMO supplementation is safe and well-tolerated in pre-term infants. Although this study was conducted in pre-term infants and may not be directly applicable to this present GRAS assessment, the findings indicate that 2'-FL is unlikely to cause adverse effects in term infants and other population groups. As this study provides further support for the safe uses of 2'-FL, it is summarized here for the sake of completeness.

In a multi-center, randomized, double-blinded, controlled, parallel group clinical trial, Parschat et al. (2021) evaluated the safety and tolerability of a mixture of five commercially prepared HMOs (2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL). In this study, healthy term infants of >14 days of age were randomized to receive exclusive feeding with an infant formula containing 5-HMOs (n=113), a control formula (n=112) or exclusive feeding with breast milk (n=116) for 4 months. With respect to the daily mean body weight gain, the formula supplemented with HMOs was considered as non-inferior to the control formula. No differences in weight, length of head circumference gain between the two formulas were noted. The formula containing the HMOs was well tolerated and the occurrence of AEs was similar across the groups. The investigators concluded that infant formula containing mixture of HMOs, including approximately 3 g/L of 2'-FL, is safe and well tolerated by infants.

In an open-label, prospective study, Roman (2020) investigated the effects of an infant formula containing HMOs (2'-FL and LNnT) on growth and tolerability in healthy term infants that were enrolled at age of 7 days to 2 months. In this study, the infants were divided in three groups: either exclusively breastfed infants (BF group; n-63), an exclusively formula-fed group (FF; n=82) who received a milk-based formula with 2'-FL and LNnT, and a group mixed fed with both formula and human milk (MF; n=62), for 8 weeks. 159 infants completed the study (66 FF, 48 MF, and 45 BF). There were no significant differences in anthropometric measures between the groups, with age appropriate growth observed in all groups. The incidence of adverse events was generally low and not significantly different among the groups. Three infants experienced potentially product-related adverse events, with 2 incidences of cow-milk intolerance (1 in formula-fed and 1 in mixed-fed groups), and 1 instance of irritability in the formula-fed group. Six serious adverse events occurred (bronchiolitis) but were not considered related to the study feeding.

In a randomized, double-blind, placebo-controlled, multi-country trial, Vandenplas et al. (2020) investigated the effects of a partially fermented infant formula on growth, safety, and tolerability. In this study, healthy, full-term, exclusively formula-fed infants less than 14 days of age at baseline were included. The reference group consisted of exclusively breastfed infants. The test and control formulas were nutritionally complete cow's milk-based formulas, with the test formula supplemented with 26% fermented formula, 2'-FL (1 g/L; source not reported), scGOS and lcFOS (9:1; 8 g/L), and milk fat (49.8% of total fat). The test and control formulas were administered from ≤14 days of age until 17 weeks of age, with anthropometric measures, gastrointestinal symptoms, and safety assessed monthly. No statistically significant differences were reported between the control and test groups with respect to growth, adverse events, serious adverse events, or gastrointestinal tolerability. The investigators concluded that the test formula supports adequate growth and is safe and well-tolerated in healthy term infants.

Leung et al. (2020) investigated the effects of standard milk formula on respiratory and gastrointestinal infections in toddlers. In this randomized, controlled, double-blind, parallel-group clinical trial, healthy Chinese children aged 1-2.5 years were recruited. The children were assigned (n=114/group) to either standard milk formula (young child formula or YCF-Ref; control) or one of three new YCFs containing bioactive proteins and/or the HMO 2'-FL (3 g/L; source of 2'-FL not reported) and/or milk fat for six months. The study formulas were consumed in two 200-ml servings/day. The subjects' caregiver recorded daily adverse events and stool frequency and consistency. Physical examinations were performed every 2 months. The investigators noted that as compared to control group, subjects consuming the formula containing 2'-FL had longer (but not more frequent) upper respiratory tract infections, more incidences of cough and runny nose, and more days with fever compared to the control formula. As compared to the control, subjects consuming the formula with bioactive proteins, milk fat, and 2'-FL had more gastrointestinal infection episodes than control subjects. However, no between-group differences in the incidence of adverse events or serious adverse events were noted and none of the reported events were considered to be product-related. No between groups differences in growth were reported. The investigators concluded that the study formulas were safe and supported normal growth in toddlers.

In a randomized, double-blinded, placebo-controlled trial, Fonvig et al. (2021) investigated the effects of 2'-FL and lacto-N-neotetraose on intestinal microbiota, safety, and digestive tolerance in children. In this study, 75 children with overweight (including obesity)

ages 6 to 12 years were randomized to receive 2'-FL, a mix of 2'-FL and lacto-N-neotetraose (Mix), or a glucose placebo orally administrated once per day for 8 weeks. Subjects were given a single-serve sachet each containing 4.5 g of either 2'-FL, 2'-FL and LNnT in a 4:1 mass ratio, or placebo. Overall, 23 (30.7%) subjects reported a total of 46 adverse events between randomization and the end of intervention. Seven, 7, and 9 subjects in the placebo group, 2'-FL group, and Mix group, respectively, reported at least one adverse event during the intervention. Blood samples for routine clinical chemistry and hematology were collected at baseline and at the end of intervention. None of the markers exhibited levels suggesting pathology at any time point for all 3 groups. Biochemical markers indicated no safety concerns, and the products did not induce digestive tolerance issues as assessed by Gastrointestinal Symptoms Rating Scale and Bristol Stool Form Scale. The investigators concluded that both 2'-FL and the Mix modulated intestinal microbiota by increasing bifidobacteria and supplementation with either 2'-FL alone or a Mix is safe and well tolerated in children.

In summary, the safety and tolerance of the supplementation of 2'-FL in infant formula alone or in combination with lacto-N-neotetraose (LNnT) or non-milk oligosaccharides (GOS or scFOS), has been previously evaluated in various GRAS notifications and a number of clinical studies conducted in full-term infants 0 to 6 months of age (Marriage et al., 2015; Goehring et al., 2016; Kajzer et al., 2016; Puccio et al., 2017; Storm et al., 2019). Based on findings from these studies, and, as described in previous GRAS notices, the available safety related evidence consistently suggest that 2'-FL supplementation at levels ranging from 0.2 to 1.0 g/L is safe and well-tolerated in infants. The new studies that appeared subsequent to the most recent GRAS notice (GRN, 1034) that received a no question letter from FDA, further supports the safety and tolerability of 2'-FL in infants. In these studies, healthy, term infants or toddlers, or children were fed formula containing 0.25 to 3 g 2'-FL/L in addition to milk fats/proteins, probiotic bacteria, and/or poorly digestible carbohydrates for 6 weeks to 6 months (Roman et al., 2020; Parschat et al., 2021; Ryan et al., 2021; Alliet et al., 2022; Wallingford et al., 2022; Hascoet et al., 2022). In these studies, some potentially test formula-related minor adverse events were reported (i.e., cow's milk intolerance and irritability), there were no significant differences in the incidences of adverse events and the investigators of these studies concluded that the formulas and foods containing 2'-FL were well tolerated, with no safety concerns noted. Although studies with 2'-FL and other oligosaccharides are described in the published literature, at this time Synaura does not intend to combine 2'-FL with other sources of poorly digested carbohydrates.

Table 7. Clinical Studies of 2'-Fucosyllactose in Infants and Children

Study Design and Population	Groups (number of participants); Duration	Safety related findings	Citation / GRN reference
Double-blind, randomized, controlled study 289 healthy formula-fed infants 60 healthy breastfed infants (reference group) Less than 14 days of age at enrollment	Control formula: Cow's milk based infant formula containing *L. reuteri* (1 x 10^7 CFU/g) Test formula: Same as control, plus 1.0 g/L 2'-FL 6 months	Investigators concluded that *L. reuteri*-containing infant formula supplemented with 2'-FL at 1.0 g/L supported age-appropriate growth and was well tolerated. Weight gain in test formula group was non-inferior to control formula as shown by a mean difference [95% CI] of 0.26 [-1.26, 1.79] g/day with the lower bound of the 95% CI above the non-inferiority margin (-3 g/day). Anthropometric Z-scores, parent-reported stooling characteristics, gastrointestinal symptoms and associated behaviors, and AEs were comparable between formula groups. Incidence of AEs was low and comparable between the formulas indicating that the test formula is safe.	Alliet et al., 2022
Double-blinded randomized controlled study Healthy term infants (n= 221; 37-42 weeks of gestation)	Infant formula without 2'-FL (n=66) Infant formula with 2'-FL – 1 g/L (n=66) Infants fed human milk (n=89) 16 weeks	The number of subjects completed the study were as follows: control n=41 (62%); test group n=56 (85%); breastfed group n=79 (89%). Adverse events reported were 4, 2, and 0, respectively. There were no notable findings in the growth data; average daily weight gain did not differ between formula groups. Safety data indicated no excess of AEs reported for the 2'-FL group. No effect on growth or incidence of adverse effects of formula-fed infants, adding evidence of safe use of this 2'-FL in the infant formula. Three subjects fed the test formula experienced AEs that led to discontinuation of the study formula: a moderate AE of projectile vomiting was considered possibly related to the study formula; a moderate AE of infant irritability and a severe AE of infantile spitting up were considered possibly related to the study formula, and one moderate AE of abdominal pain was considered probably related to the study formula. Seven subjects fed the control formula experienced AEs, which led to discontinuation of the study formula; two AEs were considered likely or definitely related to formula, whereas the others were considered unlikely related. The seven subjects had a moderate AE of GERD; a moderate AE of constipation; moderate AEs of flatulence and GERD and a severe AE of crying; a mild AE of diarrhea; mild AEs of abdominal pain and constipation; a mild AE of infantile spitting up; and a mild AE of abdominal pain.	Wallingford et al., 2022

Randomized, double-blind, placebo-controlled trial 86 preterm infants (27-33 weeks gestation, birth weight <1700 g) 43 per group Average 6 days of age at intervention initiation	Control supplement: Glucose (0.140 g/kg bw/day) Test supplement: 2'-FL and LNnT in 10:1 ratio (0.274 g/kg bw/day) Enrollment to discharge from neonatal unit	Gastrointestinal tolerance measures, incidence of gastrointestinal adverse events, incidence of necrotizing colitis, and incidence of other illnesses and infections were similar between groups. No cases of illnesses and infections were deemed related to the intervention.	Hascoet et al. (2022)
Multicenter, randomized, controlled, parallel-group clinical study 225 healthy term infants and 116 healthy breastfed infants (reference group) 112-113 per formula group ≤ 14 days of age at enrollment	Control formula: Basic infant formula Test formula: Same as control, plus a target HMO content of 5.75 g/L (2.99 g/L of 2'-FL, 1.5 g/L of LNT, 0.75 g/L of 3'-FL, 0.28 g/L of 6'-SL, and 0.23 g/L of 3'-SL) 4 months	Adverse events were equivalent in all groups. The incidence of AEs was similar in the 5HMO-Mix and IF groups: 335 AEs in 83 (80.6%) of the 5HMO-Mix infants and 289 AEs in 84 (80.8%) of the IF infants. In the BM group, 191 AEs were recorded in 73 (70.2%) of the infants. Overall, investigators concluded that infant formula supplemented with a mixture of 5 HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) at concentrations similar to those naturally occurring in human milk supported normal infant growth and was safe and well-tolerated.	Parschat et al. (2021)
Non-randomized, open-label, prospective study Healthy (n=159) term infants 7 days to 2 months old	Group 1: Formula-fed infants (n=82) Group 2: Infants consuming formula and human milk; the formula contained 1.0 g/L of 2'-FL, 0.5 g LNnT, and *Lactobacillus reuteri* (n=62) Group 3: Breast-fed infants (n=63) 8 weeks	The incidence of adverse events was low overall and was not significantly different between the groups. 16 subjects dropped out of Group 1 (six were excluded due to protocol deviations, three dropped out due adverse events (AEs), and seven were lost to follow-up). 14 subjects dropped out of Group 2 (8 were excluded due to protocol deviations, 3 dropped out due to adverse events, and 3 were lost to follow-up. 18 subjects dropped out of Group 3 (11 were excluded due to protocol deviations, 1 dropped out due to adverse events, and 6 were lost to follow-up. No significant differences between any of the groups for any of the anthropometric measures.	Roman et al., 2020

		Composite Infant Gastrointestinal Symptom Questionnaire (IGSQ) scores demonstrated low gastrointestinal distress in all feeding groups at all time points and there were no significant differences among feeding groups at baseline, 4, or 8 weeks. No significant differences among the groups in the gassiness, fussiness, crying or spitting-up/vomiting domains of the IGSQ. For the stooling domain, Group 2 were significantly different than Group 3 at baseline and 8 weeks. A total of 49 subjects experienced 58 adverse events over the course of the study. There were 19 AEs in Group 1, 21 in Group 2, and 18 AEs in Group 3. The incidence was generally low and not significantly different among the groups 3 subjects experienced potentially product-related AEs, including two instances of cow's milk intolerance (1 in Group 1 and 1 in Group 2) and 1 instance of irritability in Group 1. 6 serious adverse events occurred (4 in Group 1 and 2 in Group 2), all of which were bronchiolitis. All were considered unrelated to the study feeding	
Double-blind, randomized, controlled, multi-country trial Healthy term infants n=232; 14 days of age or less at enrollment	176 healthy formula-fed infants 56 healthy breastfed infants (reference group) Control formula: Cow's milk-based infant formula containing 8 g/L scGOS/lcFOS (9:1 ratio) Test formula: Same as control, plus 1.0 g/L 2'-FL, 0.15 g/L 3'-GL, and anhydrous milk fat About 15 weeks	The infant formula containing 2'-FL, 3'-GL, scGOS/lcFOS, and milk fat was safe and well tolerated in healthy term infants, and supportive of adequate infant growth. At least one adverse event was reported in 39.3% of infants receiving the 2'-FL test formula, 31.7% in infants receiving the control formula, and 24.6% in breastfed infants. No statistical significance was reported in regurgitation, vomiting, frequent watery stools, or infrequent hard stools between either groups receiving infant formula. 11 total serious adverse events were noted in formula-fed infants; 7 in infants who received the 2'-FL test formula and 4 in infants who received the control formula; however, this difference was not statistically significant and was determined to be unrelated to the study product by the investigators. No serious adverse events were reported in breastfed infants	Vandenplas et al., 2020
Double-blind, placebo-controlled food challenges Children with cow milk protein allergy	Treatment #1: Whey- based extensively hydrolyzed formula Treatment #2: Whey-based extensively hydrolyzed formula containing 1.0 g/L 2'-FL and 0.5 g/L LNnT Not applicable	64 children completed at least one DBPCFC 3 children were excluded due to protocol deviations (n=61) There was 1 allergic reaction to the test, and 1 to the control formula 61 of the 64 subjects completed the open-label home challenge phase with the test formula 1 subject vomited on Day 1 of the home challenge but completed the home challenge without further problems	Nowak-Wegrzyn et al., 2019 / GRN 919, page 33

		1 patient developed diarrhea on the last day of the challenge, which the site investigator attributed to gastroenteritis. No significant gastrointestinal symptoms (flatulence, abnormal stool frequency/consistency, increased spitting-up, or vomiting) were reported. No serious adverse events occurred during the entire study.	
Randomized, placebo-controlled double-blind study Healthy term infants 14 days old ±5 days.	Group 1: Formula containing *Bifidobacterium animalis* ssp *lactis* Bb12 (n=40) Group 2: Formula containing *Bifidobacterium animalis* ssp *lactis* Bb12 + 0.25 g/L 2'-FL (n=38) 6 weeks	In the 2'-FL treated group, 1 subject was lost to follow-up, 1 caregiver wished to withdraw, 3 subjects withdrew due to adverse events (AEs), and 3 subjects did not comply with feeding only the study formula. In the control group, 1 subject was lost to follow-up, 1 caregiver wished to withdraw, 3 subjects withdrew due to adverse events, and 2 subjects did not comply with feeding only the study formula. Infant gastrointestinal symptom questionnaire scores were similar in both groups at baseline and after 6 weeks of treatment. Stool frequency and consistency did not differ between the groups over the course of treatment. Significantly more stools were reported to be difficult to pass in the control group than in the test group ($p<0.05$), however, the number of infants with stools reported as difficult to pass was not different between the groups Crying, fussing duration, vomiting frequency, and the proportion of babies reported to have any spit up over the 2-day diary period were similar between the two groups. Among the babies whose caregivers reported spit-up, significantly more were reported to have spit up >5 times/day in the 2'-FL group compared to the control group. There were no serious AEs and the AEs were equally distributed among the two groups. There were significantly more subjects that experienced infections and infestations in the control group than in the 2'-FL-treated group (n=9 vs n=3; p=5). There were no effects of the 2'-FL-containing formula on anthropometric measures (body weight and lengths, and weight-for-age and length-for-age).	Storm et al., 2019 / GRN 571 supplement, page 21
Prospective, randomized, placebo-controlled study Healthy, term infants 0 to 14 days old	Group 1: Formula (n=87) Group 2: Formula with 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88)	20 infants in control and 24 infants in the HMO containing formula withdrew before the primary outcome assessment at 4 months. The dropout rate was comparable between groups. The most common reason for discontinuation was an adverse event (n=11 in control; n=12 in test). Other reasons for discontinuation before 4 months included	Puccio et al., 2017 / GRN 650, page 38

	6 months (after 6 months, al infants were switched to a non-HMO containing formula)	parent/guardian request (n=3 in control; n=6 in test); lost to follow-up/missing (n=5 in control; n=6 in test); and other (n=1 in control; n=40 in test). There was no difference in weight gain, mean weight-for-age, length-forage, head circumference-for-age, and BMI-for-age z scores between the groups. Parent-reported infant behavioral patterns including restlessness/irritability and colic were similar in the HMO and control groups except for softer stool (P=0.021) and fewer nighttime wake-ups (P = 0.036) in the test group at 2 months. Infants receiving the HMO-containing formula had significantly fewer parental reports (P = 0.004 – 0.047) of bronchitis through 4 (2.3% vs 12.6%), 6 (6.8% vs 21.8%), and 12 months (10.2% vs 27.6%); lower respiratory tract infection (adverse event cluster) through 12 months (19.3% vs 34.5%); antipyretics use through 4 months (15.9% vs 29.9%); and antibiotics use through 6 (34.1% vs 49.4%) and 12 months (42.0% vs 60.9%) compared to the infants receiving the control formula.	
Prospective, randomized, placebo-controlled study Healthy, term infants 5 days old	Group 1: Formula with GOS (n=39) Group 2: Formula with GOS + 0.2 g/L 2'-FL (n=37) Group 3: Formula with GOS + 1.0 g/L 2'-FL (n=37) Group 4: Human milk (HM)(n=42) 16 weeks	Note: This is a sub-study of the clinical study conducted by Marriage et al., 2015. The objective was to investigate the effects of feeding formulas supplemented with HMO-2'-FL on biomarkers of immune cell function Circulating plasma concentrations of inflammatory cytokines IL-1a, IL-1b, IL-16, and TNF-a and anti-inflammatory IL-1ra were significantly higher (82%, 72%, 76%, 58%, and 58%, respectively) in the group fed formula compared to the group receiving human milk ($p \leq 0.05$) Both the groups receiving the formulas containing 2'-FL exhibited profiles that were significantly different from the formula group and not different from the human milk group or each other. There were no differences in plasma cytokines IFN-a2, IFN-g, IL-10, IL-10, or RANTES between any of the group.	Goehring et al., 2016 / GRN 735, page 62
Prospective, randomized, placebo-controlled study Healthy, term infants 5 days old	Group 1: Formula with GOS (n=101) Group 2: Formula with GOS + 0.2 g/L 2'-FL (n=104) Group 3: Formula with GOS + 1.0 g/L 2'-FL (n-109) Group 4: Human milk (HM)(n=106)	338 infants completed the study (84 in the control group, 81 in the group receiving the formula containing 0.2 g/L 2'-FL, 83 in the group receiving formula containing 1.0 g/L 2'-FL and 90 in the HM group; 304 of whom completed the study on the assigned feeding or HM (79 in the control group, 70 in the group receiving the formula containing 0.2 g/L 2'-FL in the group receiving the formula containing 1.0 g/L 2'-FL and 83 in the HM group). The number of premature terminations was not statistically significant among the formula-fed groups	Marriage et al., 2015 / GRN 650, page 37

	17 weeks	Although the HM group gained significantly more weight than the group receiving 0.2 g/L 2'-FL from day 14 to 28 and the group receiving 1.0 g/L 2'-FL than the HM group from day 84 to 119, there were no significant differences (sex-specific or sex- combined) in mean weight, length, or head circumference among feeding groups during the study, and no significant differences among feeding groups in mean gains in these measures from day 14 to 119. The mean number of stools/day was significantly higher for the HM group compared to all groups receiving the formulas for the three days before the study visits at day 28, 42, and 84. The mean number of stools/day was also significantly higher for the HM group compared to the control formula group for the three days before the study visits at day 119. Although spitting-up or vomiting was significantly higher in the formula-fed groups compared to the HM group from enrollment to day 28, there were no differences after day 28. Although the mean rank stool consistency was significantly higher for the group receiving 2'-FL from enrollment to day 28 and was significantly higher in the formula-treated groups than the HM group for the remainder of the study, there was no difference among the formula-treated groups over the course of the study. There were no significant differences in the overall percentage of subjects experiencing adverse events or serious adverse events in the formula-treated groups. The control formula and the 1 g/L 2'-FL groups had significantly more subjects with reported adverse events in the "infections and infestations" category compared with the 0.2 g/L group ($p<0.05$)	
Prospective, randomized, double-blind, placebo-controlled study Healthy term infants 0 and 8 days of age	Group 1: Formula (n=42) Group 2: Formula with 0.2 g/L 2'-FL and 2 g/L scFOS (n=46) Group 3: Human milk (HM) (n=43) 5 weeks	36 (86%) subjects in the group receiving formula, 41 (89%) in the group receiving oligosaccharide and 42 (98%) in the group receiving human milk completed the study There was no difference in the mean rank stool consistency among the groups The average number of stools per day for the human milk group was significantly higher in the human milk group than both formula-fed groups There were no differences among groups for the average volume of study formula intake, number of study formula feedings/day, anthropometric data or percent feeding with spit-up/vomit. Safety endpoints not reported.	Kajzer et al., 2016 (abstract) / GRN 571, page 21

Randomized, placebo controlled, study Healthy term infants 0-14 days old	Group 1: Cow's milk based infant formula (n=87) Group 2: Cow's milk based infant formula w/ 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88) Group 3: Human milk 3 months	2'-FL and LNnT shift the stool microbiota towards that observed in breastfed infants Safety endpoints not reported.	Alliet et al., 2016 (abstract) / GRN 815, page 55
Randomized, placebo controlled, study Healthy term infants 0-14 days old	Group 1: Cow's milk based infant formula (n=87) Group 2: Cow's milk based infant formula w/ 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88) Group 3: Human milk 3 months	2'-FL and LNnT shift the stool microbiota towards that observed in breastfed infants Safety endpoints not reported.	Steenhout et al., 2016 (abstract) / GRN 735, page 62

6.2.2.2. Studies in Adult

In addition to studies in infants and children, in several studies the safety and tolerance of 2'-FL has been investigated in adults (Table 8). In one study in adult subjects, Elison et al. (2016) evaluated safety of 2'-FL, LNnT, and a mixture of the 2 HMOs (2:1 ratio of 2'-FL: LNnT) at levels of up to 20 g/day following 2-week supplementation. In this study, as compared to placebo, increased gastrointestinal symptoms were noted in individuals consuming 20 g of 2'-FL/day (nausea, rumbling, bloating, passing of gas, diarrhea, loose stools and urgency to pass stools). However, the study authors noted that scores remained low and were rated as mild discomfort, and that it was difficult to determine whether gastrointestinal symptoms were related to the test product, day-to-day variation, or increased awareness by the study participants. The investigators concluded that supplementation of 2'-FL and LNnT at daily doses up to 20 g was safe and well tolerated, as assessed using the gastrointestinal symptoms rating scale.

In an open-label trial, Ryan et al. (2021) investigated the effect of a 2'-FL-containing nutritional formula in twelve adults (n=20 recruited; 21-75 years old; BMI of 19-40 kg/m^2) with IBS or ulcerative colitis. The subjects were administered 4 g of 2'-FL in combination with micronutrients, macronutrients, amino acids, and isomaltooligosaccharide for 6 weeks. Of the 20 subjects recruited, 12 subjects completed the study. Eight subjects withdrew from the study; 2 dropped out/declined to participate; and, 3 dropped out due to non-serious adverse events. The subjects reported worsening of pre-existing gastrointestinal symptoms, gastrointestinal upset, and a non-study-related viral infection. Three were lost to follow-up. The investigators concluded that the consumption of 4 g 2'-FL/day in a nutritional formula had no adverse effects on the IBS and ulcerative colitis patient's gastrointestinal symptoms. The consumption was associated with an improvement in intra- and extra-intestinal symptoms.

In a randomized, double-blind, placebo controlled study by Iribarren et al. (2020), 60 Swedish adults with moderate irritable bowel syndrome (IBS) were assigned to consume a glucose placebo or 5 or 10 g/day of a 4:1 mixture of 2'-FL and LNnT for 4 weeks, followed by a 4-week follow-up period. In this study, IBS symptom severity, bowel habits, anxiety, and depression were assessed at baseline and at week 4 and 8. During the course of study, 2 subjects withdrew due to worsening of IBS symptoms, including 1 subject from each of the placebo and 10 g/day groups. No adverse effects on fecal microbiota were reported, and no between-group differences in severity of overall or individual gastrointestinal symptoms or symptom deterioration were reported. The investigators concluded that the study products were well tolerated and that gastrointestinal symptoms in adult IBS patients were not aggravated by consumption of up to 10 g of a 4:1 mixture of 2'-FL and LNnT/day for 4 weeks.

In a multicenter, open-label trial, Palsson et al. (2020) recruited subjects with IBS. Patients received daily orally administrated 5 g of HMOs 2'-FL and lacto-N-neotetraose in a 4:1 mix. Bowel habits, IBS symptoms, and quality of life were assessed at baseline and every 4 weeks during the 12-week intervention. In this study, a total of 317 patients (70.7% women; mean age of 44 years, range 18-93 years) received 5 g of HMOs, and 245 patients completed the trial according to protocol. The most common side effects were mild gastrointestinal symptoms such as flatulence, abdominal pain and discomfort, and distension. The authors reported that there were no incidents causing safety concerns and the patients generally reported that the intervention was well-tolerated. The investigators mentioned that supplementation with 2 selected HMOs improves IBS symptoms and quality of life without substantial side effects.

In summary, available clinical studies in adults (Elison et al., 2016; Iribarren et al., 2020;, Palsson et al., 2020; Ryan et al., 2021) show that the ingestion of up to 20 g/day of either 2'-FL, LNnT, or a combination of 2'-FL and LNnT in healthy adults and adults with IBS, ulcerative colitis, Crohn's disease, or celiac disease was well tolerated. As expected, the most common complaints were flatulence, abdominal distress, and abdominal pain. Similar results were also reported by Rasko et al. (2000), Parente et al. (2003), and Gurung et al. (2018) when the subjects ingested up to 20 g of another human milk oligosaccharide, 3'-SL/day. Thus, the publicly available evidence indicate that 2'-FL at levels up to 20 g in adults is unlikely to cause adverse effects and is considered as safe.

Table 8. Clinical Studies of Human Milk Oligosaccharides, including 2'-Fucosyllactose, in Adult Subjects

Study Design and Population	Groups and Numbers of Participants	Duration of Study	Safety Related Findings	Citation/GRN Reference
Open-label, single arm study Adults (21-75 years old) with a BMI of 19-40 kg/m^2 and with previously diagnosed inflammatory bowel disease (IBS), ulcerative colitis, Crohn's disease, or celiac disease	One group. 4 g of 2'-FL in combination with micronutrients, macronutrients, amino acids, and isomaltooligosaccharide (n=20)	6 weeks	12 subjects completed the study. 8 subjects withdrew from the study 2 dropped out/declined to participate 3 dropped out due to non-serious adverse events. They reported worsening of pre-existing gastrointestinal symptoms, gastrointestinal upset, and a non-study-related viral infection Three were lost to follow-up. Consumption of 4 g 2'-FL/day in a nutritional formula had no adverse effects.	Ryan et al., 2021
Open-label, single arm study Adult male and female patients (18 and older) with IBS	One group. 5 g of 2'-FL/LNnT (4:1 ratio) (n=317)	12 weeks	13 subjects were discontinued after completing the baseline survey because they did not start the intervention. Therefore, 273 patients completed the study. 8 subjects withdrew due to an adverse event. 4 subjects withdrew consent. 19 subjects were lost to follow-up. The authors reported that there were no incidents causing safety concerns and the patients generally reported that the intervention was well-tolerated 47 patients reported a total of 87 adverse events (AEs) in the study 61 of the AEs were related to the gastrointestinal tract. The most common side effect was passing gas, followed by abdominal distension and pain. One serious AE occurred (hospitalization due to colitis) but was determined to be unrelated to the intervention by the study's medical safety officer. No incidence of safety concerns and patients generally reported that the intervention was well-tolerated	Palsson et al., 2020
Parallel, double-blind randomized, placebo-controlled study Adult male and female patients (18 – 64 years old) with inflammatory bowel syndrome (IBS).	Group 1:Placebo (n=21) Group 2: 5 g 2'-FL/LNnT (4:1 ratio) (n=20) Group 3: 10 g 2'-FL/LNnT (4:1 ratio) (n=20)	4 weeks of treatment followed by a 4-week washout	Group 1: One patient discontinued intervention due to worsening of symptoms during the treatment period; 1 patient was lost to follow-up during the washout period Group 2: No patients left the study Group 3: One patient discontinued intervention due to worsening of symptoms during the treatment period; 1 patient was lost to follow-up during the washout period.	Iribarren et al., 2020

			There were no differences in overall gastrointestinal symptom severity among the groups at week 4 or week 8. None of the treatments aggravated the IBS symptoms. There were no significant differences among the groups in the individual domains of the Gastrointestinal Symptom Rating Scales (abdominal pain, bloating, constipation, diarrhea, and satiety). Within the groups: There was a decrease in the severity of bloating and diarrhea in Group 1 at week 4. In Group 2 and 3, there was a decrease in bloating and abdominal pain at week 8, respectively. There were no differences between groups or within the groups at week 4 or 8 regarding IBS symptom severity.	
Randomized, placebo-controlled double-blind study Healthy male and female adults ages 18-60 years	Group 1: 2 g glucose (n=10) Group 2: 5 g 2'-FL (n=10) Group 3: 10 g 2'-FL (n=10) Group 4: 20 g 2'-FL (n=10) Group 5: 5 g LNnT (n=10) Group 6: 10 g LNnT (n=10) Group 7: 20 g LNnT (n=10) Group 8: 3.3 g 2'-FL; 1.7 g LNnT (n=10) Group 9: 6.7 g 2'-FL; 3.4 g LNnT (n=10) Group 10: 13.3 g 2'-FL; 6.7 g LNnT (n=10)	1-2 week run-in period followed by a 2 week treatment period	All subjects were compliant and completed the study according to the protocol without any dropouts. 56 adverse events were reported by forty-four subjects All were judged as 'mild', and all subjects tolerated the investigational products throughout the trial period. Adverse events were usually reported as a complex of multiple symptoms such as flatulence, bloating and constipation, and were primarily reported at the end of the 2-week intervention. Most adverse events were reported by subjects taking the highest doses of 2′FL and LNnT. Gas/ flatulence was the most common adverse event reported, followed by stomach pain, diarrhea/loose stools and rumbling, but at lower frequencies. No significant difference in bowel movement was observed compared to Group 1. No change in clinical significance in any physical parameter including pulse rate and blood pressure was found during the 2-week intervention. There was no difference in clinical chemistry or hematology among the groups at the end of the 2-week intervention period.	Elison et al., 2016 /GRN 735, page 61
Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection	Group 1: Placebo (n=17) Group 2: 12 g/day 3'-SL (n=24)	4 weeks	There were no significant differences between pre- and post-dose gastrointestinal tolerance and clinical chemistry (serum biochemistry, hematology, and urine analysis) outcomes. Pre- and post-dose urea breath test values were not significantly different within or between the 3'-SL and placebo groups. Compliance and adverse events were similar between the groups.	Gurung et al., 2018 / GRN 880, pages 35, 36

Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection (dyspepsia)	Group 1: Placebo (n=21) Group 2: 10 g/day 3'-SL sodium salt (n=17) Group 3: 20 g/day 3'-SL sodium salt (n=22)	4 weeks	Five patients were excluded from analysis due to protocol violation Adverse events recorded in 6 patients were halitosis, asthenia, epigastric pain, and headache One patient dropped out due to headache associated with epigastric pain No serious adverse events were observed. *H. pylori* colonization documented by the 13C-Urea Breath Test (UBT) decreased significantly (p-value not provided) in both treatment groups and placebo but was most likely due to regression toward mean effect.	Parente et al., 2003 / GRN 766, pages 64-67
Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection	Group 1: Placebo (n=6) Group 2: 4 g 3'-SL (n=6) Group 3: 8 g 3'-SL (n=7) Group 4: 20 g 3'-SL (n=7)	56 days for control groups 1 and 2 28 days for Group 3	Oral supplementation of 3'-SL did not change Lewis antigen expression of *H. pylori* strains isolated from human gastric mucosa. No adverse effects on safety or tolerance were reported.	Rasko et al., 2000 / GRN 766, pages 64-67

6.2.3. Specific Toxicity Studies of 2'-FL

6.2.3.1. Specific Acute Toxicity Study of 2'-FL

In an acute study conducted as per National Standards of the People's Republic of China: GB 15193.3-2014 *National Food Safety Standard — Acute Oral Toxicity Test*, oral toxicity of 2'-FL was evaluated in ICR mice (CAIQTEST, 2022a). For this study, 2'-FL from Mengniu with certificate of analysis number- BJ00022000316001 and sample ID number- 2102-0215-P1, was used. In this study, 20 (10/sex) SPF ICR mice, weighing 18 - 22 g were used. Deionized water was used as the solvent and the test article was prepared immediately before use. 2'-FL was administered by a single oral gavage at a dose of 10.00 g/kg bw. Immediately after gavage, toxic signs, time to onset and resolution of signs, and time of death of the animals, if any, were observed and recorded. After gavage, the animals were observed at 0.5, 2, and 4 hours, and observed once a day thereafter for 14 consecutive days. A gross necropsy was performed on all animals, and gross pathological changes of each animal were recorded. Histopathological observation were performed, if pathological changes were observed in the gross necropsy.

Following oral gavage administration of 2'-FL, no mortality was noted. No significant toxic signs were observed during the observation period. The investigators stated that under the test conditions of the laboratory, the maximum tolerated dose (MTD) in the acute oral toxicity test of 2'-FL (the test article) in female and male ICR mice was found to be greater than 10.00 g/kg bw, and its LD_{50} was greater than 10.00 g/kg bw, indicating that the test article was actually non-toxic.

6.2.3.2. Specific Mutagenicity and Genotoxicity Studies of 2'-FL

In three separate tests, the mutagenicity and genotoxicity potentials of 2'-FL (CAIQTEST, 2022b) were investigated according to the national standards of food safety (China) guidelines for Bacterial reversion mutation test (GB 15193.4-2014), Mammalian Erythrocyte Micronucleus Test (GB 15193.5-2014), and Chromosome aberration test of mouse spermatogonia or spermatocyte (GB 15193.8-2014). For these studies, 2'-FL from Mengniu with certificate of analysis number- BJ00022000316001 and sample ID number- 2102-0215-P1, was used.

The findings from the mutagenicity and genotoxicity studies described below suggest that as evaluated by bacterial reverse mutation test, mammalian erythrocyte micronucleus test, and chromosome aberration test in mouse spermatogonia 2'-FL is unlikely to cause mutagenic or genotoxic effects.

6.2.3.2.1. Bacterial Reverse Mutation Test of 2'-FL

For the bacterial reverse mutation test, five dose groups of the test article were set at the final concentrations of 50.1, 158.1, 500, 1581.0, and 5000 µg/plate, respectively (CAIQTEST, 2022b). The test article was prepared immediately before use. A quantity of 0.50 g of the test article was suspended and diluted to 10 mL with a volume of deionized water to obtain the test solution at the highest concentration (5000 µg/plate). Test solutions at other concentrations were prepared sequentially by the $\sqrt{10}$-fold dilution method with sterile water as the solvent, and autoclaved (121°C, 20 min) for later use. In the test, a solvent (deionized water) control group, an untreated control group, and a positive control group were also set. For significant positive

results, no validation was required; for negative results, another validation would be performed at a 5-fold dose interval.

In the mutation test, five *Salmonella typhimurium* histidine-deficient strains TA97a, TA98, TA100, TA102, and TA1535, were used. Plate incorporation method: 0.1 mL of the bacterial solution, 0.1 mL of the test solution, and 0.5 mL of S_9 mixture (if metabolic activation was required) were added to the top agar (2 mL, 45°C) in turn, mixed thoroughly, and then rapidly poured onto the bottom agar. The revertant colonies per plate were counted after incubation at 37°C for 48 hours. A positive test result could be determined when the revertant colony number of the test strains TA1535 and TA98 was no less than twice that of the untreated control group, or when the revertant colony number of other test strains was no less than twice that of the untreated control group and showed a dose-response relationship or reproducible positive results at one test point. Triplicate plates were set for each dose. If the results of the first test were negative, a second test would be performed for validation at a 5-fold dose interval. The test doses were 5000.0, 1000.0, 200.0, 40.0, and 8.0 μg/plate, respectively. The whole test was repeated once (CAIQTEST, 2022b).

The background bacterial lawn of the plate in each dose group grew well without abnormalities. The results from this study revealed: the number of (spontaneous revertant) colonies in the untreated control group was within the historical normal range of the laboratory; the number of revertant colonies in the positive control group of each strain was 2 times higher than that (spontaneous revertant) in the untreated control group, indicating that the positive control was valid; the number of revertant colonies in each dose group of the test article was no more than 2 times that in the untreated control group, and no dose-response relationship was observed across dose groups. No mutagenic effects of the test article on strains TA97a, TA98, TA100, TA102, and TA1535 were observed with or without S_9 (CAIQTEST, 2022b).

6.2.3.2.2. Mammalian Erythrocytes Micronucleus Test of 2'-FL

For the *in vivo* mammalian erythrocyte micronucleus test, SPF ICR mice (25/sex; weighing 25-35 g) were used (CAIQTEST, 2022b). For this study, three dose groups of the test article were set, i.e., high dose (10.00 g/kg bw), intermediate dose (5.00 g/kg bw), and low dose (2.50 g/kg bw). The test article was prepared immediately before use in deionized water. Additionally, control group and a positive control group were included in the study. Animals in the positive control group were administered cyclophosphamide by oral gavage at 40 mg/kg bw, and those in the other groups were treated at 20 mL/kg bw. Test article (2'-FL) was administered via gavage twice within 30 hours, that is, a second dose was administered 24 hours after the first dose by oral gavage, and the animals were euthanized by cervical dislocation 6 hours after the second dose. The femoral bone marrow was harvested and diluted with calf serum to prepare the bone marrow smear, which was fixed in methanol, stained with Giemsa, and microscopically examined. For each animal, the polychromatic erythrocytes (PCEs) in 200 red blood cells (RBCs) were counted and calculated for their proportion; the micronucleus cells in 2000 PCEs were counted, and the frequency of micronucleus was calculated (‰). If there are multiple micronuclei in a PCE, only one cell is counted.

The results of this study revealed a highly significant difference between the positive control group and the control group ($P < 0.01$), indicating that the positive control was valid. The percentage of PCEs in each dose group of the test article was no less than 20% of that in the negative control group, indicating that the test article was not significantly cytotoxic at the test

dose. The frequency of micronucleus in each dose group of the test article was not statistically different from that in the control group ($P > 0.05$), indicating that the test article (2'-FL) had no micronucleus-inducing effect on mouse bone marrow cells (CAIQTEST, 2022b).

6.2.3.2.3. Chromosome Aberration Test of 2'-FL

In the *in vivo* chromosome aberration test in mouse spermatogonia, SPF ICR male mice (n=30 males; weighing 25-35 g) were used (CAIQTEST, 2022b). For this study, mice were randomly divided into 5 groups (10 in the high-dose group, and 5 in each in other four groups), on the day of administration of the test article. The test article was administered within 24 hours by a single oral gavage. Animals in the high-dose group were euthanized and sampled at two time points one at 24 hours and other at 48 hours after the last dose, while animals in other dose groups were euthanized and sampled at 24 hours after the last dose. For positive control, colchicine (prepared immediately before use; injection dose: 5.00 mg/kg bw) was injected intraperitoneally at 4 hours before euthanization of animals. After the animals were euthanized by cervical dislocation, both testes were quickly harvested, and the spermatogonia Giemsa-stained specimens were prepared according to the standard procedures and examined microscopically.

At least 1000 cells from each animal were observed to determine the spermatogonial mitotic index, which in the high-dose group should be no less than 50% of that in the solvent control group. One hundred metaphase cells (including metaphase cells with a chromosome number of $2n \pm 2$) from each animal and at least 500 metaphase cells from each dose group were observed for chromosome analysis. Parameters indicating changes in the chromosome structure [such as chromatid gap (ctg), chromosome gap (csg), chromatid break (ctb), chromosome break (csb), chromosome fragment (csf), acentric ring (r), ring chromosome (R), dicentric (dic), translocation (t), microsome (c), chromatid exchange (cte), and chromosome exchange (cse)] and in the chromosome number [such as aneuploid (u), polyploid (pol), endoreduplication (end)] were microscopically observed, analyzed, and counted. Chromosomes within the same cell that had one or more types of aberration were counted as one aberrant cell. Chromosome gaps and univalent were recorded and reported separately and not counted in the aberration rate (CAIQTEST, 2022b).

The microscopic examination showed that the spermatogonial mitotic index in all dose groups of the test article was no less than 50% as compared with the control group. The test results showed that there was a highly significant statistical difference ($P < 0.01$) between the positive control group and the solvent control group, indicating that the positive control was valid. The number and type of chromosomal aberrations and parameters of aberrant cells of mouse spermatogonia in each dose group of the test article were not statistically different from those of the solvent control group, and no dose-response relationship was observed across dose groups, indicating that there was no effect of the test article on mouse spermatogonia chromosomes (CAIQTEST, 2022b).

6.2.3.3. Specific Subchronic Toxicity Study of 2'-FL

In a 90-day repeat dose study conducted as per National Standards of the People's Republic of China: GB 15193.13-2015 *National Food Safety Standard — 90 days oral toxicity test*, effects of 2'-FL were evaluated in SD rats (CAIQTEST, 2022c). For this study, 2'-FL from Mengniu with certificate of analysis number- BJ00022000316001 and sample ID number- 2102-0215-P1, was used. In this study, 100 SPF SD rats (50/sex), weighing 50 - 100 g, were selected

and randomly divided into the main study groups [3 dose groups and a solvent control group, n = 20/group (10/sex)] and the satellite groups for the control group and high-dose group [n = 10 per group (5/sex)]. The test article (2'-FL) was mixed with the basic (standard) feed, the sample mixing amount in the low-, intermediate-, and high-dose groups was 2.5, 5.0, and 10.0% (m/m), respectively. The daily feed intake of animals was converted by 8% of the body weight, and the actual test article intake of animals was 2.0, 4.0 and 8.0 g/kg bw, respectively. In addition, the normal feed was set as the control group. The animals were fed for 90 consecutive days and had free access to drinking water. In addition to the main (control, low, mid, high) groups, an interim satellite group was included for the control group and the high-dose group that were fed for 45 days and necropsied. During the course of the study and at termination, all standard parameters, such as general clinical observation (including behavioral signs), animal weights, feed consumption, ophthalmology, urinalysis, hematology, blood biochemistry and pathological examination (gross necropsy and histopathology), relevant to the toxicity testing were measured.

During the course of study, no changes in daily clinical observations or any abnormalities were noted in the animals in the treatment groups. No mortality in any group was noted. There were no statistical differences in weekly body weight, body weight gain, food consumption, and food utilization of females between the high-dose group and the control group. At Week 6, the body weight gain and food utilization of males in the high-dose interim satellite group were significantly higher than those in the control group. These changes were not considered as biologically significant as they were within the historical control range of the laboratory. No statistical differences were seen in all other indicators. The food consumption at Week 6 was significantly higher in females in the intermediate-dose group than in the control group, at week 13, the food consumption of animals in the high-dose group significantly decreased. However, there was no dose-response relationship for any of the above indicators; the parameters were all within the historical control range of the laboratory and therefore, were not considered biologically significant. For males, there were no statistical differences in the indicators between each dose group and the control group. The authors of the report concluded that there were no adverse effects of the test article on the body weight, body weight gain, food consumption, and food utilization of the rats. Ophthalmic examinations also did not reveal any treatment related effects (CAIQTEST, 2022c).

The urinalysis parameters of females and males in each dose group showed no abnormalities in the urine color or appearance. No statistical differences were observed in other parameters (urine protein, specific gravity, pH, glucose, and urine occult blood) of females and males in each dose group of the test article compared with those of animals in the control group. Hematological parameters, including white blood cell (WBC) count and differential (at least lymphocyte (LYM), neutrophil (NEUT), and others), red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), platelet (PLT) count, prothrombin time (PT), and activated partial thromboplastin time (APTT) did not reveal any significant differences between the treatment (receiving 2.0, 4.0 and 8.0 g/kg bw/day of 2'FL) groups and control group at the end of the 90 days or in the interim group (receiving 8 g/kg bw/day of 2'-FL) at 45 days as compared to the respective control (CAIQTEST, 2022c).

The analysis of the blood biochemical parameters that included alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), Urea, creatinine (Cre), glucose (Glu), total protein (TP), albumin (Alb), total cholesterol (CHO), triglyceride (TG), chloride (Cl), potassium (K), and sodium (Na) did not

reveal any significant treatment related changes in the interim satellite groups between the high-dose group and the control group. In the main treatment groups, the Cl was significantly higher in females in the intermediate-dose group (4 g/kg bw/day) as compared to the control group. However, there was no dose-response relationship; the parameter levels were within the historical control range of the laboratory and, therefore, this change was not considered biologically significant. Additionally, there were no statistical differences in any of the other parameters between the dose groups and the control group. For males, there were no statistical differences in the parameters between the dose groups and the control group (CAIQTEST, 2022c).

Analysis of organ indicators (weight and organ to body weight ratio) of the animals in the interim satellite group revealed no statistical difference in the indicators of females and males in the high-dose group compared with those of the control group. The organs studied included, brain, thyroid gland, thymus, lung, heart, liver, spleen, kidney, adrenal gland, stomach, duodenum, jejunum, ileum, colon, rectum, pancreas, mesenteric lymph nodes, ovary, uterus, testis, epididymis, prostate, and bladder; the absolute weight of the brain, heart, thymus, adrenal gland, liver, spleen, kidney, testis, epididymis, uterus, and ovary. Similarly, analysis of organ indicators of the animals in the main study dose group revealed no statistical difference in the weights of females and males in each dose group compared with those of the control group. In conclusion, under the test conditions, the test article (2'-FL) exerted no adverse effects on the wet weight and organ/body weight ratio of the tested organs in rats. No test article-induced significant pathological changes were observed in the brain, pituitary gland, thyroid gland, thymus, lung, heart, liver, spleen, kidney, adrenal gland, stomach, duodenum, jejunum, ileum, colon, rectum, pancreas, mesenteric lymph node, ovary, uterus, testis, epididymis, prostate, or bladder of the animals in the interim satellite groups or main study groups (CAIQTEST, 2022c).

Based on the findings from this study, it is stated in the report that, according to the comprehensive analysis, no adverse effects of the test article (2'-FL) in SD rats were found under the test conditions, and the no-observed-adverse-effect level (NOAEL) was 8.00 g/kg bw/day, the highest dose tested (CAIQTEST, 2022c).

6.2.3.4. Specific Teratogenicity Study of 2'-FL

In a teratogenicity study conducted as per National Standards of the People's Republic of China: GB 15193.14-2015 *National Food Safety Standard — Teratogenicity Test*, teratogenic effects of 2'-FL were evaluated in SD rats (CAIQTEST, 2022d). For this study, unmated healthy SPF SD rats, 140 females and 70 males, weighing 220 - 260 g, were selected. The animals were divided into four groups. Based on the NOAEL (8.00 g/kg bw/day) from the 90-day feeding toxicity test, the low, intermediate and high doses (three treatment groups) for oral gavage teratogenicity study were established as 2.00, 4.00 and 8.00 g/kg bw/day, respectively. Additional groups served as controls.

On the first day of the test, female and male rats were kept in the same cage for mating at a ratio of 2:1, and the vaginal smear was observed the next morning to confirm whether conception occurred. If sperm was detected, the mating was considered successful and that day was marked as day 0 of gestation (GD0). The pregnant rats were weighed, randomly grouped and numbered, and weighed on GD6, 9, 12, 15, and 20. The test article was given to the pregnant rats once a day by oral gavage from GD6 through GD15. The rats were euthanized on GD20, with the uterus dissected out and weighed. A gross necropsy was performed to examine the

number of corpus luteum, absorbed fetuses, early stillbirths, late stillbirths, and live fetuses. The sex, body weight, and body length of the fetuses were recorded, and the appearance of the fetuses was examined for any abnormalities. For each litter, half of the live fetuses (odd or even) were sampled according to the standard method for skeletal examination and the other half for visceral examination (CAIQTEST, 2022d).

In order to investigate any effects of 2'-FL during pregnancy, SD rats were administered the test article at dose levels of 2.00, 4.00, and 8.00 g/kg bw/day by oral gavage for 10 consecutive days from GD6 through GD15. No abnormalities in the external signs, behavior, or feces, no vaginal bleeding, abortion, or other toxic signs, and no death of the pregnant rats in each dose group and the negative control group were observed during the course of study. There were no significant differences in the body weight, gestational weight gain, weight of uterus and fetus, and net weight gain of pregnant rats between each dose group and the negative control group (CAIQTEST, 2022d).

To evaluate the effects of 2'-FL on fetal developments, a gross necropsy was performed to examine the uterus and fetuses of pregnant rats that were euthanized on GD20. No significant abnormalities were observed. There were no significant differences between the low-, intermediate-, and high-dose groups and the negative control group in the number of corpus luteum, implantations, live fetuses, absorbed fetuses, and dead fetuses, as well as their mean per litter. The changes in body weight and length of fetuses in different dose groups were subjected to statistical analysis. There was no significant difference in the mean body weight and length of fetuses between each dose group of the test article and the control group (CAIQTEST, 2022d).

The effects of the test article on the fetal malformation rate did not reveal any significant differences between the dose groups of the test article and the control group, when the fetuses per litter were examined visually. There was no significant difference in the skeletal and visceral malformation characteristics and malformation rates per litter between the dose groups and the control group. In the skeletal examination, a fetus in a litter in the high-dose group showed abnormal sternum number, which was diagnosed as the absent sternum, a common developmental phenomenon in SD rats, and there was no dose-response relationship across the dose groups; therefore, it was not considered toxicologically significant. No significant abnormalities in organ size or morphological structure were observed in the visceral examination, and there were no significant differences between the dose groups of the test article and the control group (CAIQTEST, 2022d).

The authors of the study report concluded that no potential teratogenic toxicity of the test article was observed in SD rats, under the study conditions. The NOAEL of 2'-FL for teratogenic effects was established as 8.00 g/kg bw/day, the highest dose tested (CAIQTEST, 2022d).

6.2.4. Other Toxicity Studies of 2'-FL and Related Compounds

Multiple published and unpublished studies (available in public domain) that include genotoxicity (Table 9), subchronic toxicity (Table 10), neonatal piglet tolerance (Table 11) (Parschat et al., 2020; Hanlon 2020; Coulet et al., 2014; Jennewein, 2013; Jennewein, 2014a; Jennewein, 2014b; Jennewein, 2014c; Verspeek-Rip, 2015; Verbaan, 2015a; Verbaan, 2015b; van Berlo et al., 2018; Phipps et al., 2018; Pernard, 2015; Hanlon and Thorsrud, 2014) supports the safety of 2'-FL. In almost all studies, except Coulet et al. (2014) study, the 2'-FL used was produced by microbial fermentation. In the Coulet et al. (2014), 2'-FL used was produced by chemical synthesis. In several GRAS notices that received no questions letter from FDA, the

above mentioned toxicity studies have been extensively summarized. The published and unpublished studies are summarized in previous GRAS notifications include GRN 546; GRN 571; GRN 650; GRN 735; GRN 749; GRN 815; GRN 929; GRN 932; GRN 1014; and GRN 1034. The toxicity related description from these GRAS notices are incorporated in the present GRAS notice, by reference, along with GRN number and the pages where it is described, and are briefly summarized in table format in the following sections, along with the detailed summaries of the new studies. Collectively, these studies suggest that 2'-FL alone or in combination with other HMOs, is not mutagenic, clastogenic or aneugenic, has a NOAEL of at least 5 g/kg bw/day in rats, and is well-tolerated at levels up to 1.6 g/kg bw in neonatal piglets. Some of the pivotal studies of 2'-FL, including those that appeared more recently are further described below.

6.2.4.1. Genotoxicity Studies of 2'-FL

The findings from available mutagenicity and genotoxicity studies are described below and also further summarized in Table 9.

6.2.4.1.1. Bacterial Reverse Mutation and Mammalian Cell Gene Mutation Test

van Berlo et al. (2018; also described in GRN 1014- page 35) investigated the mutagenic effects of 2'-FL, produced using fermentation, in an OECD 471-compliant bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 and the tryptophan-requiring *E. coli* strain WP2 uvrA in the absence and presence of metabolic activation. In this study, five test concentrations of 2'-FL ranging from 62 to 5000 μg/plate were tested. The findings from these tests, did not reveal any dose related increase in the mean number of revertant colonies compared to background at concentrations up to 5000 μg/plate, in the presence or absence of metabolic activation. The colonies of the negative controls were within the acceptable range and positive controls showed a significant increase in the number of revertant colonies.

In another similar study, also performed as per OECD 471 guidance and described in GRN 650 (page 36), Verspeek-Rip et al. (2015) evaluated the mutagenic potentials of 2'- FL, produced by fermentation, using *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and an *E. coli* strain WP2uvrA in the presence and absence of metabolic activation. In this study, 5 concentrations of 2'- FL ranging from 52 to 5000 μg/plate were studied. There was no cytotoxicity to any of the strains tested, no significant or dose-related increases in revertant colonies, and no mutagenic effect.

In a bacterial reverse mutation test, conducted as per OECD 471 guidance, Phipps et al. (2018) investigated the effects of a mixture of 2'-FL (92.2%) and difucosyllactose (DFL) (9.70%) produced by fermentation. In this study, *S. typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* strain WP2 uvrA were exposed to concentrations of mixture of 2'-FL and DFL at levels ranging from 5 to 5000 μg/plate in the absence and presence of metabolic activation. The investigators reported no dose related increase in the number of revertant colonies in either the presence or absence of metabolic activation at concentrations up to 5000 μg/plate. Mean values for treated cultures, as well as negative and positive controls, were within respective historical control data ranges.

In another published study, Parschat et al. (2020) evaluated the mutagenicity of a mixture of HMOs containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT,

4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured using fermentation. The study was conducted as per OECD 471 bacterial reverse mutation test. In this study, five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used in two independent experiments with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test. To each plate, five concentrations (10.0, 31.6, 100, 316, or 600 mg) of the mixture of HMOs containing 2.4, 4.7, 14.9, 47.1, 148.8, and 282.6 mg 2'-FL, respectively, were applied. For negative control purified water was used. For the positive controls, depending on strain sodium azide (for TA1535 and TA100), 2-nitrofluorene (for TA98), benozo[a]pyrene 9AA (for TA1537), and mitomycin C (for TA102) were used. Cytotoxicity was defined as a reproducible reduction in the number of colonies by more than 50% compared to the solvent control and/or a scarce background lawn. Compared to the negative control, the positive controls increased the mean revertant colony numbers at least 3-fold with and without metabolic activation, verifying the validity of the test. For the HMO mixture, no cytotoxicity or mutagenicity was noted in any of the test strains up to 600 mg HMO mixture/plate (equivalent to 282.6 mg 2'- FL/plate) in either the plate incorporation or preincubation tests. These investigators concluded that the HMO mixture, and the 2'-FL contained therein, was not mutagenic under the conditions tested.

In yet another published study, conducted as per OECD 471 guideline, Coulet et al. (2014) studied the mutagenicity of synthetic 2'-FL. This study, conducted in two experiments, is further summarized in Table 9. In the first experiment using the plate incorporation method, bacterial strains of *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 were treated with 2'-FL at concentrations of 52, 164, 512, 1600, or 5000 µg/plate. In the second experiment using the pre-incubation method, the bacterial strains were incubated with 2'-FL at concentrations of 492, 878, 1568, 2800, or 5000 µg/plate. In both the experiments, treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration either in the presence of absence of S9. The positive control agents substantially induced the number of revertant colonies compared to the negative control. The findings from this study suggest that 2'-FL is non-mutagenic in the Ames test at concentrations up to 5000 µg/plate.

In addition to above described studies, Coulet et al. (2014) also investigated the mutagenic potential of synthetic 2'-FL in an *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells conducted as per OECD 476 guidelines. In the first experiment, cells were incubated with 2'-FL at concentrations ranging from 1.7 to 5000 µg/mL for 24 hours without S9. In the second experiment, cells were treated with 2'-FL at concentrations ranging from 492 to 5000 µg/mL for 4 hours with or without S9. In both the experiments, no precipitation or cytotoxicity was observed in cells treated with 2'-FL at any concentration. Also, in both the experiments, no statistically or biologically significant increases in the frequency of mutations were observed in cells treated with 2'-FL, with or without S9. In this study, methymethanesulfonate (MMS) and cyclophosphamide were used as the positive controls in the absence and presence of S9, respectively, while water was used as the negative control. The findings from this study suggest that synthetic 2'-FL is nonmutagenic in L5178Y tk +/- mouse lymphoma cells.

6.2.4.1.2. Micronucleus Test

van Berlo et al. (2018; also described in GRN 1014- page 35) investigated the potential clastogenic and aneugenic effects of 2'-FL, produced by fermentation, in an OECD 487-

compliant *in vitro* micronucleus test using cultured human lymphocytes. In this study, duplicate cultures of binucleated human lymphocytes, in the absence and presence of a metabolic activation system, were exposed to 2'-FL at levels ranging from 3.9 to 2000 μg/mL. Using the Cytokinesis-Block Proliferation Index, cytotoxicity was determined. In the first experiment, exposure was for 4 hours with a 20-hour recovery time. In the second experiment, exposure was for 20 hours with no recovery time. Findings from both of these experiments did not reveal any statistically significant, dose-related increases in cytotoxicity or in the number of binucleated cells containing micronuclei at any concentration tested. The number of binucleated cells containing micronuclei was reported to be within the test facility's historical data range. The investigators concluded that 2'-FL is not mutagenic based on the negative results of the *in vitro* micronucleus test.

In two separate unpublished studies, as summarized in GRN 650 (page 36 and 37) and in GRN 1014 (page 36), Verbann et al. (2015a; 2015b) also investigated the clastogenic and aneugenic effects of 2'-FL (chemically synthesized, as well as produced by fermentation), in OECD 487-complaint *in vitro* mammalian cell mutation assay using peripheral human lymphocytes. In the study using chemically synthesized 2'-FL, no increase in the number of micronucleated peripheral human lymphocytes at concentrations of up to 2000 μg/mL in the presence and absence of exogenous metabolic activation (S9) was observed. In the study conducted using microbially produced 2'-FL, a short-term exposure experiment was carried out in which, lymphocytes were incubated with 2'-FL at concentrations of 512, 1600, or 2000 μg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 512, 1600, or 2000 μg/mL for 24 hours in the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated cells were scored for micronuclei under each treatment condition. No significant increase in cytotoxicity or in the number of micronucleated cells in the presence or absence of metabolic activation was reported.

Phipps et al. (2018; also summarized in GRN 650 and GRN 1014) performed an OECD 487-compliant *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes and using a mixture of 2'-FL (92.2%) and DFL (9.70%) produced by fermentation. In this study, the lymphocytes were exposed to 2'-FL/DFL mixture at levels ranging from 500 to 2000 μg/plate in the presence and absence of metabolic activation for 3 hours or in the absence of metabolic activation for 20 hours. The findings from this study did not reveal any treatment related changes in clastogenicity or aneugenicity at concentrations up to 2000 μg/plate in the presence or absence of metabolic activation. The mean values for exposed cultures, as well as, negative and positive controls were within the respective historical control data ranges.

In another published study, Parschat et al. (2020) investigated the clastogenicity and/or aneugenicity of a mixture of HMOs (content as described above) as per OECD 408 guidance for *in vitro* micronucleus test using human peripheral blood lymphocytes obtained from young, healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation. Lymphocytes were exposed to 7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 3.5, 7.1, 14.1, and 28.3 mg 2'-FL/mL medium) for 4 or 24 hours in the presence and absence of metabolic activation. Purified water was used for the negative control and the positive controls were mitomycin C (0.2 μg/mL), colchicine (0.02 μg/mL), and cyclophosphamide (20 μg/mL) with and/or without metabolic activation. At least 500 cells per replicate cell culture were scored and classified as mononucleates, binucleates, or multinucleates

to estimate the proliferation index as a measure of toxicity. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI). Micronucleus frequencies were analyzed in at least 2000 binucleate cells per concentration (i.e., ≥ 1000 binucleate cells per culture; two cultures per concentration). The ability of the HMO mix to induce micronuclei was considered to be positive if there was a statistically significant and/or dose related increase compared to the negative control or if any of the results were outside the distribution of the historical negative control data (Poisson-based 95% control limits). Mitomycin C and cyclophosphamide induced significant chromosomal damage whereas colchicine induced significant ($p \leq 0.05$) damage to the cell division apparatus, both validating the tests. No chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested. The findings from this study shows that the HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (28.3 mg/mL of 2'-FL).

Table 9. Summary of Published and Unpublished Genotoxicity Studies of 2'FL

Citation	Ingredient, Source (Manufacturer ; Purity)	Test and Compliance	Test System/ Animal Species	Concentration/Dose and Controls	Results and Conclusion
Studies Reviewed and Described in Previous GRAS notices on 2'-FL					
Coulet et al. (2014); GRN 546	2'-FL, synthetic (Glycom; 99%)	Bacterial reverse mutation assay OECD Principles of GLP (OECD, 1998); OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation assay 52, 164, 512, 1600, or 5000 µg/plate (+/-S9) Pre-incubation assays 492, 878, 1568, 2800, or 5000 µg/plate (+/- S9) Negative control Water (vehicle) Positive controls + S9: 2-aminoanthracene -S9: 2-nitrofluorene (TA98), sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), t-butylhydroperoxide (TA102)	No cytotoxicity or precipitation; no biologically significant increase in the number of revertant colonies compared to the negative control Non-mutagenic under the conditions of the assay
		In vitro mammalian cell gene mutation assay OECD Principles of GLP (OECD, 1998); OECD G 476 (OECD, 1997b)	Cultured mouse lymphoma L5178Y cells	4 h exposure 492, 878, 1568, 2800, or 5000 µg/ml (+/- S9) 24 h exposure 1.7, 5.4, 17, 52, 164, 512, 1600, or 5000 µg/ml (- S9) Negative control Untreated medium Positive controls + S9: cyclophosphamide - S9: methylmethanesulfonate	No cytotoxicity or precipitation; no biologically relevant increases in mutant frequency with or without S9 No-genotoxic in mouse lymphoma cells under the conditions of the assay
Jennewein Biotechnologie (2014b) [unpublished] - In: Jennewein Biotechno	2'FL from *Escherichia coli* BL21 (Jennewein; 92.4%)	Bacterial reverse mutation assay GLP according to directive 2004/9/EC	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation and the pre-incubation assays 0, 31.6, 100, 316, 1000, 3160 or 5000 µg/plate (+/- S9) Negative control DMSO (vehicle) Positive controls + S9: sodium azide	No cytotoxicity; no increase in the number of revertant colonies compared to the negative control at any dose level with or without metabolic activation

logie (2015) GmbH (part 1); GRN 571		(EC, 2004); OECD TG 471 (OECD, 1997a)		(TA1535, TAl00), 2-nitrofluorene (TA98), 9-aminoacridine (TA1537), or mitomycin C (TA102) - S9: 2-aminoanthracene (TA1535, TA100), or benzo(a)pyrene (TA98, TA102, TA1537)	Non-mutagenic under the conditions of the assay
Jennewein Biotechnologie (2014c) [unpublished] - In: Jennewein Biotechnologie GmbH (2015) [part 1]; GRN 571		*In vivo* mammalian micronucleus assay GLP according to Directive 2004/9/EC (EC, 2004); OECD G 474 (OECD, 2016a)	Rat (*Rattus narvegicus*)/CD Crl:CD (SD) 5/sex/group	<u>24 h exposure</u> 0, 500, 1000, or 2000 mg/kg bw (single dose) <u>48 h exposure</u> 0 or 2000 mg/kg bw (single dose) Single dose (gavage) Bone marrow collected 24h (all groups) or 48h (negative control and high dose groups only) post-administration <u>Negative control</u> 0.8% aqueous hydroxypropylmethyl-cellulose (vehicle) <u>Positive control</u> 27 mg/kg bw cyclophosphamide with 0.9% NaCl solution	No systemic toxicity; no increase in the incidence of micro-nucleated PCEs compared to the negative control Non-genotoxic under the conditions of the assay
Verspeek-Rip (2015) [unpublished); GRN 650	2'-FL from *E. coli* K-12 (Glycom; 97.6%)	Bacterial reverse mutation assay OECD principles of GLP (OECD, 1998); OECD G 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA1535, and TA1537; *Escherichia coli* strain WP2uvrA	<u>Plate incorporation assay</u> 0, 52, 164, 512, 1600, or 5000 µg/plate (+/- S9) <u>Pre-incubation assay</u> 0, 492, 878, 1568, 2800, or 5000 µg/plate (+/- S9) <u>Negative control</u> Water (vehicle) <u>Positive controls</u> + S9: 2-nitrofluorene (TA98, TA1537, pre-incubation assay), methylmethanesulfonate (TA100), sodium azide (TA1535), ICR-191 (TA1537), plate incorporation assay, 9-aminoacridine (TA1537) or 4-nitroquinoline n-oxide (WP2uvrA) - S9: 2-aminoathracene	No cytotoxicity or precipitation; no biologically significant increase in the number of revertant colonies compared to the negative control Non-mutagenic under the conditions of the assay

Verbaan (2015) [unpublished]; GRN 650		*In vitro* micronucleus assay OECD principles of GLP (OECD, 1998); OECD TG 487 (OECD, 2014)	Cultured peripheral human lymphocytes	3 h exposure 512, 1600, or 2000 µg/ml (+/- S9) 24 h exposure 512, 1600, or 2000 µg/ml (- S9) Negative control Water (vehicle) Positive controls +S9: cyclophosphamide -S9: mitomycin C	No cytotoxicity or precipitation; no statistically or biologically significant increases in the frequency of mono- or bi- nucleated cells with micronuclei in cells treated with 2'-FL compared to the negative control Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay
van Berlo et al. (2018); GRN 735	2'FL from *E. coli* K-12 (Glycosyn, LLC and Friesland Campina Domo B.V.; 94%)	Bacterial reverse mutation assay OECD Principles of GLP (OECD, 1998); OECD G 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* strain WP2uvrA	Plate incorporation assay 0, 62, 195, 556, 1667, or 5000 µg/plate (+/- S9) Negative control Phosphate buffered saline (vehicle) Positive controls +S9: 2-aminoathracene (TA98, TAl00, TA1535, WP2 uvrA), or benzo(a)pyrene (TA1537) S9: sodium azide (TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), sodium azide (TA100), or N-ethyl-N-nitrosourea (WP2uvrA)	No cytotoxicity; no biologically significant increase in the number of revertant colonies compared with the negative control Non-mutagenic under the conditions of the assay
		In vitro micronucleus assay OECD Principles of GLP (OECD, 1998); OECD G 487 (OECD, 2014)	Cultured peripheral human lymphocytes	4 h exposure 0, 500, 1000, or 2000 µg/ml (+/- S9) 24 h exposure 0, 500, 1000, or 2000 µg/ml (- S9) Negative control Culture medium Positive controls + S9: cyclophosphamide - S9: vinblastine	No cytotoxicity; no statistically or biologically significant increase in the frequency of MNBCs in cells treated with 2'-FL compared to the negative control Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay
Phipps et al. (2018); GRN 815	2'-FL/DFL *E. coli* K-12 [Glycom; 82.5% (w/w) 2'-FL; 9.7%	Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537;	Plate incorporation assay 0, 5, 15, 50, 150, 500, 1500 or 5000 µg/plate (+/- S9) Pre-incubation assay 0, 50, 150, 500, 1500 or 5000 µg/plate	No biologically relevant increase in the number of revertant colonies compared to the vehicle control

	(w/w) DFL]	OECD principle of GLP (OECD, 1998); OECD G 471 (OECD, 1997a)	*Escherichia coli* strain WP2uvrA (pKM101)	(+/- S9) Negative control Water (vehicle) Positive controls + S9: 2-aminoanthracene (TA100, TA1535, WP2uvrA) or benzo[a]pyrene (TA98, TA1537) - S9: sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98) or 4-nitroquinoline-1-oxide (WP2uvrA)	Non-mutagenic under the conditions of the assay
		In vitro micronucleus assay OECD principle of GLP (OECD, 1998); OECD G 487 (OECD, 2016b)	Cultured peripheral human lymphocytes	3 h exposure 0, 500, 1000 or 2000 µg/ml (+/- S9) 24 h exposure 0, 500, 1000 or 2000 µg/ml (- S9) Negative control Water (vehicle) Positive controls + S9: cyclophosphamide - S9: colchicine and mitomycin C	No cytotoxicity; no significant increase in the frequency of MNBCs in cells treated with 2'-FL compared to the negative control Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay
Parschat et al. (2020)	HMO MIXI [47.1% dw 2'-FL; 16.0% dw 3'-FL, 23.7% dw LNT; 4.1% dw 3'-SL; 4.0% dw 6'-SL) (Jennenwein Biotechnologie)	Bacterial reverse mutation assay OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TAl00, TA102, TA1535, and TA1537	Plate incorporation assay 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (+/- S9) Pre-incubation assay 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (+/- S9) Negative control Water (vehicle) Positive controls +S9: 2-aminoanthracene (TA100, TA1535) or benzo[a)pyrene (TA98, TA102, TA1537) - S9: sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), or mitomycin C (TA102)	No cytotoxicity or mutagenicity compared to the vehicle control Non-mutagenic under the conditions of the assay
		In vitro micronucleus assay	Cultured peripheral human lymphocytes	4 h exposure 0, 7.5, 15, 30, or 60 mg/ml (+/- S9)	No indications of chromosomal damage; frequency of micronucleate

		OECD TG 487 (OECD, 2016b)		24 h exposure 0, 7.5, 15, 30, or 60 mg/ml (- S9) Negative control Water (vehicle) Positive controls + S9: cyclophosphamide - S9: colchicine and mitomycin C	cells within the historical control range for the test item and vehicle controls Non-genotoxic under the conditions of the assay
Phipps et al. (2021)	2'-FL (31.5%), LNFP-1 (59.4%), other carbohydrates (4.6%) (Glycom A/S)	Bacterial reverse mutation assay OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* WP2 uvrA	Plate incorporation assay 0, 5, 15, 50, 150, 500, 1,500, or 5,000 µg/plate (+/- S9) Pre-incubation assay 0, 50, 150, 500, 1500, or 5000 µg/plate (+/- S9) Negative control Water Positive controls + S9: benzo[a]pyrene and 2-aminoanthracene - S9: sodium azide, 9-aminoacridine, 2-nitrofluorene, and 4-nitroquinoline-1-oxide	No cytotoxicity or mutagenicity compared to the vehicle control Non-mutagenic under the conditions of the assay
Coulet et al. (2014); GRN 546		*In vitro* micronucleus assay OECD TG 487 (OECD, 2016b)	Cultured peripheral human lymphocytes	3-hour exposure 0.5, 5, 50, 500, 1000, or 2000 µg/mL (+/- S9) Additional 3-hour exposure 500, 1000, or 2000 µg/mL (+S9) 20-hour exposure 0, 0.5, 5, 50, 500, 1000, or 2000 µg/mL (- S9) Negative control Water Positive controls - S9: mitomycin C and colchicine + S9: cyclophosphamide	No indications of chromosomal damage; frequency of micronucleate cells within the historical control range for the test item and vehicle controls Not clastogenic or aneugenic under the conditions of the assay

+S9 = with metabolic activation; - S9 = without metabolic activation; 2'-FL = 2'-fucosyllactose; 3'-FL = 3'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; DMSO = dimethyl sulfoxide; dw = dry weight; FDA= Food and Drug Administration; GLP = Good Laboratory Practice; GRN = GRAS Notice; h = hours; HMO= human milk oligosaccharide; LNFP-I = lacto-N-fucopentaose I; LNT = lacto-N-tetraose; MNBC = micronucleated binucleated cell; OECD= Organization for Economic Co-operation and Development; PCE = polychromatic erythrocyte; TG = Test Guideline; U.S.= United States.

6.2.4.2. Short-term and Subchronic Toxicity Studies of 2'-FL and Related Compounds

The available short-term and subchronic toxicity studies of 2'-FL are summarized in Table 10. The short-term studies summarized in Table 10 are not further discussed. Given the importance of the subchronic toxicity studies in the safety assessment, these studies are further elaborated below.

In a published subchronic toxicity study, conducted as per OECD 480 guidance for 90-day dietary toxicity study, van Berlo et al. (2018) administered 2'-FL manufactured by fermentation in the diet at levels of 0, 3, 6, and 10% to Wistar Han IGS rats [Crl:WI(Han); 10/sex/group] for 13 weeks. Throughout the study periods, the feeds were analyzed for stability, homogeneity, and concentration of 2'-FL. With increasing age of the rats, the feed was to be decreased. Hence, the intake of 2'-FL on body weight basis decreased in all groups during the study. For the three groups, the mean 2'-FL feed intake on body weights corresponded to 2.17, 4.27, and 7.25 g/kg bw/day for males, and 2.45, 5.22, and 7.76 g/kg bw/day for females. The dietary exposure to 2'-FL for 13 weeks did not produce any exposure-related changes in mortality or clinical signs in any of the treated groups. Similarly, the functional observational battery and motor activity assessment did not indicate any neurotoxic potential for 2'-FL. No changes in feed consumption in male rats was reported. However, in female rats feed consumption in the high-dose females was significantly decreased.

In hematology analysis there were no significant changes noted, except for a significant increase in thrombocytes in the high-dose females. However, the investigators did not consider the finding related to thrombocytes to be treatment related, as the difference from controls was only slight and occurred in one sex only. No treatment related changes in clinical chemistry parameters were noted. The results of urinalysis showed a significantly decreased specific gravity in females in the high dose group. The investigators considered the change to a higher urinary excretion volume and the change was not considered toxicologically significant. As regards the organ weight, relative liver weight was significantly increased in the high dose males. Additionally, the absolute and relative weights of the filled and empty cecum were significantly increased in the mid- and high-dose group in male and female rats. Furthermore, the absolute weight of the filled cecum was significantly increased. No significant macroscopic or microscopic findings related to treatment were noted in any of the treatment groups. The investigators concluded that ingestion of 2'-FL for 13 weeks produced no treatment-related changes in male and female rats and reported a NOAEL as 7.25 g/kg bw/day in male rats and 7.76 g/kg bw/day in female rats, the highest dose tested (van Berlo et al., 2018).

Based on findings from a range finding short-term (14-day) study, in which Wistar IGS:Crl:WI (Han) rats were administered 2'-FL via gavage at doses up to 7500 mg/kg bw/day (Table 10), Coulet et al. (2014) conducted a subchronic oral toxicity study of 2'-FL with a 4-week recovery period. In this study, 2'-FL was administered via gavage at dose levels of 0, 2000, 5000, and 6000 mg/kg bw/day to juvenile rats. Fructooligosaccharides (FOS) at a daily dose of 6000 mg/kg bw served as the high dose positive control. No treatment-related adverse effected were noted, except for the death of one male and one female rat in the 6000 mg/kg bw/day dose group, and two males and one female in the 6000 mg/kg bw/day FOS dose group during the treatment period. One female in the 6000 mg/kg bw/day FOS group died during the recovery period. Oral administration of 2'-FL up to 5,000 mg/kg bw/day to rats over 90 days was not associated with any adverse effects based on clinical observations, body weight gain, food

consumption, ophthalmoscopy, clinical pathology, organ weights, and histopathology findings, although transient lower body weight gains and colored/liquid feces were observed during the first few days of the administration period. The investigators concluded that 2'-FL was well tolerated at doses of up to 5000 mg/kg bw/day with the exception of transient lower body weight gain and colored feces. However, because there were three unexplained deaths at the 6000 mg/kg bw/day dose, the authors reported a NOAEL as 5000 mg/kg bw/day (Coulet et al., 2014).

As part of a novel food submission, EFSA reviewed this (above described) evidence from the Coulet et al. (2014) study and determined an estimated NOAEL to be 2000 mg/kg bw/day on the basis of decreased kidney weights and clinical chemistry and hematological effects (EFSA, 2015). A review of the results of this study (described in the publication; also summarized in GRN 650, page 30) indicate that the hematological effects were limited to slight reductions in red blood cell count (<5%) that were not consistent between sexes and were not associated with histological or gross pathological findings. Clinical chemistry parameter changes were limited to dose responsive reductions in AST levels in both sexes. AST levels were similarly decreased by a comparable magnitude in both males and females of the FOS group (i.e., positive control). In the absence of further clinical chemistry, hematological or histopathological correlates, the reduction in AST levels were not considered adverse. Hence, the NOAEL of 5000 mg/kg bw/day is considered as appropriate.

In an unpublished subchronic toxicity study, summarized in GRN 650 (pages 31-33). Penard (2015) investigated the toxicity of a 2'- FL manufactured by fermentation. In this adapted subchronic toxicity study, groups of 7-day-old neonatal Wistar [Crl:WI(Han)] rats received 0, 2000, 4000, or 5000 mg 2'-FL/kg bw/day via gavage for 90- days. A reference group was also included that received FOS at a dose of 5000 mg/kg bw/day for 90 days. Separate recovery groups (5/sex/group) of rats were administered the control, 2'-FL, or FOS for 90 days and were terminated after a 28-day recovery period. In this study, no test article-related mortalities occurred during the study. In a majority of the animals receiving FOS (reference group), liquid feces were noted. The mid- and high-dose group animals receiving 2'-FL also revealed liquid feces, as well as soiled urogenital regions. Additionally, hypersalivation, abnormal foraging and/or pedaling were noted in animals receiving the reference compound and also in the mid- and high-dose groups receiving 2'-FL from day 35 onward, with the incidence of these clinical signs being most prominent in the high-dose 2'-FL group. Ophthalmological findings did not reveal any test related effects. No remarkable or toxicologically relevant effects on body weight, body weight gain, food consumption, or on developmental, learning, motor, or behavioral markers were observed at any dose level. Some minor differences in certain hematological parameters were deemed to be of no toxicological significance and changes in serum chemistry parameters were small in magnitude and/or within the normal historical control data range and were considered to be non-adverse. Based on these findings the author established a NOAEL of 5000 mg/kg bw/day, the highest dose tested (Penard, 2015).

In addition to the above described studies with 2'-FL alone, in the published literature some studies investigating the mixtures of HMOs, including 2'-FL, have appeared. Phipps et al. (2018; also summarized in GRN 815 and GRN 1014) conducted an OECD 408-compliant 90-day repeated dose oral toxicity study with 2'-FL/DFL, manufactured using fermentation, in male and female Sprague-Dawley rats. In this study, an 8:1 ratio mixture of 2'-FL and DFL was administered via oral gavage to neonatal rats daily at 0, 1000, 3000, and 5000 mg/kg bw/day for 90 days followed by a 28-day recovery period. The findings from this study did not reveal any

mortality or exposure-related clinical signs. No significant differences in mean body weight and feed consumption were noted between treatment groups and vehicle. The investigators reported that no treatment-related adverse effects with a dose-response relationship were observed for development and maturation, behavioral endpoints, clinical pathology, organ weights, or histopathology. The NOAEL for the 2'-FL/DFL mixture was determined as 5000 mg/kg bw/day, the highest dose tested.

In another published study on mixture of HMO mixture, Parschat et al. (2020; also described extensively in GRN 1014- pages 44-49) fed either a control diet or the same diet containing 10% of an HMO mixture to rats (10/sex/group) for 90 days. The composition of the HMO mixture contained 47.1% dry weight 2'- FL, and other constituents are as mentioned earlier, all of which were manufactured by fermentation. The overall dietary exposure to 2'-FL was 4.7% of the diet. Based on feed consumption data, the mean intake of the HMO mixture ranged from 5.01 to 6.88 g/kg bw/day for male rats and 6.26 to 7.91 g/kg bw/day for female rats. This resulted in a mean intake of 2'-FL of 2.36 to 3.24 g/kg bw/day in males and 2.95 to 3.73 g/kg bw/day in females. Overall, no signs of toxicity were observed following the administration of an HMO mixture (containing 47.1% 2'-FL by dry weight) at 10% of the diet for 13 weeks. Based on feed intake data, the NOAEL for this study for HMO mixture was 5.67 g/kg bw/day for male rats and 6.97 g/kg bw/day for female rats.

Table 10. Summary of Short-term and Subchronic Toxicity Studies of 2'-FL

Reference	Species	Test article	Dose (mg/kg bw/day) Duration	Outcome Parameters	Conclusions on Safety	GI Effects and Tolerance
Range Findings or Short-term Toxicity Studies						
Jennewein Biotechnologie (2013) [unpublished] - In: Jennewein Biotechnologie GmbH (2015), Part 1	Rat, 26 to 28 days old, Crl:CD (SD) 5 F/group	Test Article 2'-FL produced using *Escherichia coli* BL21 [Jennewein Biotechnologie GmbH] Control Standard diet	0 or 10,000 (diet)[a] 7 days	BW, food intake, stool consistency, mortality, clinical signs	No deaths and no changes in behavior, appearance, food consumption, or bw	Stools were of normal consistency
Coulet et al. (2014)	Rat, 7 days old, Wistar Crl:Wl (Han) 5/sex/group	Test Article 2'-FL produced synthetically [Glycom A/S] Vehicle control Water Reference control FOS: 7500 mg/kg bw/day	0, 2000, 5000, or 7500 (gavage) 14 days	Mortality, clinical signs, bw, macroscopy	Well tolerated at the low-dose; limited effects at the mid-dose (transient lower bw gain and liquid feces); effects more marked at the high-dose (though similar effects were observed in the FOS group) and mortality in 2 of 5 females (cause of death not determined).	Liquid stools with occasional erythema in the urogenital area observed in rats from the mid- and high-dose groups and the FOS group - Days 1 to 3 and up to Days 9 to 11
Glycosyn, LLC and Friesland Campina Domo B.V. (GRN 723)	Rat, 6 to 7 weeks old, Wistar outbred (Crl:Wl(Han)) 4 M/group	Test article 2'-FL produced using *Escherichia coli* K-12 [Friesland Campina] Control Cereal-based (closed formula) diet	0, 2560, 5080, or 7990 (diet) 14 days	Clinical signs, bw, food intake, macroscopy, organ weights	No treatment-related effects with regard to clinical signs, bw, food intake, and macroscopic examination; decreased relative liver weights in the mid- and high-dose groups (not considered toxicologically significant); increased absolute and relative cecal weights in the mid- and high-dose groups (likely from physiological adaptation to the test material)	NR

Flaxmer, 2017 [unpublished]	Rat, neonatal Crl:CD*(SD) 8/sex/group	Test Article 82.5% (w/w) 2'-FL and 9.7% (w/w) DFL mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control Water	0, 4000, or 5000 (gavage) 14 days	Mortality, clinical signs, bw, gross macroscopic necropsy	One death reported on Day 15 in a male from the high dose 2'-FL/DFL group, determined to be non-treatment related by the study authors; no biologically relevant differences in body weight between groups; no macroscopic abnormalities reported	Red and/or yellow staining around the anus in some animals treated with 2'-FL/DFL, but these were transient (absent at the end of the observation period) and considered to be non-adverse
Subchronic Toxicity Studies						
Jennewein Biotechnologie (2014a) [unpublished] - In: Jennewein Biotechnologie GmbH (2015), Part 2	Rat, 26 to 28 days old Crl:CD*(5D) 10/sex/group	Test Article 2'-FL produced using *Escherichia coli* BL21 [Jennewein Biotechnologie GmbH] Control Standard diet	Males 0 or 7660 (diet) Females 0 or 8720 (diet) 90 days	Bw, bw gain, food intake, stool consistency, mortality, clinical signs, behavior, hematology, clinical biochemistry, urinalysis, ophthalmological examination, organ weights, histopathology	No substance-related adverse effects; the study authors reported NOAELs of 7660 mg/kg bw/day for females and 8720 mg/kg bw/day for males	Pale stools in 7 of 10 M and 4 of 10 F in the 2'-FL group (Days 9 to 69) - study authors concluded that this was due to undigested 2'-FL excreted in the feces
Coulet et al. (2014)	Rat, juvenile Wistar Crl:Wl(Han) 10/sex/group	Test article 2'-FL produced synthetically [Glycom A/S] Vehicle control Water Reference control FOS: 6000 mg/kg bw/day	0, 2000, 5000 or 6000 (gavage) 90 days, followed by a 28-day recovery period (5/sex/group)[b]	Mortality, clinical signs, ophthalmology, bw, food intake, hematology, coagulation, clinical chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5000 mg/kg bw/day as any changes observed were determined to be of no biological or toxicological significance', whereas EFSA reported a NOAEL of 2000 mg/kg bw/day based on the decrease in the relative kidney weight in the high-dose F group and significant changes in hematological parameters,	Diarrhea observed in a few rats from the low-dose group, and all rats from the mid- and high-dose groups and the FOS group (Day 1 until Days 12 to 13) - associated with erythema in the urogenital area of rats from the high-

					unexplained deaths in 2 rats (lM, lF) from the high-dose group, and changes in clinical blood parameters in the mid- and high-dose groups	dose group and the FOS group
Penard (2015) [unpublished); taken from GRN 650 (Glycom A/S, 2016)	Rat, 7 days old Crl:Wl(Han) 10/sex/group	Test Article 2'-FL produced using *Escherichia coli* K-12 [Glycom A/S] Control NR Reference control FOS: 5000 mg/kg bw/day	0, 2000, 4000, or 5000 (gavage) 90 days, followed by a 28-day recovery period (5/sex/group)[b]	Mortality, bw, clinical signs, ophthalmology, hematology, coagulation, clinical chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5000 mg/kg bw/day	Liquid stools observed in rats from the mid- and high-dose groups (also observed in all rats from the FOS group)
Phipps et al. (2018)	Rat, 7 days old Crl:CD*(SD) 10/sex/group	Test Article 82.5% (w/w) 2'-FL and 9.7% (w/w) DFL mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control Water Reference control FOS: 5000 mg/kg bw/day	0, 1000, 3000, or 5000 (gavage) 90 days, followed by a 4-week recovery period (5/sex/group)[b]	Mortality, clinical signs, ophthalmoscopy, bw, food consumption, sexual maturation, pre-weaning development, ulna growth, neurobehavior, hematology, coagulation, blood chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5000 mg/kg bw/day for the mixture	NR
Phipps et al. (2021)	Rat, 7 days old SD 10/sex/group	Test Article 31.5% (w/w) 2'-FL and 59.4% (w/w) LNFP-1 mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control	0, 1000, 3000, or 5000 (gavage) 90 days, followed by a 4-week recovery period (5/sex/group)[b]	Mortality, clinical signs, bw, food consumption, sexual maturation, neurobehavior, estrous cycle, hematology, blood chemistry,	The study authors reported a NOAEL of 5000 mg/kg bw/day for the mixture	NR

		Water Reference control FOS: 5000 mg/kg bw/day		urinalysis, organ weight, histopathology		
van Berlo et al. (2018)	Rat, Crl:Wl(Han) 10/sex/group	Test article 2'-FL produced using *Escherichia coli* K-12 [Friesland Campina] Control Cereal based rodent diet	0, 3, 6, or 10% (diet) for 90 days Males 0, 2170, 4270, or 7250 mg/kg bw/day Females 0, 2450, 5220, or 7760 mg/kg bw/day	Animal condition, mortality, behavior, motor activity, ophthalmoscopic observations, bw food and water consumption, hematology, clinical chemistry, urinalysis, organ weights, and histopathology	2'-FL did not induce adverse effects in any test group, the study authors reported a NOAEL of ≥7250 mg/kg bw/day in M and ≥7760 mg/kg bw/day in F	NR

2'-FL = 2'-fucosyllactose; bw = body weight; DFL = difucosyllactose; EFSA = European Food Safety Authority; FOS = fructooligosaccharides; GI = gastrointestinal; GRN = GRAS Notice; LNFP-1 = lacto-N-fucopentaose I; NOAEL = no-observed-adverse-effect level; NR = not reported.

[a] Rats were administered 0 or 10% 2'-Fl in the diet. Unit conversion was calculated using U.S. FDA (1993), assuming young rats.

[b] Control, FOS, and high-dose 2'-FL groups only.

[c] The authors noted that small statistically significant effects were observed in some of the hematological and clinical chemistry parameters, but that individual values generally remained within the historical control ranges, were without dose-response relationships, were often limited to 1 sex, and generally also occurred in the FOS reference group. Furthermore, the authors noted that none of the statistically significant changes were supported by findings from clinical parameters or histopathological observations.

6.2.4.3. Toxicity Studies of 2'-FL in Piglets

In addition to above described subchronic toxicity studies in rats, supporting animal toxicity studies of 2'-FL ingredients have been conducted in piglet, a sensitive neonatal rodent model. In a published study, Hanlon and Thorsrud (2014; summarized in GRN 571- pages 31-32) evaluated the safety and tolerance of a 2'-FL, while in another published study Hanlon (2020; also summarized in GRN 1014- pages 50-51) investigated the safety and tolerance of a mixture of HMOs containing 2'-FL, 3'-FL, LNT, 3'-SL, and 6'-SL.

In the study by Hanlon and Thorsrud (2014), 2-day old Yorkshire piglets were administered a typical milk replacer or the replacer supplemented with 200 (low), 500 (mid) or 2000 (high) mg 2'-FL/L (via a feeding bowl) for 21 days. The corresponding doses were 29.37, 72.22, or 291.74 mg/kg bw/day in males and 29.30, 74.31, and 298.99 mg/kg bw/day in females, respectively. All piglets survived to scheduled necropsy on Day 22. There were no reported dose-related adverse clinical findings during the dosing period. Both male and female piglets showed good growth based on body weight gain and feed efficiency. No treatment-related adverse effects on the clinical pathology parameters evaluated, including hematology, clinical chemistry, coagulation and urinalysis were reported. Also no treatment-related adverse macroscopic and microscopic findings, including intestinal pH were reported. In one male and one female of the high dose group and one female in mid dose group, microscopic findings included mild to moderate inflammation within the keratinized portion of the squamous epithelium in the nonglandular part of the stomach. The one male in the high dose group also showed focal loss/thinning in the keratinized portion of the squamous epithelium, associated with inflammation but without ulceration. There were no macroscopic findings associated with the observation. All other microscopic findings were considered incidental and were within the range of typical observations in swine of this age and strain. The investigators concluded that the daily dietary administration of 2'-FL to neonatal piglets for three weeks following birth at levels up to 2000 mg 2'-FL/L was well tolerated and did not produce any adverse treatment-related effects on growth and development.

Table 11. Summary Conclusion of 2'-FL Tolerance Studies in Neonatal Piglet

Substance studied	Study details	Safety conclusion	Reference
2'-FL	21-day in neonatal piglet tolerance study	NOAEL: males = 0.292 g/kg/day; females = 0.299 g/kg/day	Hanlon and Thorsrud (2014)
2'FL, 3'FL, LNT, 3'-FL, and 6'-SL	21-day in neonatal piglet tolerance study	NOAEL: males = 1.6 g/kg/day; females = 1.7 g/kg/day	Hanlon (2020)

In another study, Hanlon (2020) administered a mixture of HMOs containing 2'-FL, 3'-FL, LNT, 3'-SL, and 6'-SL to three groups of two-day-old Yorkshire crossbred piglets (n=12/group) for 21 days. The treatment groups received either a control diet, a diet containing 5.75 or 8.0 g/L HMO MIX 1. The control diet was Specialty Milk Replacer. The HMO mix contained 49.1% 2'-FL, 10.4% 3-FL, 19.9% LNT, 3.5% 3'-SL, and 4.2 % 6'-SL on a dry weight basis. One male piglet in the 8.0 g/L dosing group was euthanized on day 7 for humane reasons. In this animal, presence of *E. coli* was noted in the feces. Veterinary observations of yellow, discolored feces occurred in all three treatment groups, including the control group, and

antibiotics were administered to these animals presenting with these clinical signs. The distribution of these clinical signs supports the conclusion that the unscheduled death of the male piglet in the 8.0 g/L dosing group was not related to HMO MIX 1, but rather due to an underlying infection that was distributed evenly between the animals in all dosing groups. All of the remaining animals survived until the scheduled study termination on day 22. The clinical pathology values, and macroscopic and microscopic findings in the remaining animals did not reveal a relationship to the HMO Mix 1 treatment. The findings from this study indicate that daily dietary administration of HMO Mix 1 to neonatal piglets for 3 weeks at concentrations up to 8.0 g/L with calculated intakes of 3.6 and 3.7 g/kg bw (1.8 and 1.8 g 2'-FL/kg bw) in males and females, respectively, was well-tolerated, and did not produce adverse effects on growth and development.

6.2.5. Allergenicity

2'-FL, the subject of this GRAS assessment is unlikely to cause adverse allergic reactions as the content of residual protein in the Synaura's 2'-FL is ≤ 100 mg/kg (as mentioned in the specifications- Table 3). Secondly. none of the introduced gene products were found to have homology in amino acid sequences with those of allergenic proteins when the allergenic potential was screened using the databases: NCBI Entrez (http://www.ncbi.nlm.nih.gov/); Uniprot (https://www.uniprot.org/). Thirdly, no production microorganisms and residual DNA were present in Synaura's 2'-FL.

6.2.6. Safety of Production Strain

The safety of the host organism, *Escherichia coli* BL21 (DE3), is summarized in several GRAS notices, such as GRN 1015 (3'-sialyllactose sodium salt), GRN 925 (3-fucosyllactose), GRN 919 (lacto-N-neotetrose), GRN 815 (2'-FL), GRN 571 (2'-FL), all of which received "no questions" letters from the FDA (complete GRAS notice along with FDA response available at FDA GRAS Notices Inventory). Both GRN 815 and GRN 571 described the use of *E. coli* BL21 (DE3) as the host organism in the production of 2'-FL. This information suggest that *E. coli* is a suitable and safe strain in the production of food ingredients.

The available information shows that *E. coli* are commensal residents of the gut microflora of humans and several other animal species. Based on the sequence similarity of housekeeping genes, different strains of *E. coli* are taxonomically grouped into five different phylogroups, such as A, B1, B2, D, and E (Archer et al., 2011). Commensal strains present in human are typically found in Group A or B1, with non-related pathogenic strains classified under Group B2, D, and E. According to their relative pathogenicity for healthy adult humans, 3 group A laboratory strains, as well as strains K-12, B, C, and their derivatives are designated as Risk Group 1 organisms (Archer et al., 2011; Daegelen et al., 2009). As per recent National Institutes for Health (NIH) guidelines for research involving recombinant or synthetic nucleic acid molecules, Risk Group 1 organisms "are not associated with disease in healthy adult humans" (NIH, 2023). Among these strains, *E. coli* K-12 and the B derivatives (e.g., BL21) are commonly used for production of industrial, pharmaceutical, and food biotechnology preparations.

In several comprehensive studies, the safety of *E. coli* BL21 (DE3) has been demonstrated. The *E. coli* BL21 (DE3) does not carry the well-recognized pathogenic components required by *E. coli* strains that cause the majority of enteric infections. Hence, *E. coli* BL21 (DE3) is considered to be nonpathogenic (non-virulent) and unlikely to survive in host tissues or to cause disease (Chart et al., 2000). This strain has been the first organisms for which

whole genome sequence assembled. Only marginally it differs from another widely used production strain, i.e., *E. coli* K-12 (Studier et al., 2009). The whole genome sequencing revealed the absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence (Jeong et al., 2009). The findings from an acute oral toxicity study revealed that the *E. coli* BL21 (DE3) endotoxin produced no toxicity in mice, even at the highest dose of 1,000,000 EU (3.3 mg/kg bw) (Harper et al., 2011).

It should be noted that *E. coli* EB011065 was engineered with genes with known functions, which do not confer toxicogenicity, virulence, or DNA, using site-specific homologous recombination; it is commonly accepted that *E. coli* EB011065 is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile (non-pathogenic) as *E. coli* BL21 (DE3).

Based on the comprehensive characterization of this strain and its widespread use in protein production, the use of *E. coli* BL21 (DE3) as the host strain and *E. coli* EB011065 as the production strain are not expected to result in any safety concerns.

6.2.7. Regulatory Agency Review of 2'-FL

The available information from the FDA GRAS Inventory (Table 6) suggest that 2'-FL, chemically synthesized or derived from microbial fermentation, is GRAS for use in non-exempt infant formulas at levels up to 2.4 g/L, exempt infants formulas at levels up to 2.0 g/L, selected conventional foods and beverages and enteral tube feeding formulas at levels ranging from 0.28 to 1.2 g/serving (GRN 546; GRN 571; GRN 650; GRN 735; GRN 749; GRN 852; GRN 897; GRN 929; GRN 932; GRN 1014; GRN 1034). In a majority of these GRAS notices, 2'-FL was produced by microbial fermentation, except for the first GRAS notice (GRN 546), in which 2'-FL was chemically synthetized. As of now 16 GRAS Notifications have been filed with FDA, twelve of which have received "no questions" letters. FDA's review or evaluation of two of the 2'-FL GRAS Notifications (GRN 859; GRN 987) were ceased, one due to deficiencies and other was due to questions regarding support for an intended use level of 3.64 g/L. Among the 12 GRAS notices that received no question letter, one was on mixture of 2'-FL and difucosyllactose for use in infant formula, toddler formula, drinks for young children and selected conventional foods and beverages. Given the structural, chemical and compositional (Table 4) similarity of the subject of this present GRAS, 2'-FL, with those described in FDA GRAS notices that received a no question letter, the safety data summarized in these GRAS notices is applicable to this present GRAS determination and supports the safe use of 2'-FL at the proposed use levels.

In the European Union, 2'-FL is a Novel Food and is approved for use in infant formula and selected foods, alone or in combination with lacto-N-neotetraose (LNnT), at levels up to 1.2 g/L and 200 g/kg, respectively. 2'-FL is also a Novel Food in Canada. In several other countries, such as Malaysia, Taiwan, Singapore, Israel, and the Philippines, 2'-FL is authorized for uses in food. Additionally, in Australia and New Zealand, 2'-FL and LNnT are currently the subjects of a Novel Food application and the Food Standards of Australia and New Zealand has concluded that there are no public health and safety concerns associated with the addition of 2'-FL, alone or in combination with LNnT, to infant formula products and follow-on formula and formulated supplementary foods for young children (FSFYC) at the requested levels, or at higher estimated levels of dietary intakes based on 2.4 g/L 2'-FL (Food Standards Australia New Zealand, 2018).

6.3. Summary, Discussion and Conclusion

At the request of Synaura Biotechnology (Shanghai) Co., Ltd., China (Synaura), this assessment was undertaken by Soni & Associates Inc., to evaluate the Generally Recognized As Safe (GRAS) status of 2'-fucosyllactose (2'-FL), produced by fermentation with recombinant *Escherichia coli* EB011065 strain, for use as a food ingredient in milk and soy-based, non-exempt infant formula for term infants at a maximum level of 2.4 g/L of formula as consumed; in toddler formulas (intended for children >12 months of age) and meal replacement drinks for children ages 1-3 years at a maximum level of 2.4 g/L, as consumed; in infant and toddler foods at maximum levels of 10.0 g/L in drinks, 10.9 g/kg in cereals and desserts, 57 g/kg in dry snacks; in beverages (sports and "energy" drinks, flavored waters, fruit juices and drinks, milk drinks, dairy analogs, milk-based meal replacements) at maximum levels ranging from 0.8-6 g/L; and in the following foods, at maximum levels ranging from 4.8-80 g/kg: breakfast cereals; frozen dairy desserts; puddings, fillings, mousses; yogurt; meal replacement and snack bars; syrups; and jams and jellies. A comprehensive search of the scientific literature for safety and toxicity information on 2'-FL and related compounds, such as other HMOs, was conducted through April 2023 and was used in the preparation of this dossier. The available information was critically evaluated and summarized in this dossier.

The subject of this GRAS assessment is a spray-dried, white to off-white powdered food ingredient that contains more than 94% 2'-FL dry weight. The remaining components of the final products include carbohydrate by-products, ash, and moisture. 2'-FL is a neutral, fucosylated oligosaccharide found in human milk. Synaura's 2'-FL is produced by current Good Manufacturing Processes (cGMP) by fermentation with recombinant *E. coli* BL21 (DE3) (EB011065) strain. Appropriate food grade specifications for 2'-FL have been established. The final product contains >94% 2'-FL and other byproducts such as L-fucose, difucosyllactose, 2'-fucosyl-D-lactulose. In several studies, the safety of byproducts (as constituent of 2'-FL) has been investigated along with 2'-FL and these byproducts are considered as safe. Analytical data from three nonconsecutive lots of 2'-FL demonstrates that the manufacturing process produces a consistent product that meets the defined specifications that include parameters on its identity/composition, and established limits for heavy metal and microbiological contaminants. Specification limits for 2'-FL purity and related carbohydrate byproduct are similar to those established for other 2'-FL ingredients that have been concluded to be GRAS, demonstrating that Synaura's 2'-FL is compositionally the same as other 2'-FL ingredients that received no question letters from FDA. 2'-FL has been demonstrated by HPAEC-PAD to be structurally and chemically identical to 2'-FL that is naturally present in human breast milk. Additionally, identity of 2'-FL has been confirmed with other techniques such as LC-MS/MS, IR and NMR spectra.

All raw materials, processing aids, and food contact substances employed in the manufacturing of 2'-FL are GRAS and/or conform to the specifications stated in 21 CFR and/or the FCC. The genetically engineered strain of *E. coli* BL21 (DE3) used by Synaura in the production of 2'-FL, is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile as *E. coli* BL21 (DE3). Process controls and product specifications are in place to control the levels of residual impurities and carbohydrate by-products, as well as heavy metals, microbes, and production organism-derived DNA and endotoxin, ensuring a consistent, food-grade finished ingredient.

The proposed uses of 2'-FL in different population groups at the above specified levels will result in estimated dietary exposures (eaters-only) at mean and 90th percentile to be 2.06 and 3.17 g/person/day (0.33 and 0.53 g/kg bw/day), respectively, for infants 0-5 months of age, and 2.60 and 4.95 g/person/day (0.29 and 0.53 g/kg bw/day), respectively, for infants 6-11 months of age. For children 1-3 years of age, the estimated dietary exposures to 2'-FL at the mean and 90th percentile will be 1.45 and 2.23 g/person/day (0.12 and 0.19 g/kg bw/day), respectively. For the total population (all ages), estimates of dietary exposure at the mean and 90th percentile will be 1.77 and 3.59 g/person/day (0.034 and 0.074 g/kg bw/day), respectively. Given the similarity of cumulative dietary exposure estimates (proposed and background uses) to the dietary exposure estimates from the intended uses only, it is concluded that inclusion of the potential uses outside the scope of this GRAS does not impact the overall dietary exposure to 2'-FL. The intended use of 2'-FL is substitutional to other sources of 2'-FL currently on the US market.

Given the structural and chemical (identity) similarity of 2'-FL, the subject of this GRAS assessment, to that present in human milk, the natural background dietary exposure to 2'-FL from the consumption of human milk is the primary consideration in the assessment of the safety of Synaura's 2'-FL. The amount of 2'-FL in human milk ranges from 0 to 9.5 g/L. Human milk oligosaccharides (HMOS), including 2'-FL, are resistant to the digestive enzymes in the gastrointestinal tract, poorly absorbed, and pass through the gastrointestinal tract where they are either fermented by the microbiota or excreted unchanged. The available safety related information on 2'-FL is extensively summarized in the 12 GRAS notices that received "no question" letters from FDA. The safety data and information discussed in the previous 2'-FL GRNs, includes three published 90-day toxicological studies in young rats and supports safety of 2'-FL at proposed uses. In two studies, 2'-FL administrated by gavage did not produce any treatment-related adverse effects at doses up to 5000 mg/kg bw/day. In the third study, 2'-FL did not cause any toxicologically relevant effects at concentrations up to 10% in the diet, equivalent to an oral dose of higher than 7250 mg/kg bw/day. Published tolerance studies in neonatal piglets suggest that the ingestion of 2'-FL at levels up to 3.92 g/L alone or in the presence of other HMOs was well-tolerated and supported normal growth in neonatal piglets. The findings from available multiple genotoxicity studies shows that 2'-FL is not mutagenic or genotoxic.

In a series of specifically designed unpublished studies conducted with Synaura's 2'-FL, no adverse effects of 2'-FL were noted. In the acute toxicity study, the LD_{50} of 2'-FL was greater than 10 g/kg bw, indicating that the test article (2'-FL) was actually non-toxic. The findings from three separate mutagenicity and genotoxicity studies suggest that as evaluated by bacterial reverse mutation test, mammalian erythrocyte micronucleus test, and chromosome aberration test in mouse spermatogonia, Synaura's 2'-FL is unlikely to cause mutagenic or genotoxic effects. Based on the findings from a subchronic toxicity study in rats, the NOAEL for Synura's 2'-FL was determined as 8 g/kg bw/day, the highest dose tested. In a teratogenicity study in SD rats, the NOAEL of 2'-FL for teratogenic effects was established as 8 g/kg bw/day, the highest dose tested.

In a number of clinical studies conducted in full-term infants, 0 to 6 months of age, the safety and tolerance of the supplementation of 2'-FL in infant formula alone, or in combination with lacto-N-neotetraose (LNnT) or non-milk oligosaccharides [galactooligosaccharides (GOS) or short-chain fructooligosaccharides (scFOS)] has been previously evaluated. The findings from these studies consistently suggest that 2'-FL supplementation at levels ranging from 0.2 to 1.0 g/L is safe and well-tolerated in infants. In addition to these previously evaluated studies, in

recent published studies healthy, term infants or toddlers were fed formula containing 0.25 to 3 g 2'-FL/L for 6 weeks to 6 months did not reveal any significant differences in the incidences of adverse events, except for some potentially test formula-related minor adverse events (i.e., cow's milk intolerance and irritability). In these studies, 2'-FL was well tolerated, with no safety concerns noted. The available clinical studies in adults show that the ingestion of up to 20 g/day of either 2'-FL, LNnT, or a combination of 2'-FL and LNnT in healthy adults and adults with IBS, ulcerative colitis, Crohn's disease, or celiac disease was well tolerated. As expected, the most common complaints were flatulence, abdominal distress, and abdominal pain. The publicly available evidence indicate that 2'-FL at levels up to 20 g in adults is unlikely to cause adverse effects and is considered to be safe.

All pivotal data and information described in this dossier and used to establish the safety of Synaura's 2'-FL under its intended conditions of use are "generally available" (i.e., in the public domain). From the data and information presented herein, Synaura concludes that 2'-FL produced with a genetically engineered strain of *E. coli* BL21 (DE3) is GRAS for its intended uses in infants, toddlers and children, and the general population, based on scientific procedures.

In conclusion, the totality of the available evidence from *in vitro,* animal and human studies, and the exposure to 2'-FL from human milk, support the safety-in-use of 2'-FL, for its intended use as an ingredient in milk and soy-based, non-exempt infant formula for term infants at a maximum level of 2.4 g/L of formula as consumed; in toddler formulas (intended for children >12 months of age) and meal replacement drinks for children ages 1-3 years at a maximum level of 2.4 g/L, as consumed; in infant and toddler foods at maximum levels of 10.0 g/L in drinks, 10.9 g/kg in cereals and desserts, 57 g/kg in dry snacks; in beverages (sports and "energy" drinks, flavored waters, fruit juices and drinks, milk drinks, dairy analogs, milk-based meal replacements) at maximum levels ranging from 0.8-6 g/L; and, in the following foods, at maximum levels ranging from 4.8-80 g/kg: breakfast cereals; frozen dairy desserts; puddings, fillings, mousses; yogurt; meal replacement and snack bars; syrups; and jams and jellies. On the basis of scientific procedures[1], and exposure from human milk, the consumption of 2'-FL as an added food ingredient is considered safe at the proposed use levels and resulting intake for infants, toddlers, children and the general population. The intended uses are compatible with current regulations, *i.e.,* 2'-FL is used in selected food categories described in this document and is produced according to current good manufacturing practice (cGMP).

[1] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

7. PART VII- LIST OF SUPPORTING DATA AND INFORMATION

Alliet P, Vandenplas Y, Roggero P, Jespers SNJ, Peeters S, Stalens JP, Kortman GAM, Amico M, Berger B, Sprenger N, Cercamondi CI, Corsello G., 2022. Safety and efficacy of a probiotic-containing infant formula supplemented with 2'-fucosyllactose: a double-blind randomized controlled trial. Nutr J. 21(1):11. doi: 10.1186/s12937-022-00764-2.

Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D., 2013. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology 23:664-676.

Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY, Nielsen LK.. 2011. The genome sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of E. coli. BMC Genomics. 12:9. doi: 10.1186/1471-2164-12-9.

Brand-Miller JC, McVeagh P, McNeil Y, Messer M, 1998. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. J Pediatr 133(1):95-98.

CAIQTEST, 2022a. Chinese Academy of Inspection and Quarantine. Acute oral toxicity test of 2'-Fucosyllactose. Test acceptance No.: BJ00022000316001. Study completion date: April 12, 2022. Applicant: China Mengniu Dairy Company Limited. Study report provided by Synaura Biotechnology (Shanghai) Co., Ltd.

CAIQTEST, 2022b. Chinese Academy of Inspection and Quarantine. Bacterial reverse mutation test, mammalian erythrocyte micronucleus test, and chromosome aberration test in mouse spermatogonia of 2'-Fucosyllactose. Test acceptance No.: BJ00022000316001. Study completion date: October 13, 2022. Applicant: China Mengniu Dairy Company Limited. Study report provided by Synaura Biotechnology (Shanghai) Co., Ltd.

CAIQTEST, 2022c. Chinese Academy of Inspection and Quarantine. Teratogenicity Test of 2'-Fucosyllactose. Test acceptance No.: BJ00022000316001. Study completion date: October 13, 2022. Applicant: China Mengniu Dairy Company Limited. Study report provided by Synaura Biotechnology (Shanghai) Co., Ltd.

CAIQTEST, 2022d. Chinese Academy of Inspection and Quarantine. 90-day Oral Toxicity Test of 2'-Fucosyllactose. Test acceptance No.: BJ00022000316001. Study completion date: October 13, 2022. Applicant: China Mengniu Dairy Company Limited. Study report provided by Synaura Biotechnology (Shanghai) Co., Ltd.

Castanys-Munoz E, Martin MJ, and Prieto PA., 2013. 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71:773-789

Chart H, Smith HR, La Ragione RM, Woodward MJ., 2000. An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5alpha and EQ1. J Appl Microbiol. 89(6):1048-1058.

Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, and Newburg DS, 2001. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11:365-372.

Coulet M, Phothirath P, Allais L, Schilter B, 2014. Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'FL). Regul Toxicol Pharmacol. 68(1):59-69. doi: 10.1016/j.yrtph.2013.11.005.

Daegelen P, Studier FW, Lenski RE, Cure S, Kim JF., 2009. Tracing ancestors and relatives of *Escherichia coli* B, and the derivation of B strains REL606 and BL21(DE3). J Mol Biol. 394(4):634-643.

Dotz V, Rudloff S, Blank D, Loch nit G, Geyer R, Kunz C, 2014. ^{13}C-labeled oligosaccharides in breastfed infants' urine: individual-, structure-and time-dependent differences in the excretion. Glycobiology 24(2):185-194.

EFSA, 2015. Scientific opinion on the safety of 2'-0-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 258/97 (EFSA Panel on Dietetic Products, Nutrition and Allergies/NOA) (Question no: EFSA-Q-2015-00052, adopted: 29 June 2015 by European Food Safety Authority). EFSA J 13(7):4184 [32pp]. DOl:10.2903/j.efsa.2015.4184. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/4184.

EFSA, 2019. Scientific Opinion on the safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283 (EFSA Panel on Dietetic Products, Nutrition and Allergies/NOA) (Question no: EFSA-Q-2018-00374; adopted: 15 May 2019 by European Food Safety Authority). EFSA J 17(6):5717 [23pp]. DOl: 10.2903/j.efsa.2019.5555. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5717

Elison E, Vigsnaes LK, Rindom Krogsgaard L, Rasmussen J, Sørensen N, McConnell B, Hennet T, Sommer MO, Bytzer P., 2016. Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. Br J Nutr. 116(8):1356-1368. doi: 10.1017/S0007114516003354.

Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H, 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71(6): 1589-1596.

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, Ruiz-Palacios G, Hilty MD, Pickering LK, Prieto PA., 2000. Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 30:181–192

Flaxmer J., 2017. Report. 2'-O-Fucosyllactose and difucosyllactose (2'-FL/DFL): 14-day Dose Range Finding Study in the Neonatal Crl:CD(SD) Rat by Oral (Gavage) Administration. (Envigo Study No: RW47RS; 30 October 2017). Prepared by

Huntingdon, Cambridgeshire, UK: Envigo CRS Limited for Hørsholm, Denmark: Glycom A/S. Cited in GRN 815.

Fonvig CE, Amundsen ID, Vigsnæs LK, Sørensen N, Frithioff-Bøjsøe C, Christiansen M, Hedley PL, Holm LA, McConnell B, Holm JC., 2021. Human Milk Oligosaccharides Modulate Fecal Microbiota and Are Safe for Use in Children With Overweight: A Randomized Controlled Trial. J Pediatr Gastroenterol Nutr. 73(3):408-414.

Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila, C, Giuliani F, Bertino E, Fabris C, Coppa GV, 2011. Preterm milk oligosaccharides during the first month of lactation. Pediatrics 128:e1520-1531.

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, Santoro L, Gabrielli O, Volpi N, 2012. On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2- aminoacridone. Anal Biochem 430:97-104.

Gehring U, Spithoven J, Schmid S, Bitter S, Braun-Fahrländer C, Dalphin JC, Hyvärinen A, Pekkanen J, Riedler J, Weiland SK, Büchele G, von Mutius E, Vuitton DA, Brunekreef B, PASTURE study group. 2008, Endotoxin levels in cow's milk samples from farming and non-farming families - the PASTURE study. Environ Int. 34(8):1132-1136.

Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S, 2000. Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12):3014-3020.

Gnoth MJ, Rudloff S, Kunz C, Kinne RK, 2001. Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatographymass spectrometry technique. J Biol Chem 276(37):34363-34370.

Goehring KC, Kennedy AD, Prieto PA, Buck RH, 2014. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PloS ONE 9(7):e101692 [11 pp].

Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG and Buck RH., 2016. Similar to Those Who Are Breastfed, Infants Fed a Formula Containing 2'-Fucosyllactose Have Lower Inflammatory Cytokines in a Randomized Controlled Trial. J Nutr 146:2559-2566.

Gurung RB, Woo J, Cho SS, Hong JH and Kim DH, 2018. Gastrointestinal tolerance and safety of 3’- sialyllactose in subjects positive with Helicobacter pylori: a pilot study. EC Nut 13:600-608.

Hanahan D., 1983. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166(4):557-580.

Hanlon PR, Thorsrud BA., 2014. A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets. Food Chem Toxicol 74:343-348.

Harper MS, Carpenter C, Klocke DJ, Carlson G, Davis T, Delaney B, 2011. *E. coli* Lipopolysaccharide: acute oral toxicity study in mice. Food Chem Toxicol. 49(8):1770-1772.

Hegar B, Wibowo Y, Basrowi RW, Ranuh RG, Sudarmo SM, Munasir Z, Atthiyah AF, Widodo AD, Supriatmo, Kadim M, Suryawan A, Diana NR, Manoppo C, Vandenplas Y., 2019. The Role of Two Human Milk Oligosaccharides, 2'-Fucosyllactose and Lacto-N-Neotetraose, in Infant Nutrition. Pediatr Gastroenterol Hepatol Nutr. 22(4):330-340.

Hirschmugl B, Brandl W, Csapo B, van Poppel M, Köfeler H, Desoye G, Wadsack C, Jantscher-Krenn E., 2019. Evidence of Human Milk Oligosaccharides in Cord Blood and Maternal-to-Fetal Transport across the Placenta. Nutrients. 11(11):2640. doi: 10.3390/nu11112640.

Hascoet JM, Chevallier M, Gire C, Brat R, Rozé JC, Norbert K, Chen Y, Hartweg M, Billeaud C., 2022. Use of a Liquid Supplement Containing 2 Human Milk Oligosaccharides: The First Double-Blind, Randomized, Controlled Trial in Preterm Infants. Front Pediatr. 10:858380. doi: 10.3389/fped.2022.858380.

Iribarren C, Törnblom H, Aziz I, Magnusson MK, Sundin J, Vigsnaes LK, Amundsen ID, McConnell B, Seitzberg D, Öhman L, Simrén M., 2020. Human milk oligosaccharide supplementation in irritable bowel syndrome patients: A parallel, randomized, double-blind, placebo-controlled study. Neurogastroenterol Motil. 32(10):e13920. doi: 10.1111/nmo.13920.

Jantscher-Krenn E, von Schirnding L, Trötzmüller M, Köfeler H, Kurtovic U, Fluhr H, Müller A, Bagci S., 2022. Human Milk Oligosaccharides Are Present in Amniotic Fluid and Show Specific Patterns Dependent on Gestational Age. Nutrients 14(10):2065. doi: 10.3390/nu14102065.

Jantscher-Krenn E, Aigner J, Reiter B, Köfeler H, Csapo B, Desoye G, Bode L, van Poppel MNM., 2019. Evidence of human milk oligosaccharides in maternal circulation already during pregnancy: a pilot study. Am J Physiol Endocrinol Metab. 316(3):E347-E357.

Jennewein Biotechnologie, 2013 (unpublished). As described in GRN 571.

Jennewein Biotechnologie, 2014a (unpublished). As described in GRN 571.

Jennewein Biotechnologie, 2014b (unpublished) [unpublished]. As described in GRN 571.

Jennewein Biotechnologie, 2014c (unpublished). As described in GRN 571.

Jennewein Biotechnologie GmbH, 2015. (unpublished). As described in GRN 571.

Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH, Couloux A, Lee SW, Yoon SH, Cattolico L, Hur CG, Park HS, Ségurens B, Kim SC, Oh TK, Lenski RE, Studier FW, Daegelen P, Kim JF., 2009. Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3). J Mol Biol. 394(4):644-52. doi: 10.1016/j.jmb.2009.09.052.

Kajzer J, Oliver J, Marriage B, 2016. Gastrointestina l tolerance of formula supplemented with oligosaccharides. FASEB J 30(1, Suppl.) (abstract 671.4).

Kuntz S, Kunz C, Barsch C, Vazquez E, Buck R, Reutzel M, et al., 2019. Metabolic fate and distribution of 2'-fucosyllactose: direct influence on gut microbial activity but not on brain. Mal Nutr Food Res 63(13):e1900035 [8pp].

Leung TF, Ulfman LH, Chong MKC, Hon KL, Khouw IMSL, Chan, PKS, Delsing DJ, Kortman GAM, Bovee-Oudenhoven IMJ, 2020. A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers. Pediatric Allergy and Immunology, 31(7):745–754. https://doi.org/10.1111/pai.13276

Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA, 2015. Infants Fed a Lower Calorie Formula With 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. J Pediatr Gastroenterol Nutr 61:649-658.

McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, Prentice AM, Kvist LJ, Otoo GE, Brooker SL, Price WJ, Shafii B, Placek C, Lackey KA, Robertson B, Manzano S, Ruíz L, Rodríguez JM, Pareja RG, Bode L., 2017. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. Am J Clin Nutr. 105(5):1086-1100.

Musumeci M, Simpore J, D'Agata A, Sotgiu S, Musumeci S., 2006. Oligosaccharides in colostrum of Italian and Burkinabe women. J Pediatr Gastroenterol Nutr. 43(3):372-378.

NIH. 2023. National Institute of Health. Risk Groups. Available at: https://www.phe.gov/s3/BioriskManagement/biosafety/Pages/Risk-Groups.aspx

Nowak-Wegrzyn A, Czerkies L, Reyes K, Collins B, Heine RG, 2019. Confirmed Hypoallergenicity of a Novel Whey-Based Extensively Hydrolyzed Infant Formula Containing Two Human Milk Oligosaccharides. Nutrients. 11(7):1447. doi: 10.3390/nu11071447. PMID: 31248026; PMCID: PMC6682865.

Obermeier S, Rudloff S, Pohlentz G, Lentze MJ, Kunz C, 1999. Secretion of ^{13}C-labelled oligosaccharides into human milk and infant's urine after an oral ^{13}C-galactose load. Isotopes Environ Health Stud 35(1/2):119-125.

Palsson OS, Peery A, Seitzberg D, Amundsen ID, McConnell B, Simrén M., 2020. Human Milk Oligosaccharides Support Normal Bowel Function and Improve Symptoms of Irritable Bowel Syndrome: A Multicenter, Open-Label Trial. Clin Transl Gastroenterol. 11(12):e00276. doi: 10.14309/ctg.0000000000000276.

Parente F, Cucino C, Anderloni A, Grandinetti G, Bianchi Porro G., 2003. Treatment of Helicobacter pylori infection using a novel antiadhesion compound (3'sialyllactose sodium salt). A double blind, placebo-controlled clinical study. Helicobacter. 8: 252-256.

Parschat K, Melsaether C, Jäpelt KR, Jennewein S., 2021. Clinical Evaluation of 16-Week Supplementation with 5HMO-Mix in Healthy-Term Human Infants to Determine Tolerability, Safety, and Effect on Growth. Nutrients 13(8):2871. doi: 10.3390/nu13082871.

Parschat K, Oehme A, Leuschner J, Jennewein S, Parkot J., 2020. A safety evaluation of mixed human milk oligosaccharides in rats. Food Chem Toxicol 136:111-118.

Penard L., 2015. 2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-Free Period. (unpublished study described in GRN 650)

Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, Mikš MH, Röhrig CH, 2018. Safety evaluation of a mixture of the human-identical milk oligosaccharides 2'-fucosyllactose and difucosyllactose. Food Chem Toxicol 120:552–565.

Phipps KR, Lynch B, Stannard DR, Gilby B, Baldwin N, Miks MH, Lau A, Rohrig CH., 2021. Genotoxicity and neonatal subchronic toxicity assessment of a novel mixture of the human-identical milk oligosaccharides lacto-N-fucopentaose I and 2'-fucosyllactose. J Appl Toxicol. 41(4):632-649.

Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, et al., 2017. Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. J Pediatr Gastroenterol Nutr. 64:624–631.

Rasko DA, Wilson TJ, Zopf D, Taylor DE., 2000. Lewis antigen expression and stability in Helicobacter pylori isolated from serial gastric biopsies. J Infect Dis. 181(3):1089-95. doi: 10.1086/315354.

Roman E, Moreno Villares JM, Domínguez Ortega F, Carmona Martínez A, Picó Sirvent L, Santana Sandoval L, Casas Rivero J, Alshweki A, Cercamondi C, Dahbane S, Vidal Guevara ML, 2020. Real-world study in infants fed with an infant formula with two human milk oligosaccharides. Nutr Hosp. 37(4):698-706. English. doi: 10.20960/nh.03084. PMID: 32698596.

Rudloff S, Kunz C, 2012. Milk oligosaccharides and metabolism in infants. Adv Nutr 3, 398s-405s.

Rudloff S, Obermeier S, Barsch C, Pohlentz G, Hartmann R, Brosicke H, Lentze MJ, Kunz C, 2006. Incorporation of orally applied (13)C-galactose into milk lactose and oligosaccharides. Glycobiology 16:477-487.

Rudloff S, Pohlentz O, Diekmann L, Egge H, Kunz C, 1996. Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. Acta Paediatr 85(5):598-603.

Rudloff S, Pohlentz G, Barsch C, Lentze MJ, Kunz C, 2012. Urinary excretion of in vivo ^{13}C-labelled milk oligosaccharides in breastfed infants. Br J Nutr 107(7):957-963.

Ryan JJ, Monteagudo-Mera A, Contractor N, Gibson GR., 2021. Impact of 2'-Fucosyllactose on Gut Microbiota Composition in Adults with Chronic

Gastrointestinal Conditions: Batch Culture Fermentation Model and Pilot Clinical Trial Findings. Nutrients. 13(3):938. doi: 10.3390/nu13030938.

Steenhout P, Sperisen P, Martin F-P, Sprenger N, Wernimont S, Pecquet Sand Berger B., 2016. Term Infant Formula Supplemented with Human Milk Oligosaccharides (2'Fucosyllactose and Lacto-N-neotetraose) Shifts Stool Microbiota and Metabolic Signatures Closer to that of Breastfed Infants. The FASEB Journal 30:275-277 (abstract).

Storm HM, Shepard J, Czerkies LM, Kineman B, Cohen SS, Reichert H, Carvalho R., 2019. 2'-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula With *Bifidobacterium lactis*: A Randomized Controlled Trial. Glob Pediatr Health. 6:2333794X19833995. doi: 10.1177/2333794X19833995.

Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF., 2009. Understanding the differences between genome sequences of Escherichia coli B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. J Mol Biol. 394(4):653-80.

Thurl S, Munzert M, Boehm G, Matthews C, Stahl B., 2017. Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev. 75(11):920-933.

Townsend S, Caubilla Barron J, Loc-Carrillo C, Forsythe S., 2007. The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. Food Microbiol. 24(1):67-74. doi: 10.1016/j.fm.2006.03.009.

Urashima T, Asakuma S, Leo F, Fukuda K, Messer M, Oftedal OT., 2012. The predominance of type I oligosaccharides is a feature specific to human breast milk. Adv Nutr. 3(3):473S-82S.

van Berlo D, Wallinga AE, van Acker FA, Delsing DJ, 2018. Safety assessment of biotechnologically produced 2'-Fucosyllactose, a novel food additive. Food Chem Toxicol 118:84-93.

Vandenplas Y, de Halleux V, Arciszewska M, Lach P, Pokhylko V, Klymenko V, et al., 2020. A partly fermented infant formula with postbiotics including 3'-GL, specific oligosaccharides, 2'-FL, and milk fat supports adequate growth, is safe and well-tolerated in healthy term infants: a double-blind, randomised, controlled, multi-country trial (On Behalf of the Voyage Study Group). Nutrients 12(11):3560 [17pp, plus supplementary data]. DOI:10.3390/nu12113560.

Verbaan IAJ, 2015a. An *In Vitro* Micronucleus Assay with 2'-O- Fucosyllactose In Cultured Peripheral Human Lymphocytes (Laboratory Project Identification: Project 507398; Substance 206096/A). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. Unpublished. Summarized in GRN 650, page 36.

Verbaan IAJ, 2015b. An *In Vitro* Micronucleus Assay with 2'FL in Cultured Peripheral Human Lymphocytes (Laboratory Project Identification: Project 507433; Substance

206374/B). Prepared by DD ‘s-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. Unpublished. Summarized in GRN 650, pages 36 and 37.

Vazquez E, Santos-Fandila A, Buck R, Rueda R, Ramirez M, 2017. Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. Br J Nutr 117(2):237-247.

Verspeek-Rip CM, 2015. Evaluation of the Mutagenic Activity of 2’FL in the *Salmonella Typhimurium* Reverse Mutation Assay and the *Escherichia Coli* Reverse Mutation Assay. Unpublished. Summarized in GRN 650, page 36.

Wallingford JC, Neve Myers P, Barber CM., 2022. Effects of addition of 2-fucosyllactose to infant formula on growth and specific pathways of utilization by Bifidobacterium in healthy term infants. Front Nutr. 9:961526. doi: 10.3389/fnut.2022.961526.

8. Appendix A - Test Methods

A.1 Content of 2'-Fucosyllactose (as dried basis)

A.1.1 Method summary

2'-Fucosyllactose was quantified by area normalization method using a differential refractive index detector.

A.1.2 Instruments and equipment

High performance liquid chromatograph (differential refractive index detector), and electronic analytical balance

A.1.3 Reagents and test solutions

Acetonitrile (HPLC), purified water, 2'-Fucosyllactose reference standard

A.1.4 Chromatographic conditions

A.1.4.1 Column: Merck Supelco apHera NH_2, 250 mm × 4.6 mm, 5 μm

A.1.4.2 Mobile phase: Acetonitrile: water = 72 : 28 (V/V)

A.1.4.3 Column temperature: 25°C;

A.1.4.4 Detector temperature: 40°C;

A.1.4.5 Flow rate: 1.1 mL per min;

A.1.4.6 Injection volume: 5 μL;

A.1.4.7 Run time: 45 min.

A.1.5 Analytical procedure

A.1.5.1 Preparation of solutions

A.1.5.1.1 Blank solution/diluent: water;

A.1.5.1.2 Reference solution: Accurately weigh a suitable amount of 2'-Fucosyllactose reference standard, and transfer into a suitable volumetric flask, dissolve in water and dilute to volume, and shake well to obtain a reference solution containing 50 mg of 2'-Fucosyllactose per mL (expiration period: 4 weeks at a temperature of 4°C–8°C);

A.1.5.1.3 Test solution: Accurately weigh a suitable amount of sample, and transfer into a suitable volumetric flask, add a suitable amount of water, oscillate to dissolve and dilute with water to volume, and shake well to obtain a solution containing 50 mg of the sample per mL. Consecutively inject the test solution for three times, to calculate the average value as the test results.

A.1.6 System suitability requirements

Consecutively inject the reference solution for at least three times, and the following requirements should be met:

(1) The relative standard deviation of the retention time of the sample peak from three consecutive injections of the reference solution is less than 1.0%;

(2) The relative standard deviation of the response value of the sample peak from three consecutive injections of the reference solution is less than 1.0%;

(3) The chromatogram of the eluent should be a pure baseline.

Note: The high performance liquid chromatogram of 2'-Fucosyllactose reference standard is shown in Appendix C.1.

A.1.7 Calculation

Calculate the content by area normalization method:

$$w_1 = \frac{A_1}{S_1} \times 100\%$$

In which:

A_1——Peak area of 2'-Fucosyllactose from the test solution;

S_1——Sum of the peak areas of all components from the test solution except the solvent peaks.

A.2 Related substances

A.2.1 Method summary

The related substances, including D-lactose, 2'-Fucosyllactose, difucose lactose, L-fucose, D-glucose, and other total impurities were quantified by area normalization method using a differential refractive index detector.

A.2.2 Instruments and equipment

High performance liquid chromatograph (differential refractive index detector), and electronic analytical balance

A.2.3 Reagents and test solutions

Acetonitrile (HPLC), purified water, D-lactose reference standard, 2'-Fucosyllactose reference standard, difucose lactose reference standard, L-fucose reference standard, and D-glucose reference standard

A.2.4 Chromatographic conditions

A.2.4.1 Column: Merck Supelco apHera NH_2, 250 mm × 4.6 mm, 5 μm

A.2.4.2 Mobile phase: Acetonitrile: water = 72 : 28 (V/V)

A.2.4.3 Column temperature: 25°C;

A.2.4.4 Detector temperature: 40°C;

A.2.4.5 Flow rate: 1.1 mL per min;

A.2.4.6 Injection volume: 5 μL;

A.2.4.7 Run time: 45 min.

A.2.5 Analytical procedure

A.2.5.1 Preparation of solutions

A.2.5.1.1 Blank solution/diluent: water;

A.2.5.1.2 Reference solution: Accurately weigh a suitable amount of D-lactose reference standard, 2'-Fucosyllactose reference standard, difucoyl lactose reference standard, L-fucose reference standard, and D-glucose reference standard respectively, and transfer into a suitable volumetric flask, dissolve in water and dilute to volume, and shake well to obtain a reference solution containing about 0.5 mg per mL (expiration period: 4 weeks at a temperature of 4°C–8°C);

A.2.5.1.3 Test solution: Accurately weigh a suitable amount of sample, and transfer into a suitable volumetric flask, add a suitable amount of water, oscillate to dissolve and dilute with water to volume, and shake well to obtain a solution containing 50 mg of the sample per mL. Consecutively inject the test solution for three times, to calculate the average value as the test results.

A.2.6 System suitability requirements

Consecutively inject the reference solution for at least three times, and the following requirements should be met:

(1) The relative standard deviation of the retention time of the sample peak from three consecutive injections of the reference solution is less than 1.0%;

(2) The relative standard deviation of the response value of the sample peak from three consecutive injections of the reference solution is less than 1.0%;

(3) The chromatogram of the eluent should be a pure baseline.

Note: 1. The reference solutions of D-lactose, L-fucose and D-glucose are selected for system suitability test, and 2'-Fucosyllactose and difucosyl lactose are selected for qualitative test.

2. The high performance liquid chromatograms of the reference solutions of D-lactose, 2'-Fucosyllactose, difucosyl lactose, L-fucose, and D-glucose are shown in Appendix C.2, C.3, C.4, C.5 and C.6, respectively.

A.2.7 Calculation

Calculate the content by area normalization method:

$$w_2 = \frac{A_x}{S_2} \times 100\%$$

In which:

A_x——Peak area of each impurity from the test solution;

S_2——Sum of the peak areas of all components from the test solution except the solvent peaks.

9. Appendix B - Content of Residual Proteins

B.1 Basis for Method Establishment

The analytical procedure refers to the determination of total residual proteins in the new food additive 2'-Fucosyllactose (comment version). Coomassie Brilliant Blue Dye reacted with the proteins, and the absorbance measured at a wavelength of 595 nm was used for the determination of proteins. In order to prevent the interference of the sample matrix on the color development reaction, the sample solution was mixed with bovine serum albumin (BSA) reference solution at different concentrations for color development, and a quadratic standard curve was plotted to calculate the protein content in the sample. The limit of detection (LOD) of this method was 17 mg/kg.

B.2 Determination Range

Determination range of this method: ≥ 17 mg/kg

B.3 Instruments and Reagents

Instruments: Electronic analytical balance (Sartorius (SQP)), Shimadzu UV spectrophotometer (Shimadzu UV-1900 i), and vortex mixer (IKA VORTEX 3)

List of reagents: BSA (Beijing Solarbio Life Science Co., Ltd., Lot No. 1229Z051), Bradford reagent (Bioengineering (Shanghai) Co., Ltd., Lot No. HA12DB0002)

B.4 Test Procedure

B.4.1 Preparation of solutions

(1) BSA stock solution

Weigh 20.0 mg of bovine serum albumin RS in a 10 mL volumetric flask, dissolve with water to volume, and mix well to obtain a solution at a concentration of 2 mg/mL.

(2) BSA reference solution

Measure 100 μL of the above stock solution into a 10 mL volumetric flask, dilute with water to volume, and mix well to obtain a solution at a concentration of 20 μg/mL.

(3) Test solution

Weigh 200 mg of sample in a 5 mL volumetric flask, dissolve with water to volume, and mix well to obtain a solution at a concentration of 40 mg/mL.

B.4.2 Analytical procedure

According to Table B.1, directly add the test solution, water, BSA, and Coomassie Brilliant Blue Dye into the cuvette, mix well, and allow to stand at room temperature for 10 min. Measure the absorbance value of the mixed solution successively at 595 nm using water as the reference.

Table B.1 Determination system of Bradford method

	BSA concentration (mg/L)	Sample solution (μL)	Water (μL)	BSA reference solution (μL)	Coomassie brilliant blue reagent (μL)	Final concentration of solution
Blank solution 1	0	0	800	0	200	0
Blank solution 2	0	0	800	0	200	0
Mixed solution 0	0	600	200	0	200	0 mg/L BSA+24mg/mL 2'-FL
Mixed solution 1	1	600	150	50	200	1 mg/L BSA+24mg/mL 2'-FL
Mixed solution 2	2	600	100	100	200	2 mg/L BSA+24mg/mL 2'-FL
Mixed solution 3	3	600	50	150	200	3 mg/L BSA+24mg/mL 2'-FL
Mixed solution 4	4	600	0	200	200	4 mg/L BSA+24mg/mL 2'-FL

B.4.3 Content of residual proteins

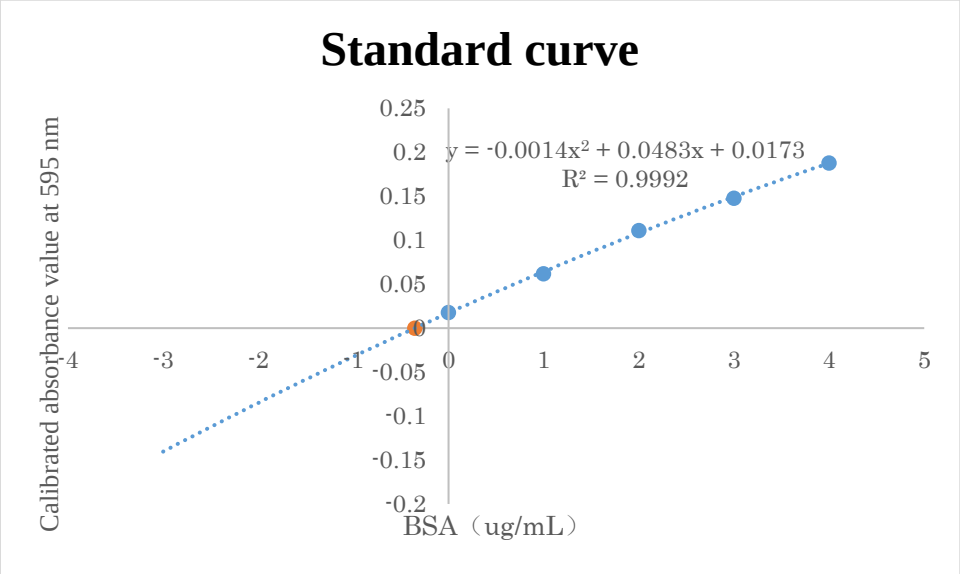
The calibrated absorbance values of the spiked sample solution obtained by subtracting the average absorbance values of blank solutions from the absorbance values of the mixed solution are shown in Table B.1. With the calibrated absorbance values as the y-axis and the concentration of BSA reference solutions as the x-axis, a quadratic standard curve through the intersection of the left half axis of the abscissa was plotted. The absolute value of the concentration corresponding to the intersection of the standard curve and the left half axis of the abscissa is the protein concentration of the sample.

Example 1: The test sample 2102-0215-P1 is shown in following Table B.2, and the quadratic standard curve is shown in the figure below.

Table B.2. Quadratic standard curve of spiked test sample 2102-0215-P1 determined by Bradford method

Sample (μg/mL)	0	1	2	3	4
Calibrated absorbance value at 595 nm	0.018	0.062	0.111	0.148	0.188

Figure of quadratic standard curve of spiked test sample 2102-0215-P1 determined by Bradford method



Calculate the protein content ω of the sample according to the following formula, with the result expressed in mg/kg.

In which:

$$\omega = \frac{-1 \times c \times v}{0.6 \times m} \times f$$

c——Concentration value corresponding to the intersection of the standard curve and the left half axis of the abscissa; the value is negative, and expressed in micrograms per milliliter (μg/mL);

$-1 \times C$——Protein concentration of mixed solution determined by the standard curve, in micrograms per milliliter (μg/mL);

v——Constant volume of sample solution, in milliliters (mL);

f——Dilution factor;

m——Weight of the sample, in milligrams (mg);

0.6——Volume of the sample solution in 1 mL of the mixed solution is 0.6 mL.

The LOD of the method is 17 mg/kg (17 ppm). If the result is below LOD, the result is expressed as < 17 mg/kg, or not detected.

10. Appendix C-1 - High Performance Liquid Chromatograph

C.1 High performance liquid chromatogram of 2'-Fucosyllactose reference standard

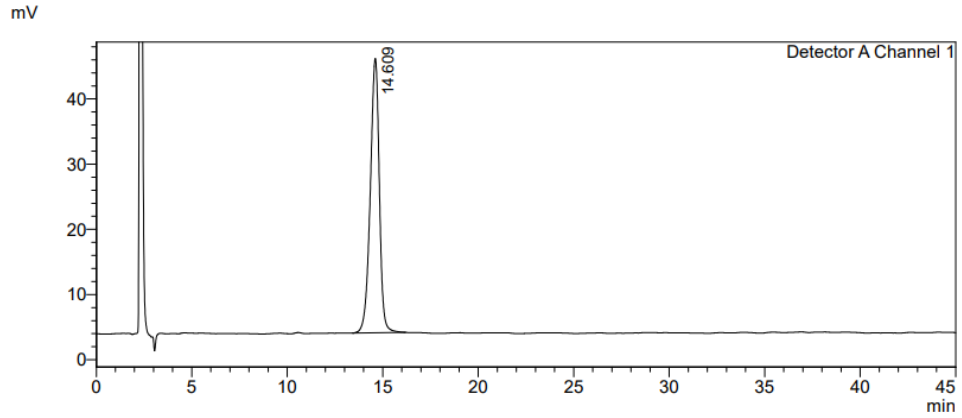


Figure C.1 High performance liquid chromatogram of 2'-Fucosyllactose reference standard

C.2 High performance liquid chromatogram of D-Lactose reference standard

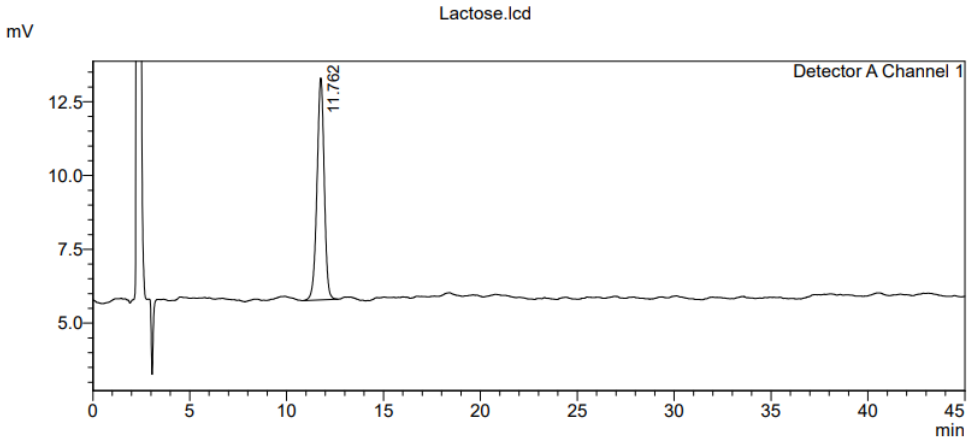


Figure C.2 High performance liquid chromatogram of D-Lactose reference standard

C.3 High performance liquid chromatogram of 2'-Fucosyllactulose reference standard

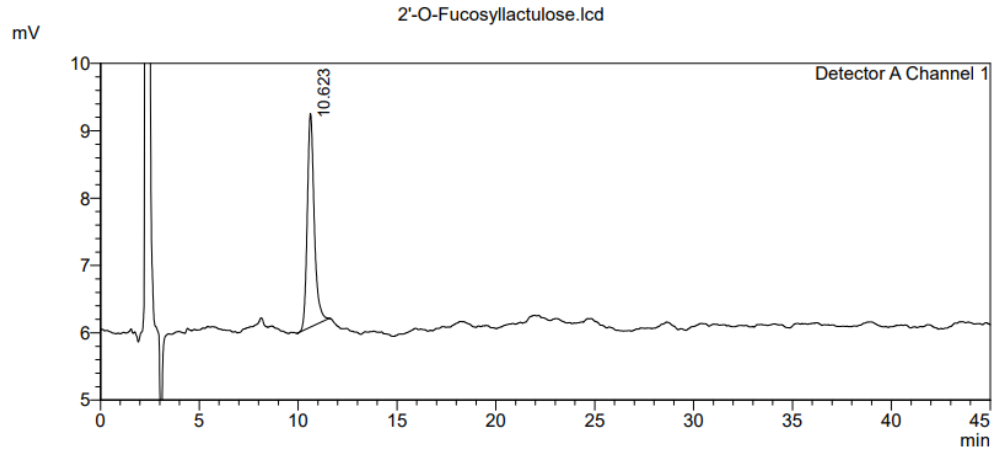


Figure C.3 High performance liquid chromatogram of 2'-Fucosyllactose reference standard

C.4 High performance liquid chromatogram of Difucosyllactose reference standard

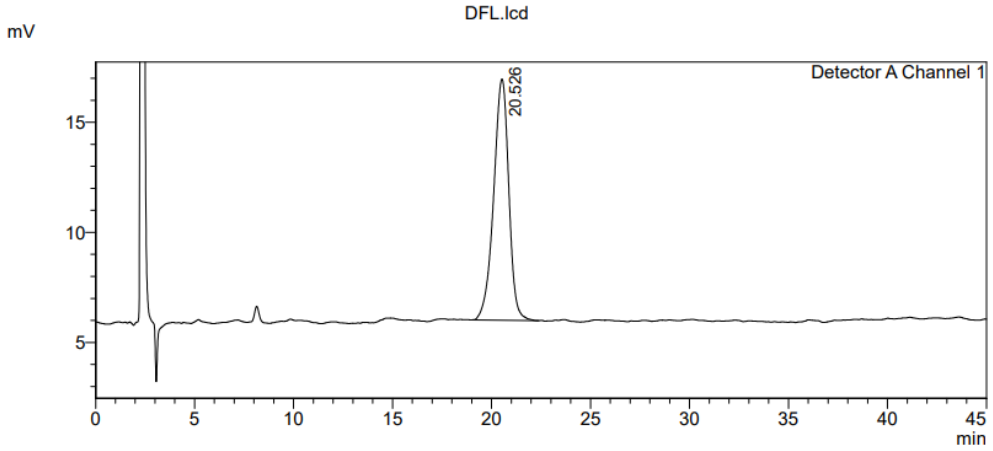


Figure C.4 High performance liquid chromatogram of Difucosyllactose reference standard

C.5 High performance liquid chromatogram of L-fucose reference standard

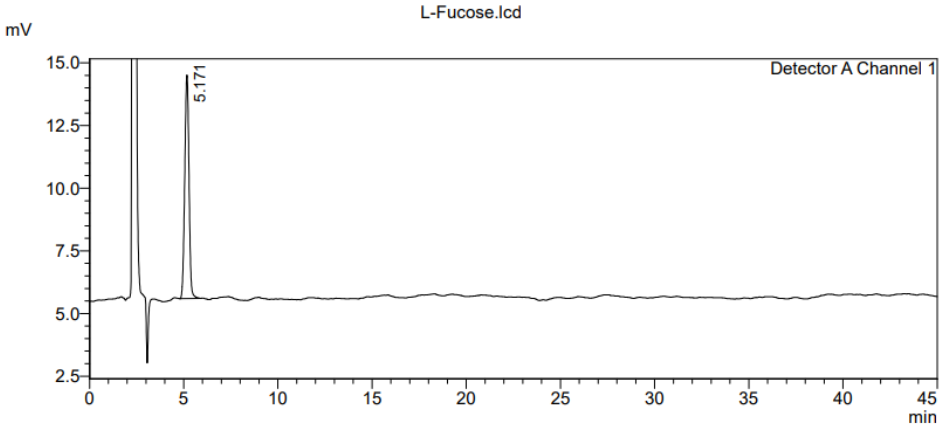


Figure C.5 High performance liquid chromatogram of L-fucose reference standard

C.6 High performance liquid chromatogram of D-glucose reference standard

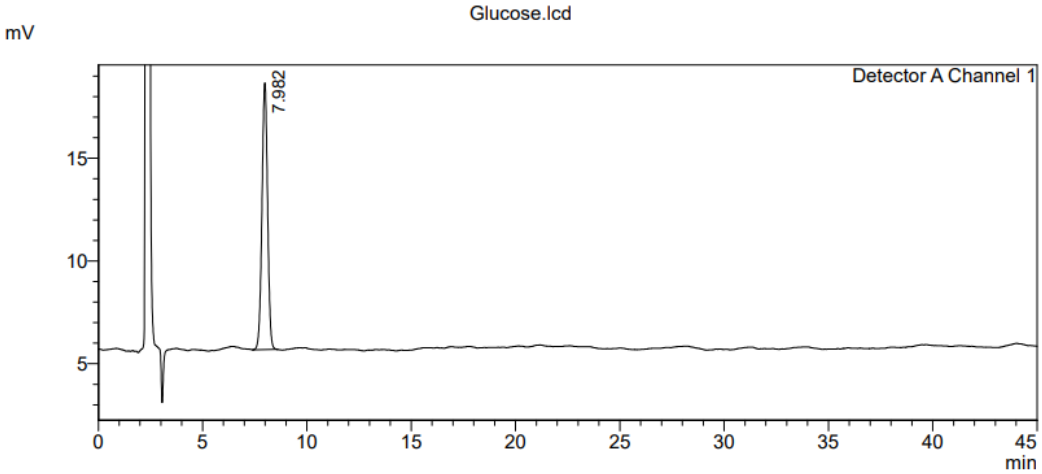


Figure C.6 High performance liquid chromatogram of D-glucose reference standard

Table C.1. Retention times of each compound

Chemical compound	RT(min)
Water (solvent)	2–3
L-Fucose	5.2
D-Glucose	8.0
2'-fucosyllactulose	10.6
D-Lactose	11.7
2'-Fucosyllactose	14.6
Difucosyllactose	20.5

11. Appendix C-2 - Chromatograms from HPAEC-PAD method

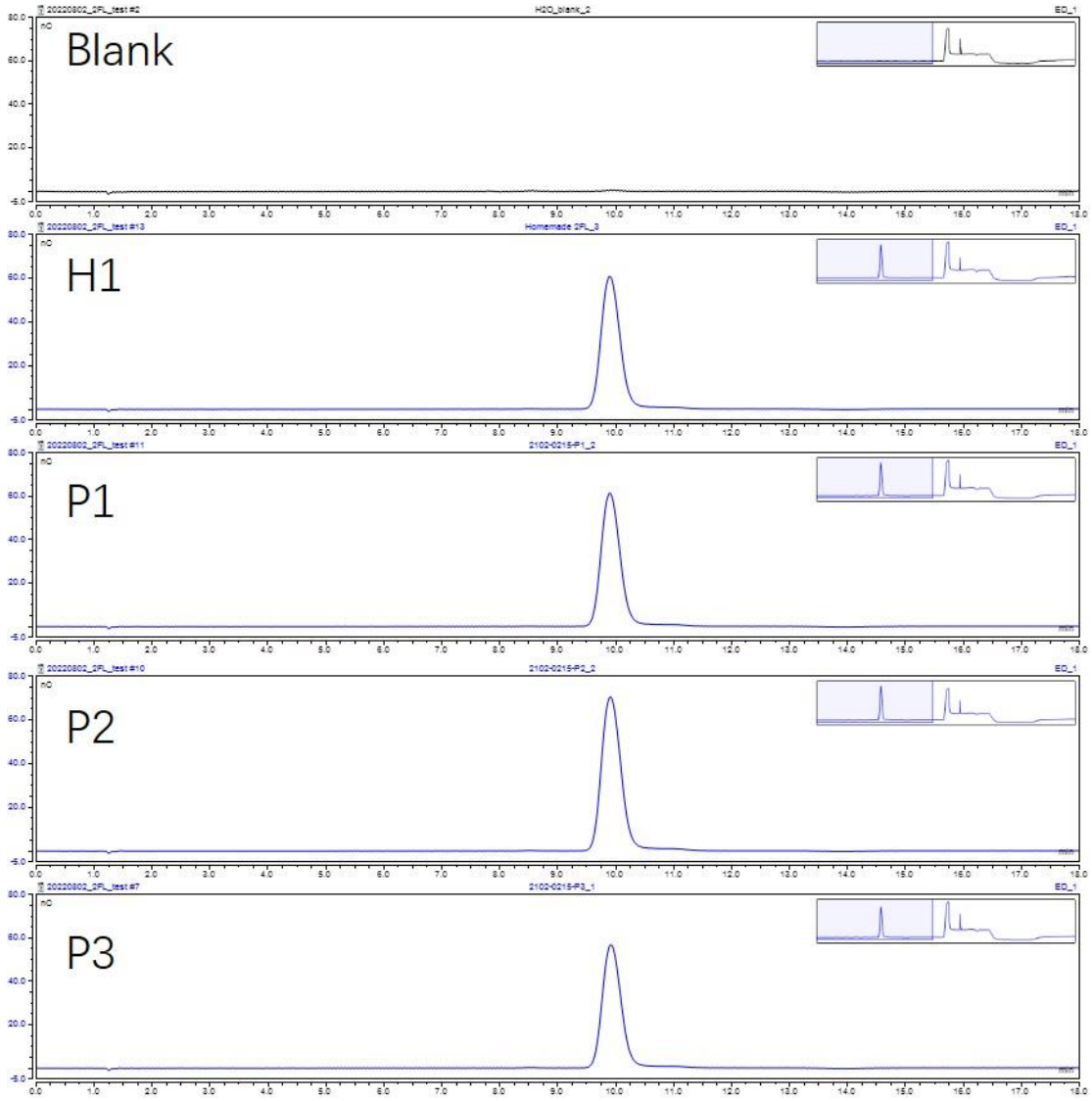


Figure C.1 Chromatograms from HPAEC-PAD method

Note:
H1 refers to the reference 2'-FL. P1, P2 and P3 refer to the three batches of Synaura's 2'-FL.

12. Appendix D - Production Strain Construction

1. Host strain

The host strain was *Escherichia coli* (*E. coli*) BL21 (DE3), purchased from Novagen sku: 69450-M. *E. coli* BL21 (DE3), a gram-negative Brevibacterium with blunt round ends belonging to strain B of *Escherichia coli*.

E. coli B strain is one of the four laboratory model strains of *E. coli*, and the other three are *E. coli* K12, *E. coli* W and *E. coli* C. Due to the lack of adverse effects on humans and mammals (NIH, 2023) and the ability to colonize in the human gut (Bauer et al., 2008), these four model *E. coli* strains are classified as laboratory microorganisms with safety level I (Archer et al., 2011; ATCC, 2018).

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

The whole genome sequence of *E. coli* BL21 (DE3) is clear and available on the NCBI website; see the link below:

https://www.ncbi.nlm.nih.gov/nuccore/CP001509.3?report=fasta#opennewwindow

Due to the advantages of short growth cycle, low difficulty in strain construction, and relatively low cost, currently, most of the mainstream manufacturers of 2'-FL also use *E. coli* as the production strain (Parschat et al., 2020). For instance, the German company Jennewein uses *E. coli* BL21 as the parent strain, both Glycom A/S (Danish) and Glycosyn LLC (American) all use *E. coli* K-12 as the parent strain. The *E. coli* BL21 (DE3) used in this project may be the most widely used strain in the overexpression of heterologous and homologous recombinant proteins, and has been used in the production of many drug proteins such as granulocyte colony stimulating factor.

2. Introduced genes for 2'-fucosyllactose (2'-FL) biosynthesis

Several genetic manipulations were performed in the genome of recipient *E. coli* BL21 (DE3) by Synaura Biotechnology (Shanghai) Co., Ltd., which included knock-out of *lac*Z, *fuc*Ik, *ara*A, *rha*A, *wca*J, and *pfk*A genes, and knock-in of *lac*Y gene, respectively. In addition, two plasmids (plasmid pBCGW expressing *gmd*, *wca*G, *man*C and *man*B genes from *E. coli* and plasmid pGT065 expressing a codon-optimized *gt065* gene derived from *Neisseria*) were transferred into *E. coli* BL21 (DE3), thus a recombinant *E. coli* EB011065 capable of efficiently producing 2'-FL was developed.

Two plasmids were introduced into *E. coli* BL21 (DE3). *manB, manC, gmd* and *wcaG* genes were carried by plasmid pCDFduet-1 via scarless cloning to obtain plasmid pBCGW; Synthetic *gt065* gene was carried by plasmid pET28a to obtain plasmid pGT065.

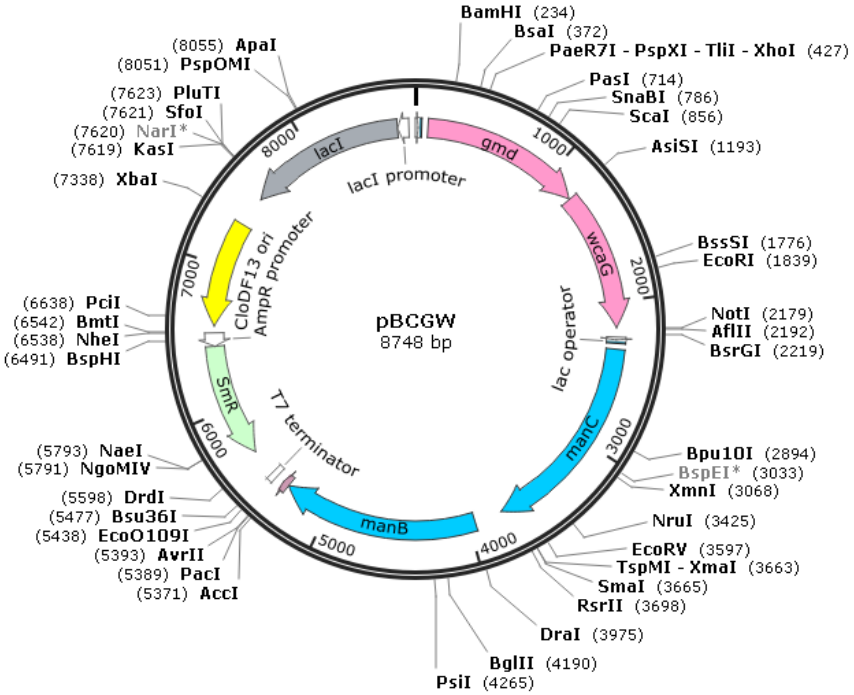


Fig D. 1 Plasmid map of pBCGW

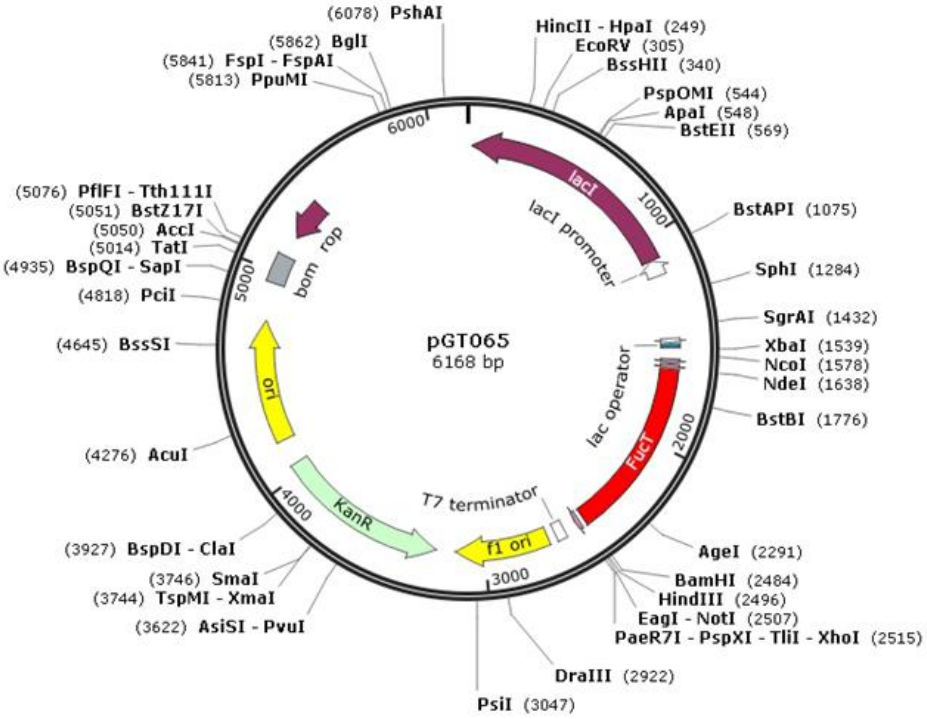


Fig D. 2 Plasmid map of pGT065

Table D.1 Descriptions and positions of the introducted genetic elements in plasmid pBCGW

Name of gene or element	Base position (bp)	Source	Function
T7 promoter	1-87	Bacteriophage T7	T7 promoter
gmd	88-1209	*E. coli* BL21	GDP-mannose-4,6-dehydrase
*wca*G	1210-2177	*E. coli* BL21	GDP-fucose synthase
Plasmid DNA	2178-2242	pCDFduet-1	Interval sequence
T7 promoter	2243-2325	Bacteriophage T7	T7 promoter
*man*C	2326-3763	*E. coli* BL21	Mannose-1-phosphate-guanosine transferase
Plasmid DNA	3764-3954	*E. coli* BL21	Interval sequence
*man*B	3955-5325	*E. coli* BL21	Phosphomannose mutase
S-Tag	5326-5388	pCDFduet-1	Analytical marker
T7 terminator	5389-5469	Bacteriophage T7	T7 terminator
Plasmid DNA	5470-5651	pCDFduet-1	Interval sequence
SmR	5652-6443	*Staphylococcus aureus*	Streptomycin resistance gene
ampR promoter	6444-6535	pCDFduet-1	Ampicillin resistance gene promoter
Plasmid DNA	6536-6582	pCDFduet-1	Interval sequence
CloDF13 ori	6583-7321	pCDFduet-1	Replicon
Plasmid DNA	7322-7530	pCDFduet-1	Interval sequence
*lac*I	7531-8625	*E. coli*	Lactose operon repressor
lacI promoter	8626-8703	*E. coli*	Lactose operon promoter
Plasmid DNA	8704-8748	pCDFduet-1	Interval sequence

TableD.2 Descriptions and positions of the introducted genetic elements in plasmid pGT065

Name of gene or element	Base position (bp)	Source	Function
Vector DNA	1-23	pET28a	Interval sequence
lacI	24-1106	*E. coli*	Lactose operon repressor
lacI promoter	1107-1184	*E. coli*	Lactose promoter
Vector DNA	1185-1492	pET28a	Interval sequence
lacO	1493-1579	*E. coli*	Lactose operon
Synthetic *gt065*	1580-2497	*Neisseria*	α-1,2-fucosyltransferase
Vector DNA	2502-2604	pET28a	Interval sequence
T7 terminator	2605-2652	Bacteriophage T7	T7 terminator
Vector DNA	2653-2687	pET28a	Interval sequence
f1 ori	2689-3144	F1 phage	f1 origin of replication
Vector DNA	3145-3236	pET28a	Interval sequence
kanR	3237-4052	*Klebsiella*	Kanamycin resistance gene
Vector DNA	4053-4173	pBR322	Interval sequence
pUC ori	4174-4762	pBR322	pUC origin of replication
Vector DNA	4763-4947	pBR322	Interval sequence
Vector DNA	4948-5191	pBR322	Interval sequence
rop	5192-5383	pBR322	Controlled low copy
Vector DNA	5384-6186	pBR322	Interval sequence

13. Appendix E - Genetic Stability testing

Synaura Biotechnology (Shanghai) Co., Ltd. (Synaura ") knock out genes *lac*Z, *fucI*k, *ara*A, *rha*A, *wca*J, and *pfkA*, and knock in gene *lac*Y in the genome of *Escherichia coli* BL21 (DE3) (receptor). Moreover, Synaura constructed the expression vector pBCGW with exogenous genes *gmd*, *wca*G, *man*C, and *man*B and the expression vector pGT065 with gene *gt065*. The constructed expression vectors were then transformed into *E. coli* BL21 (DE3) to develop the recombinant *E. coli* EB011065 that can efficiently produce 2'-Fucosyllactose ("2'-FL").

In this report, the specific DNA fragments of two exogenous plasmids (pBCGW and pGT065) were determined by fluorescence quantitative PCR (qPCR), using gene *16S r*RNA in the genome of the strain as the reference gene. The copy number stability of plasmid pBCGW and pGT065 of recombinant *E. coli* EB011065 for five passages was then determined, which demonstrated the passage stability of the target genes of exogenous plasmids of recombinant *E. coli* EB011065 for five passages (target genes of pBCGW: *gmd*, *wca*G, *man*C, and *man*B; target gene of pGT065: *gt065*).

1. Test Method

(1) Culture of recombinant *E. coli* EB011065 for five passages

Take out the frozen strain (stored in the strain preservation solution) from the strain library, streak the LB agar plate, invert the plate, and incubate at 37 °C for 24 h. Select a single colony, inoculate in liquid LB medium, and incubate at 37 °C with shaking (220 rpm) for 6 h. The resulting strain is the first-passage production strain of recombinant *E. coli* EB011065. Add 0.5 mL of 40% (v/v) glycerol to 0.5 mL of culture solution to obtain the final glycerol concentration of 20% (v/v). Preserve the first-passage production strain of recombinant *E. coli* EB011065 in the glycerol preservation solution and store at -80 °C.

Completely thaw the glycerol preservation solution of the first-passage strain on ice, transfer to the liquid medium, and incubate at 37 °C with shaking (220 rpm) for 6 h. The resulting strain is the second-passage strain, which shall be stored at -80 °C using the method mentioned above. Repeat the same procedure to obtain the strain preservation solutions for the third-, fourth-, and fifth-passage strains of recombinant *E. coli* EB011065.

Select the strains of the recombinant *E. coli* EB011065 (a total of 5 strains from the first passage to the fifth passage) and the wild-type *E. coli* BL21 (DE3) (receptor), and then extract the genomic DNA for PCR amplification using the Ezup Column Genomic DNA Extraction Kit (Sangon Biotech (Shanghai) Co., Ltd., Cat. No.: B518255).

(2) Primer design

To analyze plasmid copy number, a specific gene shall be first selected for copy number characterization. Four endogenous genes (*gmd*, *wca*G, *man*C, and *man*B) of plasmid pBCGW have identical DNA coding sequences as those on chromosome, which may lead to misinterpretation of corresponding plasmid copy number. Therefore, the resistance gene *Sm*R was used for characterization.

Second, it is necessary to distinguish the plasmid of interest from other plasmids in the host strain that also exhibit specificity. The host strain carries two different plasmids, pBCGW and pGT065. While the basic framework of both plasmids has similarities, the pGT065 plasmid carries

the specific exogenous gene *gt065*. Consequently, Plasmid pGT065 is characterized using the specific exogenous gene *gt065.*

Multiple pairs of primers were designed for the gene 16S rRNA and the plasmid vectors pBCGW and pGT065 using Primer3Plus. The primers were screened using conventional Gel-PCR.

Table E.1. Primers of reference gene and target genes

Primer name	Sequence (5' to 3')	Fragment length	Location on a gene/plasmid
16S-5-F	GCCACACTGGAACTGAGACA	238 bp	310-330bp
16S-5-R	TTGCACCCTCCGTATTACCG		528-547bp
*Sm*R-2-F	GGTGATCTCGCCTTTCACGTAG	122 bp	5670-5691bp
*Sm*R-2-R	CCCAGTATCAGCCCGTCATACT		5770-5791bp
gt065-4-F	AAAAGATCTGGTGGAAAGTGTTCAC	134 bp	1872-1896bp
gt065-4-R	GAAGTATTTTTCGCTCTGCCAGTAG		1981-2005bp

(3) Assessment of gene amplification efficiency using the standard curve

The use of control group ensures that the genetic expression changes were comparable among various body tissues and experimental groups for the comparative Ct method for relative quantification. The application of this method should meet two requirements: (1) the target gene and reference gene shall have similar amplification efficiency, and (2) the amplification efficiency shall be as high as possible (close to 100%).

The gene amplification efficiency can be determined by drawing standard curves for gene 16S rRNA and plasmids pBCGW and pGT065. The method is as follows:

a. Preparation of standard solutions with different concentrations

Use the extracted and purified genomic DNA from BL21(DE3) as the gene 16S rRNA standard (Ezup Column Genomic DNA Extraction Kit, Sangon Biotech (Shanghai) Co., Ltd., Cat. No.: B518255-0050). Use the in-house purified plasmids pBCGW (pCDFduet-1 backbone) and pGT065 (pET28a backbone) as standard plasmids (SanPrep Column Plasmid Mini-Preps Kit, Cat. No.: B518191-0100). Measure the nucleic acid concentration using a NanoDrop spectrophotometer.

Preparation of standard solutions with different concentrations for genomic DNA: Dilute with deionized water to 10^{1}–10^{-4} ng/μL according to the dilution gradient and use as a qPCR template.

Preparation of standard solutions with different concentrations for plasmid pBCGW: Dilute with deionized water to 10^{-1}–10^{-6} ng/μL according to the dilution gradient and use as a qPCR template.

Preparation of standard solutions with different concentrations for plasmid pGT065: Dilute with deionized water to 10^{-1}–10^{-5} ng/μL according to the dilution gradient and use as a qPCR template.

b. qPCR system and procedure

Table E.2. qPCR reaction system

Reagent	Volume	Final concentration
TB Green Premix Ex Taq (Tli RNaseH Plus) (2X) (TAKARA)	5 μL	1×
qPCR-M2-FPrimer(10 μM)	0.2 μL	0.2 μM
qPCR-M2-RPrimer(10 μM)	0.2 μL	0.2 μM
DNA template (<100 ng)	0.8 μL	
Sterile water	3.8 μL	
Total	10 μL	

Prepare three replicate wells for each sample. The Ct value difference between the replicate wells is no more than 0.5.

qPCR procedure: 95 °C, 30 s (95 °C, 5 s; 60 °C, 30 s) for 40 cycles, (60 °C, 95 °C) 15 s, end.

qPCR instrument brand/model: Jena qTOWER3.

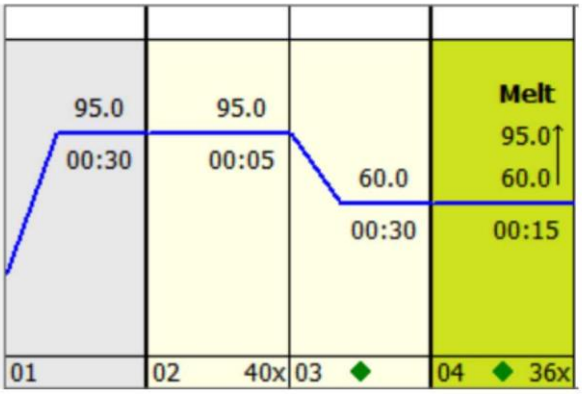


Figure E.1. qPCR amplification procedure

(4) Determination of plasmid copy numbers of five transgenic strains by qPCR

Determine the Ct values of the reference gene and the target gene in the total DNA of each passage of strain by qPCR. Take the first-passage strain as the control group and the second- to fifth-passage strains as the experimental groups, detect the expression differences between the experimental groups and the control group. Use the comparative Ct method to calculate the ΔΔCt value, under the precondition that the amplification efficiency is similar and close to 100% for the reference and target genes. Next, use the formula $F=2^{-\Delta\Delta Ct}$ to determine the relative expression of the target gene compared to the reference gene.

Perform ultrasonic lysis for the first- to fifth-passage strains and measure the nucleic acid concentration using a NanoDrop spectrophotometer to ensure that the nucleic acid concentration in each passage is between 100 and 200 ng/μL.

Sample preparation: Dilute the total DNA sample with water by the ratio of 1:10,000 and 1:100,000, and use the diluted samples as templates for qPCR. Use water as the negative control, genomic standard as the positive control for the reference gene, pBCGW plasmid standard as the positive control for pBCGW, and pGT065 plasmid standard as the positive control for pGT065.

The qPCR system and procedure are the same as that stated in section (2).

The calculation method of relative expression is as follows:

ΔCt (experimental group) = Ct (target gene of experimental group) - Ct (reference gene of experimental group);

ΔCt (control group) = Ct (target gene of control group) - Ct (reference gene of control group);

ΔΔCt = ΔCt (experimental group) - ΔCt (control group);

$F=2^{-\Delta\Delta Ct}$

According to the ΔΔCt value, the multiple of expression difference can be calculated as $F=2^{-\Delta\Delta Ct}$, which represents the fold change of the expression of the target gene of the experimental group relative to that of the control group. The expression of the target gene relative to the reference gene can be obtained directly using this method.

2. Test Results

(1) Assessment of gene amplification efficiency using the standard curve

Plot a standard curve with the logarithm of the concentration of the standard as the X-axis and the Ct value of qPCR as the Y-axis.

a. Standard curve for gene 16S rRNA (reference gene)

Table E.3. Standard curve for reference gene by qPCR

Logarithm of the concentration of the standard $10^{(N)}$	-4	-3	-2	-1	0	1
CT	26.1	23.24	19.68	16.22	12.83	9.76

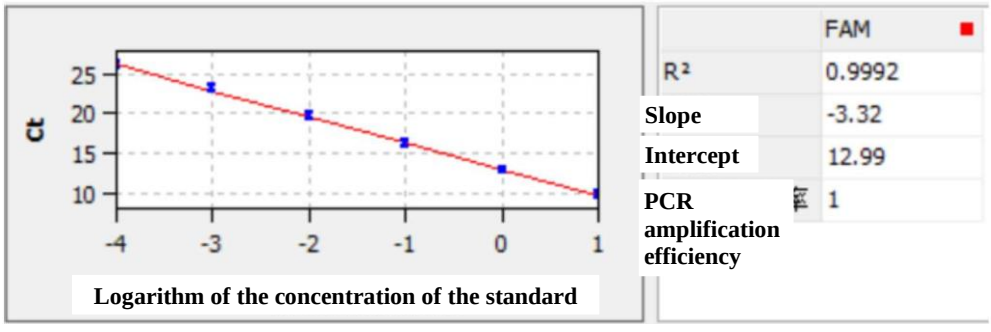


Figure E.2. Standard curve and amplification efficiency for reference gene by qPCR

b. Standard curve for plasmid pBCGW

Table E.4. Standard curve for plasmid pBCGW by qPCR

Logarithm of the concentration of the standard $10^{(N)}$	-6	-5	-4	-3	-2	-1
CT	25.99	23.17	20.12	16.36	12.79	9.55

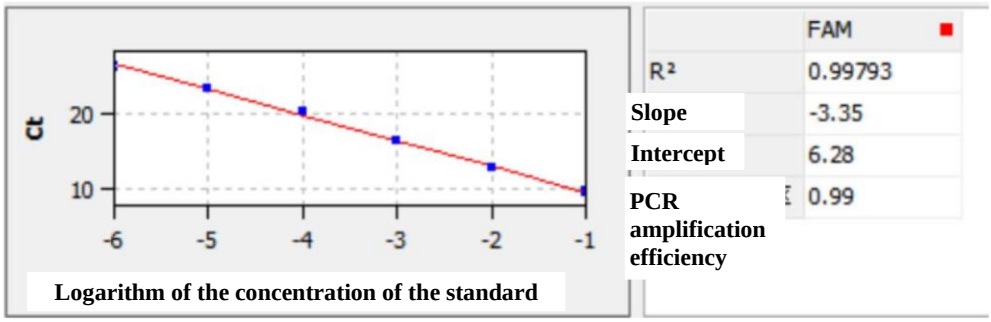


Figure E.3. Standard curve and amplification efficiency for plasmid pBCGW by qPCR

c.Standard curve for plasmid pGT065

Table E.5. Standard curve for plasmid pGT065 by qPCR

Logarithm of the concentration of the standard $10^{(N)}$	-5	-4	-3	-2	-1
CT	24.41	20.98	18.19	13.97	10.48

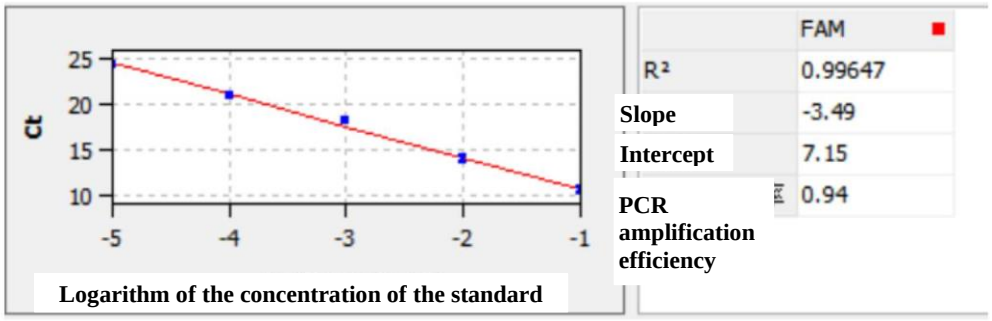


Figure E.4.Standard curve and amplification efficiency for plasmid pGT065 by qPCR

The results showed that the amplification efficiency of the target gene and the reference gene was similar and within the range of 90%–105%. It demonstrates the feasibility of the comparative Ct method in determining the passage stability of plasmids.

(2)Determination of the stability of the plasmid copy numbers of five transgenic strains by Qpcr

Table E.6. Ct value and CV (coefficient of variation) value of the target gene *Sm*R and the reference gene 16S in the same run of experiments by qPCR

Passage number	*Sm*R		16S	
	Ct	CV	Ct	CV
First	16.49	0.008	19.16	0.005
Second	17.24	0.003	19.7	0.000
Third	17.15	0.005	19.87	0.006
Fourth	16.38	0.001	18.99	0.006
Fifth	16.83	0.004	19.63	0.005

Table E.7. Ct value and CV value of the target gene *gt065* and the reference gene 16S in the same run of experiments by qPCR

Passage number	*gt065*		16S	
	Ct	CV	Ct	CV
First	19.31	0.007	22.58	0.020
Second	19.78	0.005	23.01	0.007
Third	19.80	0.007	23.17	0.009
Fourth	19.53	0.003	22.68	0.002
Fifth	20.15	0.003	23.46	0.007

Table E.8. Copy number of the target gene relative to the reference gene by qPCR

Passage number	$2^{-\Delta\Delta Ct}$	
	pBCGW (*Sm*R)	pGT065 (*gt065*)
First	1.00	1.00
Second	0.86	0.97
Third	1.04	1.07
Fourth	0.96	0.92
Fifth	1.09	1.03
CV	8.9%	5.7%

Note:

1. The first-passage strain is used as the control, and therefore the copy number ratio of the first-passage strain is 1.00.

2. There is no significant difference if the CV value is less than 15%.

The $2^{-\Delta\Delta Ct}$ results indicate that plasmids pBCGW and pGT065 exhibit a basically consistent trend among various passages, ensuring that the strain remains stable during passage.

3.Conclusion

In this report, we developed a method for determining the passage stability of plasmids pBCGW and pGT065. The comparative Ct method for relative quantification (by qPCR) was utilized. With the gene *16S r*RNA used as the reference gene, the primers for the reference and target genes were designed and screened. A standard curve was plotted under the precondition that the amplification efficiency was similar and close to 100% for the reference and target genes, ensuring that the method mentioned above is feasible.

Based on this, qPCR results show basically consistent expression differences between the target gene and the reference gene in passage strains. As a result, the copy numbers of plasmids pBCGW and pGT065 remain stable relative to the reference gene during passaging, thereby ensuring the stability of the target genes copy numbers (*gmd*, *wca*G, *man*C, and *man*B for plasmid pBCGW; *gt*065 for plasmid pGT065) on the plasmids.

From: 于艳艳
To: Anderson, Ellen
Cc: "yaofei@mengniu.cn"; 余瑶盼
Subject: [EXTERNAL] Re:Re:GRN 001157
Date: Wednesday, February 14, 2024 11:19:45 PM
Attachments: image001.png
Responses on the Questions Regarding GRN 001157_final.pdf

Dear Ellen,

Please kindly find the attached document for the responses on the questions regarding GRN 001157.

If you have any question, please kindly let me know. Thank you.

All my best regards,

Wing Yu
Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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From: "于艳艳" <wing.yu@cirs-group.com>
Date: 2024-02-04 17:27:39
To: "Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
Cc: "'yaofei@mengniu.cn'" <yaofei@mengniu.cn>,"余瑶盼" <yyp@cirs-group.com>
Subject: Re:GRN 001157

> Dear Ellen,

Thank you for your prompt response and the assessment feedback regarding our substance. We will address the issues raised as quickly as possible.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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发件人："Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
发送日期：2024-02-01 04:20:46
收件人："'yaofei@mengniu.cn'" <yaofei@mengniu.cn>
抄送人："'wing.yu@cirs-group.com'" <wing.yu@cirs-group.com>
主题：GRN 001157

Dear Fei,

Our review of GRN 001157 is complete. We respectfully ask you to respond to the questions listed in the attached document. Please do not hesitate to contact me if you have any questions about our request.

Sincerely,
Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

Dear Dr. Anderson,

RE: Questions Regarding GRN 001157 (2'-Fucosyllactose)

This responds to your email of January 31, 2024, regarding your queries that need to be addressed for 2'-Fucosyllactose GRAS Notice (GRN 1157) submitted by Synaura Biotechnology (Shanghai) Co., Ltd. We are providing a point-by-point response to all of your queries along with some additional relevant clarifications/discussion.

INTENDED USES

FDA Query: (1) On page 19, the notifier declares an intended use as "milk and soy-based, nonexempt infant formula… as consumed (ready-to-drink or reconstituted formula prepared from powder)." On page 20, in Table 5 the notifier declares an intended use as infant formula "including ready-to-drink formula or reconstituted formula prepared from powder." Please clarify if formula prepared for consumption from concentrated liquid infant formula is included among the intended uses.

Response: At this point, Synaura does not intend to use 2'-Fucosyllactose in concentrated liquid formula.

FDA Query: (2) The intended uses represent a subset of current GRAS uses of 2′-FL (summarized in GRN 001051). Please confirm that it is your intention to substitute for uses described in GRNs 000735 and 000932 and not for all current uses of 2′-FL.

Response: It is our intention to substitute for uses described in GRNs 000735 and 000932 only. We note that GRN 0001051 was not available at the time of preparation of our GRAS notice (GRN 1157) and we were not aware of uses summarized in GRN 001051. As per FDA GRAS inventory website GRN 001051 is still pending or under review. We note that GRN 001051 is now avaialable on FDA's website (as of 01-31-2024) [GRAS Notices (fda.gov)]. We have reviewed the available content and can state that our uses are also described in GRN 001051 as well.

FDA Query: (3) On page 19, the notifier mentions use in "flavored waters" in the text, although it is not included in Table 5 (page 20). "Flavored waters" were noted parenthetically in the response letter for GRN 000932; however, this was not one of the food categories listed in either GRN 000735 or GRN 000932. For clarity, FDA often refers to the beverage and water-based food categories listed in the What We Eat in America classification system.[1] In this system, flavored or enhanced waters are subdivided into "flavored or carbonated water" (e.g., flavored seltzer, Capri Sun® Roarin' Waters) and "enhanced or fortified water" (e.g., Propel® water, Glacéau Vitaminwater®). "Enhanced or fortified waters" were included in GRNs 000546, 000897, 001051, and 001091. Carbonated beverages were included among the intended uses in GRNs 000546 and 0001051. If either "enhanced or fortified waters" or "flavored or carbonated waters" are among the intended uses in GRN 001157, then the uses would not be strictly substitutional for GRNs 000735

[1] https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/1718/Food_categories_2017-2018.pdf

and 000932. That said, there are recent cumulative estimates of dietary exposure in GRN 001051 that include use in such beverages that might be cited in GRN 001157. We request that you clarify if one or either of these categories (flavored/carbonated or enhanced water) are included among the intended uses in GRN 001157.

Response: Thank you for bringing to our attention that flavored waters were not included in GRN 000735 or GRN 000932. We are sorry that we missed that similar question was raised for GRN 000932. As indicated above, at the time of preparation of our GRAS notice (GRN 001157), we had no access to details of GRN 001051 and this GRAS notice is still pending as per FDA GRAS notice inventory. Similar to GRN 000932, Synaura intend to use 2'-FL in additional food categories as described in "GRN 932 amendments" on page 3 and Table 2.1. on page 4 (page 60 and 61 of the amendment in pdf format). Thus, the proposed uses and use levels of Synaura's 2'-FL is in the same food and at the same use levels as described in GRN 000932.

In the GRN 000932 amendment, we noted that revised intake analysis was provided that includs 4 additional food categories. This analysis revealed that the cumulative estimated daily intakes (EDIs) were not significantly increased to meaningfully affect the overall estimates of exposure to 2′-FL in the total diet. We are including all this revised intake analysis data (Tables 2.4. and 2.5), along with food categories and intended uses of 2'-FL for our GRN 001157. The 90th percentile EDIs of 2'-FL in all users in all ages were 3.32 and 3.59 g/person/day under the conditions of intended uses and cumulative uses, respectively. These levels correspond to 71 and 74 mg/kg bw/day, respectively. The cumulative EDI values do not affect the safety conclusions.

FDA Query: (4) The notifier includes the category of "infant meal replacement products" among the intended uses. However, we note that this category is not included among the current uses of 2'-FL, and such products would fall under the category of "infant formula." Although "infant meal replacement products" were originally noted in GRNs 000735, 000852, and 000932, the example product was Pediasure, which is not an infant formula and is intended for children ages 2-13. This category was later clarified in these notices to be "milk-based meal replacement beverages for children." Please confirm that the intended use in GRN 001157 is in meal replacement drinks for children and not "infant meal replacement products".

Response: Sorry for our oversight and thank you for bringing this to our attention. We confirm that the intended use of 2'-FL is in meal replacement drinks for children and not for infants.

FDA Query: (5) The notifier includes "milk formula for toddlers and children aged 12-36 months" among the intended uses. Please clarify if milk substitute-based formulas for young children are also included among the intended uses.

Response: Synaura intends to use 2'-FL in milk substitute-based formulas for young children as well.

Method of manufacture

FDA Query: 6. Please specify if *E. coli* EB011065 is deposited in a recognized culture collection (e.g., CGMCC, CCTCC, ATCC, etc.) or if you maintain the strain in an in-house repository.

Response: Synaura maintains the strain in an in-house repository.

FDA Query: 7. The notifier states that IPTG (isopropyl β-D-1-thiogalactopyranoside) is used as an inducer in the production of 2′-FL by fermentation. Please clarify which of the purification steps described in the method of manufacture ensures the removal of IPTG.

Response: In the Crystallization step, the product is concentrated to above 40% (w/v) at certain temperature, then ethanol is added dropwise with stirring until a white powder precipitates. The product is then cooled down to ambient temperature, and the product is filtered and dried. By doing this, the product purity is improved simultaneously. IPTG is removed by crystallization below the detection limit.

FDA Query: 8. The notifier states in the purification summary (page 10) that "the sequence and selection of purification steps can be adjusted."

FDA Query: a. Please clarify which steps are considered optional and the sequence adjustments that might be made in the production of 2′-FL.

Response: In order to avoid complications, Synaura has decided to use only one process in the GRAS ingredient manufacturing, meaning they will adopt a single process flow without any adjustments or optional steps. The process information is included as Appendix I.

FDA Query: b. Depending on the type of adjustments that are made to the purification procedure, it maybe considered an alternate method of manufacture, where results of batch analyses (minimum of 3 non-consecutive lots) would be needed to show that the product can still meet the stated specifications.

Response: Based on the above question (FDA query: a) response, as Synaura will use only one process. Given this, we think there is no need to provide data from additional lots.

FDA Query: 9. Please clarify the later steps of purification and concentration/drying, including:

FDA Query a. If there are two crystallization steps in the purification process. Figure 3 (page 11) indicates a crystallization step prior to germ filtration. In the text describing the method on pages 11-12, this crystallization step is not included, although the final crystallization and drying steps are noted. If two crystallization steps are used, please clearly describe this in the text summary and indicate any additional solvent use.

Response: As indcated above, please note the second crystallization step is removed. This is also summarized in Appendix I.

FDA Query: b. If both crystallization and spray drying steps are used after the germ filtration step in the manufacture of 2′-FL, as suggested by the process flow diagram (page 11).

Response: The crystallization step is removed. Only spray drying step is used (Appendix I).

FDA Query: c. If batch analyses were conducted on 2′-FL produced using crystallization or spray drying or both processes.

Response: As mentioned above (FDA query: a) the crystallization process is removed and 2'-FL is produced by spray drying only (Appendix I).

FDA Query: 10. The notifier states on page 12 that "none of the raw materials used in the fermentation are major allergens or are derived from a major allergen." As a general comment, lactose is derived from a major allergen (milk) and may contain residual milk protein.

Response: Thank you for bringing this to our attention and we agree that lactose is derived from a major allergen (milk). As a carbohydrate, lactose is not considered allergenic. However, concentration of residual milk protein present in the finished ingredient is not significant and unlikely to be of allergenic concern.

Methods of analysis

FDA Query: 11. The notifier states (page 13) that, based on high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analyses and observed retention times, the "Synaura 2′-FL is substantially equivalent or is identical to the 2′-FL reference." The notifier also states that the identity of the product was also confirmed by nuclear magnetic resonance (NMR) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Given that the latter two techniques are more often used to confirm chemical and structural identity, we request that you provide additional clarifying information on these analyses.

a. Confirm that the NMR and LC-MS/MS methods are validated and fit for purpose.
b. If the NMR and LC-MS/MS methods are published, please provide citations.

Response: We confirm that the NMR and LC-MS/MS mehtods are validated. The references are listed below.

The objective of this experiment is to confirm that Synaura's 2'-FL is structurally identical to the 2'-FL found in human breast milk, which is a qualitative study. Both the liquid chromatography-mass spectrometry (LC-MS) method and the nuclear magnetic resonance (NMR) method used in references 1 and 2 can conclude that our 2'-FL is structurally consistent with that in breast milk.

1. Based on LC-MS/MS analytical method: Ref1-LC-MS/MS (

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In recent years, the LC-MS/MS strategy has been widely used for the qualitative and quantitative analysis of human milk oligosaccharides. Using "Ref1-LC-MS/MS" as a reference, ACN and H2O were selected as the mobile phase solvents. The target ion with a mass-to-charge ratio (m/z) of 487.16, identified as [M-H]^- in the primary mass spectrometry, is consistent with the literature; secondary mass spectrometry fragments further verified that 487.16 is the structure of 2'-FL.

2. Based on NMR analytical method: Ref2-NMR(Urashima T, Hiramatsu Y, Murata S, Nakamura T, Messer M., 1997. Identification of 2'-fucosyllactose in milk of the crabeater seal (Lobodon carcinophagus). Comp Biochem Physiol B Biochem Mol Biol. 116(3):311-314.)

Referencing "Ref2-NMR" and using DMSO as the solvent, the chemical shifts of key groups in the hydrogen spectrum are about 0.3 ppm different from those reported in the literature.

Moreover, this experimental report includes characterization with carbon spectra and other two-dimensional spectra (Dept135, COSY, HSQC, HMBC, HSQC-TOCSY).

FDA Query: 12. Please provide a comparison of the specifications provided in Table 2 (page 13) to those in the monograph for 2′-fucosyllactose in the 13th Edition of the Food Chemicals Codex (FCC, 2023).

Response: The comparisons of the specifications for 2'-FL in the FCC and our specifications for our 2'_FL is as follows:

Comparison of 2'-Fucosyllactose Specifications from Synaura with Food Chemical Codex

Parameters	Specification	FCC Specifications
Color	White to off-white	White to off-white
Form	Powder	Powder
2'-Fucosyllactose (calculated on the dried basis), *w/*%	≥ 94%	NLT 92%
pH (5% in water)	3.2–7.0	3.0-7.5
Sulfated ash, *w/*%	≤ 0.2%	NMT 2.0%
Water content, *w/*%	≤ 9.0%	NMT 9.0%
Content of residual proteins	≤ 100 mg/kg	NA
Residual DNA	Negative	NA
Residual ethanol	≤1000 ppm	NA
Related substances		
D-Lactose	≤ 3.0%	NMT 8.0% calculated as 2'-fucosyllactose on the anhydrous basis
2'-Fucosyl-D-lactulose	≤ 1.0%	NMT 2.0% calculated as 2'-fucosyllactose on the anhydrous basis
Difucosyllactose	≤ 1.0%	NA
L-Fucose	≤ 1.0%	NMT 3.0% calculated as 2'-fucosyllactose on the anhydrous basis
D-Glucose	≤ 1.0%	NA
Total impurities	≤ 5%	NA
Heavy metals		
Arsenic (calculated as As)	≤ 0.1 mg/kg	NMT 0.2 mg/kg, calculated on the anhydrous basis
Cadmium (Cd)	≤ 0.1 mg/kg	NA
Mercury (Hg)	≤ 0.05 mg/kg	NA

Lead (Pb)	≤ 0.02 mg/kg	NMT 0.1 mg/kg, calculated on the anhydrous basis
Aflatoxins		
Aflatoxin B_1	≤ 0.1 μg/kg	NA
Aflatoxin B_2	≤ 0.1μg/kg	NA
Aflatoxin G_1	≤ 0.1μg/kg	NA
Aflatoxin G_2	≤ 0.1μg/kg	NA
Aflatoxin M_1	≤ 0.05μg/kg	NA
Microbial limits		
Aerobic plate count	≤ 500 cfu/g	NA
Molds and yeasts count	≤ 100 cfu/g	NA
Coliforms count	< 3 MPN/g	NA
Enterobacteriaceae	< 10 cfu/g	NA
Salmonella/(25 g)	Absent	NA
Cronobacter (*Enterobacter sakazakii*)/(100 g)	Absent	NA
Shigella/(25 g)	Absent	NA
Staphylococcus aureus/(25 g)	Absent	NA
Bacterial endotoxins	≤ 300EU/g	NA

ChP = Chinese Pharmacopoeia; CFU = colony forming unit; MPN = most probable number; GB = Chinese Nations standard (Guojia Biaozhun); NA = not available

a. For 2′-FL and "related substances" expressed as a percentage in GRN 001157, please clarify if the units are presented in the same units (i.e., percent weight of ingredient (dry basis)).

Response: a. Yes, the units of "related substances" presented in the same units (percent weight of ingredient (dry basis).

b. Please clarify if "difucosyllactose" refers to 3,2′-difucosyl-D-lactose.

Response: b. Yes, "difucosyllactose" refers to 3,2′-difucosyl-D-lactose.

FDA Query: 13. Please confirm that the methods of analysis listed in Table 2 (pages 13-14) are validated and fit for purpose.

Response:

1. The methods for 2'-FL, D-lactose, and difucosyllactose have been validated by the Synaura's laboratory. Other relevant substances are calculated using the area normalization method. Once 2'-FL has been validated, the impurities can be quantified as well.

2. The method for residual protein content has been validated by the client's laboratory.

3. Other methods listed in Table 2 follow the Chinese Pharmacopoeia 2020 or relevant Chinese national standards (GB standards), which are statutory methods, that are validated and are fit for purpose.

FDA Query: 14. For methods cited as Chinese Pharmacopeia, "ChP" (year), or Chinese Nations standard, "GB" (method number-year), please briefly describe the type of analyses conducted.

Response: The different methods are listed in the table below. It is noted that these methods are typical of routine laboratory methods and are familiar with scientists involved in these types of analyses.

Please note: There is a mistake in the method's year code for Shigella within the submitted dossier; it should be 2012 instead of 2016. Sorry for our oversight.

Parameters	**Method**	**Briefly describe of the method**
pH (5% in water)	General Chapter <0631>, Volume IV, ChP 2020	Determination by pH meter (acidimeter).
Sulfated ash, *w*/%	GB 5009.4-2016	Incineration and weighing
Water content, *w*/%	GB 5009.3-2016	Karl Fischer Titration
Residual DNA	<3407>, Volume IV, ChP 2020	Fluorescent staining method.
Residual ethanol	<0861>, Volume IV, ChP 2020	Gas Chromatography (GC)
Arsenic (calculated as As)	GB 5009.11-2014	ICP-MS
Cadmium (Cd)	GB5009.15-2014	Atomic Absorption Spectroscopy
Mercury (Hg)	GB 5009.17-2014	ICP-MS
Lead (Pb)	GB 5009.12-2017	ICP-MS
Aflatoxin B_1	Method I, GB 5009.22-2016	ID-LC-MS/MS
Aflatoxin B_2	Method I, GB 5009.22-2016	ID-LC-MS/MS
Aflatoxin G_1	Method I, GB 5009.22-2016	ID-LC-MS/MS
Aflatoxin G_2	Method I, GB 5009.22-2016	ID-LC-MS/MS
Aflatoxin M_1	Method I, GB 5009.24-2016	ID-LC-MS/MS
Aerobic plate count	GB 4789.2-2016	Plate Count method

Molds and yeasts count	Method I, GB 4789.15-2016	Plate Count method
Coliforms count	Method I, GB 4789.3-2016	Most Probable Number (MPN) Method.
Enterobacteriaceae	Method I, GB 4789.41-2016	Plate Count method
Salmonella/(25 g)	GB 4789.4-2016	Biochemical tests and serological identification
Cronobacter (*Enterobacter sakazakii*)/(100 g)	Method I, GB 4789.40-2016	Qualitative analysis (colony morphology and biochemical characteristics)
Shigella/(25 g)	GB 4789.5-2012*	Biochemical tests and serological identification
Staphylococcus aureus/(25 g)	Method I, GB 4789.10-2016	Qualitative analysis
Bacterial endotoxins	General Chapter <1143>, Volume IV, ChP 2020	Gel-Clot Method
Listeria monocytogenes (new addition)	Method I GB 4789.30-2016	Qualitative analysis (Biochemical tests and hemolysis tests.)

FDA Query: 15. For the results of the batch analyses (Table 3, page 15), please clarify the detection limits for residual DNA and bacterial endotoxins.

Response:

1. The detection limits of Residual DNA is 0.06 ppm.

2. The detection method for bacterial endotoxins uses the Gel-Clot Method, as described in the Chinese Pharmacopoeia 2020, which is a non-quantitative detection method. The expression "not detected" used in our document has caused ambiguity; we request to revise the result expression to "Conforms to specifications (Conformed)".

FDA Query: 16. Please provide the following clarifications and/or corrections for the appendices included in GRN 001157:

a. Appendix A:

i. In Section A2, 2′-FL is included among the list of "related substances". Please clarify if this should be 2′-fucosyllactulose or another related substance.

Response: yes, it is a mistake, it should be 2′-fucosyllactulose.

ii. Please clarify if the generic terms "difucose lactose" (Sections A.2.1 & A.2.3, p. 81) and "difucoyl lactose" (Sections A.2.5.1.2, p. 82) refer to 3,2′-difucosyl-D-lactose.

Response: yes, it refers to “Difucosyllactose”, or called “3,2′-difucosyl-D-lactose.”

iii. Please clarify the term “test solution” described in Sections A.1.5.1.3 (page 80) and A.2.5.1.3 (page 82), including whether the term refers to an extract of a sampled production lot of 2′-FL.

Response: The term "test solution" refers to the "test sample solution," which contains the sampled production lot of 2'FL to be tested. For the specific preparation method, refer to sections A.1.5.1.3 and A.2.5.1.3.

b. Appendix B:

i. The determination range for the protein analysis method is given as “≥17 mg/kg”. Please clarify the validated range of the analytical method.

Response: The LOD of the method is 17 mg/kg (17 ppm), and the validated range of the analytical method is 17mg/kg~167mg/kg.

ii. Please clarify the term “test solution” described in B.4.1. (page 84), including whether the term refers to an extract of a sampled production lot of 2′-FL.

Response: The term "test solution" refers to the "test sample solution”, which contains the sampled production lot of 2'-FL to be tested.

c. Appendix C: In Table C.1. (page 89), the retention times for 2′-FL and 2′-fucosyllactulose are given as 14.6 min and 10.6 min, respectively. If these retention times are correct, it appears that the “Figure C.3” label (page 88) is misidentified. Please confirm for the record.

Response: Yes, the retention times for 2′-FL and 2′-fucosyllactulose are given as 14.6 min and 10.6 min, respectively. The “Figure C.3” label is misidentified, and it shall be “Figure C.3 High performance liquid chromatogram of **2′- fucosyllactulose** reference standard”.

Specifications

FDA Query: 17. Given that the production organism is non-toxigenic and the 2′-FL is produced under controlled fermentation with food-grade materials, we would not expect the notifier to provide a specification for aflatoxin. Please comment on the source of aflatoxin. If there is no source of aflatoxin in the starting material, we suggest removal of the aflatoxin specifications (page 14, Table 2).

Response: There is no source of aflatoxin in the starting material and the production process. We agree to remove the aflatoxin specifications.

FDA Query: 18. The notifier does not include specifications for *Listeria monocytogenes*. Please provide a sample size for the absence of this microorganism, method used for detection, and confirmation that the method is validated for the sample size.

Response:

1. *Listeria monocytogenes* can be included, the specification is Absent/25 g, and the test method is GB 4789.30-2016 Method I. The method is validated for the sample size.
2. The test reports from three batches for *Listeria monocytogenes* conducted at Eurofins (Dalian,Liaoning, China) confirmed the absence/25 g sample.

FDA Query: 19. Please confirm the method for *Cronobacter sakazakii* is validated for the sample size indicated (100 g).

Response: Yes, according to the relevent Chinese national standard GB 4789.40-2016 Method I, the sample size is 100 g. The method is validated for the sample size of 100 g.

FDA Query: 20.We request that you consider reducing the specifications for arsenic and cadmium (each ≤0.1 mg/kg) to more closely reflect the results of batch analyses for these heavy metals (cadmium <limit of detection (LOD) 0.003 mg/kg; arsenic <LOD 0.01 mg/kg).

Response:

Synaura requests to maintain the limit of ≤0.1 mg/kg for both of arsenic and cadmium. The reasons are as follows,

1. For arsenic, the FCC Specifications stipulate a limit requirement of 0.2 ppm, and refer to several FDA GRAS notices (e.g., GRN.932, GRN.735, GRN.571), they are all ≤0.1 mg/kg or ≤0.2 mg/kg.

2. For cadmium, the FCC Specification does not set a limit requirement, and refer to GRN.571, it is ≤0.1 mg/kg. Meanwhile, we have also referred to the EU-authorised novel food. According to the approval notice for 2′-FL, the cadmium limit for 2′-FL produced using genetically modified strain of *Escherichia coli* BL-21 is 0.1 mg/kg (powder and liquid).

FDA Query: 21. For bacterial endotoxins, please compare the results of the batch analyses (page 15), where the results were stated as "not detected" and the limit of quantification (LOQ) is not stated, to the specified limit for endotoxins (Table 2, page 14). We generally suggest specifications be based on the results of the batch analyses for an ingredient produced in accordance with current good manufacturing practices (cGMPs).

Response: The detection method for bacterial endotoxins uses the Gel-Clot Method, as described in the Chinese Pharmacopoeia 2020, which is a non-quantitative detection method. The expression "not detected" used in our document has caused ambiguity; we request to revise the result expression to "Conforms to specifications (Conformed)".

FDA Query: 22. The specification for residual ethanol (≤1000 mg/kg) is higher than results of batch analyses (24.2-26.4 mg/kg). Please consider reducing this specification to more closely reflect results of batch analyses.

Response: Synaura requests to maintain the original limit since ethanol is a solvent used in the production process, and generally, ≤1000 mg/kg is considered a relatively low limit. At the same time, we have also referred to the EU- authorised novel foods. According to the authorization for 2′-FL, the quality specifications for 2′-FL produced using genetically modified strain of *Corynebacterium glutamicum* ATCC 13032 also set a limit for ethanol, which is required to be ≤1000 mg/kg.

FDA Query: 23. The notifier provides a specification of total impurities (Table 2, page 14) in the 2′- FL ingredient. Although this specification is under the heading of "related substances", it is not clear if the total impurities specification includes identified or unidentified substances. Please clarify the composition of "total impurities".

Response: The total impurities include D-lactose (retention time: 11.7 min), 2'-fucosyllactulose (10.6 min), Difucosyllactose (20.5 min), L-fucose (5.2 min), and D-glucose (8.0 min). No other impurities have been detected in this product apart from the aforementioned impurities, thus the total impurities are specified as these known impurities.

ESTIMATES OF DIETARY EXPOSURE

FDA Query: (24) If any of the intended uses (e.g., flavored or carbonated water, enhanced or fortified water) in GRN 001157 are outside the scope of uses included in GRN 000932, the discussion of dietary exposure (citing GRN 000932 estimates) should be modified accordingly.

Response: As indicated above (FDA query 3), the intended uses are not outside the scope of uses included in GRN 000932. The intended uses are in the same food categories and at same use levels to those described in GRN 000932.

SAFETY NARRATIVE

FDA Query: (25) On page 28, the notifier states, "In the available studies, healthy, term infants or toddlers were fed formula containing 0.25 to 3 g 2'-FL/L for 6 weeks to 6 months" but conclude that "available information suggest that HMOs are well tolerated in infants at levels up to 1 g/day". It is not clear how you derived your conclusion regarding the expected exposure (e.g., 1 g/day) for infants from concentration levels cited (e.g., 0.25 to 3 g/L). We also note that many of the cited references are studies with older children and/or adults (with or without various confounders); thus, they would not be relevant to support safety of 2'-FL as an ingredient in infant formula at levels above those previously concluded to be GRAS (e.g., 2.4 g/L). Please provide a statement(s) clarifying what data and information support the proposed use as an ingredient for infant formula and those that support safety of 2'-FL for use as an ingredient for conventional foods.

Response: Sorry for the confusion, the conclusion statement is general and is for HMOs at levels 1 g/day. Although, this was mentioned in a few GRAS notices (GRN 001015; GRN 001017), inadvertently we included in GRN 001157. As described in the GRAS notice the estimated maximum (90^{th} percentile) intake in infants (0-5 months) is determined as 3.17 g/person/day (0.53 g/kg bw/day). We apologies for the wrong conclusion. We agree that several of the cited studies are for older children and adults and may not be relevant to support safety of 2'-FL at higher levels in infant formula.

Similar to our GRAS notice (GRN 001157), in several recent GRAS notices (GRNs 000546, 000650, 000735, 000749, 000852, 000932), 2'-FL is intended to be used at levels up to 2.4 g/L. In some infant studies, 2'-FL is used at higher levels: Parschat et al. (2021) in which 2'-FL is used at 2.99 g/L; Leung et al. (2019) in which 2'-FL is used at 3 g/L. Additionally, as described in the GRAS notice, the maximum use level of 2'-FL of 2.4 g/L in term infant formulas is based on providing a similar level of 2'-FL as that which occurs in mature human breast milk, which may be up to 9 g/L. Furthermore, there are several animal and human studies (described in GRN 001157) that corroborate the safety of 2'-FL in infants at use levels of 2.4 g/L in infant formula. Also as described in the GRAS dossier, in studies in adults, the daily supplementation of up to 20 g 2'-FL is found to be safe and well-tolerated in adults. The GRAS determination for use of 2'-FL in infant formula and in conventional foods is based on the totality of the available evidence.

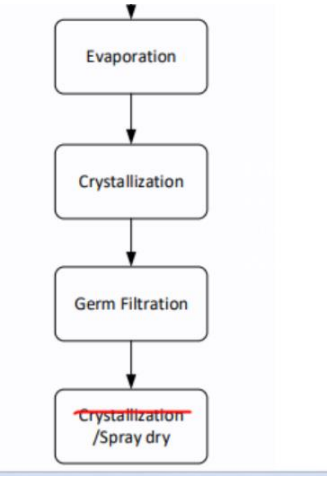
Appendix I

Update of the production process information_ B. Purification

1. What has been updated.

1) We has opted to declare only one process, eliminating the selective steps in the purification part; thus, we have removed the crystallization step from the last part of the process chart.

2) Due to discrepancies between the process description and the flowchart in Part B, the process description has been updated accordingly, as detailed below.



Here is the complete updated version of Part B, with the updated sections highlighted.

B. Purification

1) Production strain removal

The purpose of this step is to remove production strain from fermentation broth. For this aim, flocculation, centrifugation and membrane filtration are carried out. The filtrate is subjected for subsequent purification operations.

After this process, the production strains are removed. As a result, the final product doesn't contain viable producer strain.

2) Ultra-Filtration

The molecular weight of 2'-FL is 488.4. Based on this property, the permeate is obtained by ultrafiltration using a suitable ultrafiltration membrane. In this way, the macromolecules like protein, DNA, endotoxin and other substances, are removed, and the product is also clarified during this process.

3) Ion exchange

To remove anions and cations contained, the product is passed through an activated cation exchange resin and an anion exchange resin, respectively; first the cation column and then anion column.

Impurities such as salts, amino acids, and pigments can be removed in this step.

4) Decolorization

To take advantage of the strong adsorption capacity of activated carbon, the product is also treated with activated carbon for decolorization at room temperature.

This step can remove the pigment and other impurities in the product.

5） Evaporation

In this step, the liquid containing the product is concentrated to >30%, to remove the water by membrane evaporator.

6） Crystallization

In the Crystallization step, the product is concentrated to above 40% (w/v) at certain temperature, then ethanol is added dropwise with stirring until a white powder precipitates. The product is then cooled down to ambient temperature, and the product is filtered and dried. By doing this, the product purity is improved simultaneously and IPTG is removed by crystallization below the detection limit.

7） Germ filtration

In this step, the product is dissolved in water at concentration of ~40%, then is filtered through a sterilizing filter. It can remove foreign particles and reduce the bioburden as well. This step will be carried out before the final spray drying operation.

8） Spray dry

In this step, the germ filtrated liquid containing the product is dried by spraying under vacuum at certain temperature. The final product is sieved and packaged before shipping. By this step, the water is removed, and the final product is produced with desired particle size.

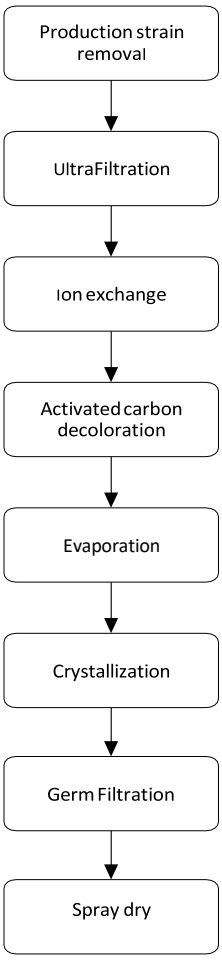


Figure 3. 2'-FL Purification process flow

From: 于艳艳
To: Anderson, Ellen
Cc: 姚斐(研发组); yyp@cirs-group.com
Subject: [EXTERNAL] Re:Follow-up questions for GRN 001157
Date: Wednesday, April 17, 2024 9:10:21 PM
Attachments: image001.png
Response on follow-up questions for GRN 001157_Final.pdf

Dear Ellen,

Please kindly find the attached document for the response on follow-up questions for GRN 001157.

If you have any question, please kindly let me know. Thank you.

All my best regards,

Wing Yu
Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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发件人："Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
发送日期：2024-04-10 23:17:28
收件人："于艳艳" <wing.yu@cirs-group.com>
抄送人："姚斐(研发组)" <yaofei@mengniu.cn>,"yyp@cirs-group.com" <yyp@cirs-group.com>
主题：Follow-up questions for GRN 001157

> Hello Wing,

Thank you for your patience as we completed our review of the amendment for GRN 001157 received on February 14, 2024 (attached for your reference). We have a few follow-up questions noted below:

1. In response to question 7, Synaura states that "IPTG [isopropyl β-D-1-thiogalactopyranoside] is removed by crystallization below the detection limit". Please state the method used to analyze for IPTG and the limit of detection for this method.

2. In response to question 7, Synaura states that "the product is concentrated to above 40% (w/v) at certain temperature, then ethanol is added dropwise with stirring until a white powder precipitates. The crystals are then filtered and dried." It is our understanding that residual ethanol is removed in the drying step, but this should be stated for the record. We request that you provide a statement describing removal of ethanol from the final product and indicate if the drying step uses heat and/or vacuum.

3. In response to question 9, Synaura notes that the second crystallization step is removed, and 2'-FL is produced by spray drying only. However, it is still unclear if the batch analyses were performed on 2'-FL produced in accordance with Appendix I (one crystallization step before germ filtration) or using two crystallization steps (with a second step after germ filtration). Please confirm that the batch analyses presented in the notice are for the batches that were manufactured using the crystallization step before germ filtration with no second crystallization step after germ filtration.

4. In response to Question 11, Synaura notes that the chemical shifts of key groups in the hydrogen spectrum are about 0.3 ppm different from those cited in Urashima et al. (1997). Please provide a brief explanation for this difference in the chemical shift. If you recorded the NMR spectra for their ingredient and the standard under the same conditions in your in-house analyses, please briefly describe this comparison.

5. In response to question 12, Synaura lists specifications for 2'-FL, including the specifications for minor components in the ingredient. Table 1, shown below, is an excerpt of the specifications included in the table provided by Synaura in response to question 12. We have additional clarifying questions for Synaura.

Table 1: Specifications for 2'-fucosyllactose and minor components in GRN 001157 and the Food Chemicals Codex (FCC 13th edition, 2023).

Parameter	**GRN 001157 Specification (w/w% anhydrous basis)**	**FCC Specifications cited by Synaura (w/w% anhydrous basis)**	**FCC (2023) specifications (w/w% anhydrous basis)**
2'-Fucosyllactose	≥ 94%	NLT 92%	Same as cited by Synaura, determined as sum of 2'-fucosyllactose + L-fucose + D-lactose + 3,2'-difucosyl-D-lactose
D-Lactose	≤ 3.0%	NMT 8.0% calculated as 2'-fucosyllactose	Same as cited by Synaura
2'-Fucosyl-D-lactulose	≤ 1.0%	NMT 2.0% calculated as 2'-fucosyllactose	Same as cited by Synaura
Difucosyllactose	≤ 1.0%	NA	NMT 7.0% 3,2'-difucosyl-D-lactose calculated as 2'-fucosyllactose
L-Fucose	≤ 1.0%	NMT 3.0% calculated as 2'-fucosyllactose	Same as cited by Synaura
D-Glucose	≤ 1.0%	NA	--
Total impurities	≤ 5%	NA	--

a. In responses 12b and 16ii, Synaura confirms that difucosyllactose is synonymous with 3,2′-difucosyl-D-lactose. However, the FCC specification is reported by Synaura as "NA" when it should be "NMT 7.0% 3,2′-difucosyl-D-lactose calculated as 2′-fucosyllactose on the anhydrous basis." Please correct the cited FCC specification accordingly.

b. Please confirm, based on your response to question 23, that the specification for total impurities of ≤5% (anhydrous basis) refers to ≤5% (anhydrous basis) of the ingredient calculated as the sum of L-fucose + D-lactose + 3,2′-difucosyl-D-lactose + 2′-fucosyl-D-lactulose + D-glucose.

c. Please clarify if the specification for 2′-FL is for 2′-FL only or for the sum of 2′-FL and the minor components (i.e., 2′-fucosyllactose + L-fucose + D-lactose + 3,2′-difucosyl-D-lactose) as indicated in the FCC monograph. If the former, given the specification of ≥94%, please clarify if the specification for total impurities should be ≤6% instead of ≤5%.

d. In response to question 17, Synaura states that the aflatoxin specifications are removed. However, these specifications are included in the specifications table provided in response question 14. Please confirm the aflatoxin specifications are no longer included for 2′-FL.

We would appreciate receiving a response at your earliest convenience. Please contact me if you have any questions about this request.

Sincerely,

Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

From: 于艳艳 <wing.yu@cirs-group.com>
Sent: Wednesday, February 14, 2024 11:19 PM
To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Cc: 'yaofei@mengniu.cn' <yaofei@mengniu.cn>; 余瑶盼 <yyp@cirs-group.com>
Subject: [EXTERNAL] Re:Re:GRN 001157

Dear Ellen,

Please kindly find the attached document for the responses on the questions regarding GRN 001157.

If you have any question, please kindly let me know. Thank you.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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From: "于艳艳" <wing.yu@cirs-group.com>
Date: 2024-02-04 17:27:39
To: "Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
Cc: "'yaofei@mengniu.cn'" <yaofei@mengniu.cn>,"余瑶盼" <yyp@cirs-group.com>
Subject: Re:GRN 001157

Dear Ellen,

Thank you for your prompt response and the assessment feedback regarding our substance. We will address the issues raised as quickly as possible.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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发件人："Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
发送日期：2024-02-01 04:20:46
收件人："'yaofei@mengniu.cn'" <yaofei@mengniu.cn>
抄送人："'wing.yu@cirs-group.com'" <wing.yu@cirs-group.com>
主题：GRN 001157

Dear Fei,

Our review of GRN 001157 is complete. We respectfully ask you to respond to the questions listed in the attached document. Please do not hesitate to contact me if you have any questions about our request.

Sincerely,

Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

Dear Dr. Anderson,

RE: Follow-up questions for GRN 001157

This responds to your email of April 10, 2024, regarding your follow-up questions that need to be addressed for the amendment for GRN 001157 submitted by Synaura Biotechnology (Shanghai) Co., Ltd. We are providing a point-by-point response to all of your questions along with some additional relevant clarifications/discussion.

1. In response to question 7, Synaura states that "IPTG [isopropyl β-D-1-thiogalactopyranoside] is removed by crystallization below the detection limit". Please state the method used to analyze for IPTG and the limit of detection for this method.

Response: We have established an IPTG detection method based on HPLC-MS/MS. The instrument used is the LCMS-8050, with an Acquity UPLC HSS T3 chromatography column. The mobile phase consists of 0.1% formic acid in water with 5mM ammonium acetate solution and methanol in a 95:5 (V/V) ratio, at a flow rate of 0.4 mL/min and an injection volume of 10 µL. Mass spectrometry was performed in the positive ESI mode using multiple reaction monitoring (MRM), with the precursor ion selected at m/z 256.1 and the quantifier ion at m/z 163.1. The detection limit of the IPTG standard solution was 0.2 ng/mL, and the quantification limit was 0.5 ng/mL. For three batches of 2'-FL samples at a concentration of 1 mg/mL, the quantification limit for IPTG translates to 0.5 ng/mg, equivalent to 0.5 ppm. IPTG was not detected in any of the three batches of samples.

2. In response to question 7, Synaura states that "the product is concentrated to above 40% (w/v) at certain temperature, then ethanol is added dropwise with stirring until a white powder precipitates. The crystals are then filtered and dried." It is our understanding that residual ethanol is removed in the drying step, but this should be stated for the record. We request that you provide a statement describing removal of ethanol from the final product and indicate if the drying step uses heat and/or vacuum.

Response: We confirm the drying step uses heat (40℃~50℃) and vacuum (-0.09~-0.10MPa). Please see the statement regarding the removal of ethanol from the final product as attached in Appendix A.

3. In response to question 9, Synaura notes that the second crystallization step is removed, and 2'-FL is produced by spray drying only. However, it is still unclear if the batch analyses were performed on 2′-FL produced in accordance with Appendix I (one crystallization step before germ filtration) or using two crystallization steps (with a second step after germ filtration). Please confirm that the batch analyses presented in the notice are for the batches that were manufactured using the crystallization step before germ filtration with no second crystallization step after germ filtration.

Response: Yes, Synaura confirms that the batch analyses presented in the notice are for the batches that were manufactured using the crystallization step before germ filtration with no second crystallization step after germ filtration the batch analyses presented in the notice are for the batches that were manufactured using the crystallization step before germ filtration with no second crystallization step after germ filtration.

4. In response to Question 11, Synaura notes that the chemical shifts of key groups in the hydrogen spectrum are about 0.3 ppm different from those cited in Urashima et al. (1997). Please provide a brief explanation for this difference in the chemical shift. If you recorded the NMR spectra for their ingredient and the standard under the same conditions in your in-house analyses, please briefly describe this comparison.

Response: The sample was dissolved in DMSO, whereas in the literature it was dissolved in water. The resulting chemical shift is due to the solvent effect. In a recent publication Chavelas-Hernández et al. (2020) reported that 1H NMR chemical shifts of organic molecules can vary depending on the solvent used. When a compound is dissolved in an anisotropic solvent such as benzene, the solvent causes NMR signals to shift upfield in comparison with their shifts in an isotropic solvent such as tetrachloromethane. We are also attaching in-house NMR spectra in Appendix B.

Reference: Chavelas-Hernández et al., 2020. A New Approach Using Aromatic-Solvent-Induced Shifts in NMR Spectroscopy to Analyze β-Lactams with Various Substitution Patterns. Synlett 2020; 31(02): 158-164 DOI: 10.1055/s-0039-1691498

5. In response to question 12, Synaura lists specifications for 2′-FL, including the specifications for minor components in the ingredient. Table 1, shown below, is an excerpt of the specifications included in the table provided by Synaura in response to question 12. We have additional clarifying questions for Synaura.

Response: In response to query 12, we included the specification retrieved (Feb 12, 2024) from the search of USP database. As we were not sure of the volume and year from the print out, we mentioned recent edition. As described below, we are sorry for the oversight for some parameters.

Table 1: Specifications for 2′-fucosyllactose and minor components in GRN 001157 and the Food Chemicals Codex (FCC 13th edition, 2023).

Parameter	GRN 001157 Specification (w/w% anhydrous basis)	FCC Specifications cited by Synaura (w/w% anhydrous basis)	FCC (2023) specifications (w/w% anhydrous basis)
2′-Fucosyllactose	≥ 94%	NLT 92%	Same as cited by Synaura, determined as sum of 2'-fucosyllactose + L-fucose + D-lactose + 3,2'-difucosyl-D-lactose
D-Lactose	≤ 3.0%	NMT 8.0% calculated as 2′-fucosyllactose	Same as cited by Synaura
2′-Fucosyl-D-lactulose	≤ 1.0%	NMT 2.0% calculated as 2′-fucosyllactose	Same as cited by Synaura
Difucosyllactose	≤ 1.0%	NA	NMT 7.0% 3,2′-difucosyl-D-lactose calculated as 2′-fucosyllactose
L-Fucose	≤ 1.0%	NMT 3.0% calculated as 2′-fucosyllactose	Same as cited by Synaura
D-Glucose	≤ 1.0%	NA	--
Total impurities	≤ 5%	NA	--

a. In responses 12b and 16ii, Synaura confirms that difucosyllactose is synonymous with 3,2′-difucosyl-D-lactose. However, the FCC specification is reported by Synaura as "NA" when it should be "NMT 7.0% 3,2′-difucosyl-D-lactose calculated as 2′-fucosyllactose on the anhydrous basis." Please correct the cited FCC specification accordingly.

Response: Thank you for bringing this to our attention, we are sorry for the oversight. It should have been NMT 7.0% 3,2′-difucosyl-D-lactose calculated as 2′-fucosyllactose on the anhydrous basis.

b. Please confirm, based on your response to question 23, that the specification for total impurities of ≤5% (anhydrous basis) refers to ≤5% (anhydrous basis) of the ingredient calculated as the sum of L-fucose + D-lactose + 3,2′-difucosyl-D-lactose + 2′-fucosyl-D-lactulose + D-glucose.

Response: We confirm the 5% total impurities refer to refers to ≤5% of the ingredient calculated as the sum of L-fucose + D-lactose + 3,2′-difucosyl-D-lactose + 2′-fucosyl-D-lactulose + D-glucose. We are sorry for not making this clear in our earlier response.

c. Please clarify if the specification for 2′-FL is for 2′-FL only or for the sum of 2′-FL and the minor components (i.e., 2′-fucosyllactose + L-fucose + D-lactose + 3,2′-difucosyl-D-lactose) as indicated in the FCC monograph. If the former, given the specification of ≥94%, please clarify if the specification for total impurities should be ≤6% instead of ≤5%.

Response: The specification for 2′-FL is for 2′-FL only. And the limit for total impurities is set at ≤5% in order to strictly control the quantity of impurities. We accept to set this to 6%.

d. In response to question 17, Synaura states that the aflatoxin specifications are removed. However, these specifications are included in the specifications table provided in response question 14. Please confirm the aflatoxin specifications are no longer included for 2′-FL.

Response: Sorry for our oversight. We confirm that the aflatoxin specifications are no longer included for 2'-FL.

Appendix A- statement regarding the removal of ethanol from the final product

Statement Regarding the Removal of Ethanol from the Final Product

To Whom It May Concern :

In compliance with the request for detailed clarification on the removal of ethanol from our final product of 2' -fucosyllactose (2' -FL), we hereby provide the following statement:

During the manufacturing process, after the crystallization step where ethanol is used, the crystals are then filtered and dried. This drying step uses heat and vacuum, which ensure the remove of residual ethanol from the crystals. The residual ethanol is reduced to levels compliant with the established specifications for the final product.

Fei Yao

Application R&D Director

Synaura Biotechnology (Shanghai) Co., Ltd.

Signature:_____

Appendix B - NMR spectra data In-house

The ^{1}H chemical shifts for biosynthetic 2'-FL named P1~P3 and 2'-FL (H1) isolated from human milk are shown in Table B-1 and Fig.1-4. The ^{1}H chemical shifts of biosynthetic ones were essentially identical with that of isolated one. The H-1 signals of alpha and beta anomers of reducing glucose are found at δ 4.90 and δ 4.33, which were exactly the same between biosynthetic P1~P3 and isolated H1. The resonances at δ 5.01 and δ 4.32 have to be assigned to the H-1 protons of fucose α1-2 and galactose β1-4 residues, respectively. The chemical shifts of H-1 for fucose and galactose were also exactly the same. The characteristic resonances of H-5 and H-6 protons of fucose α 1-2 residue were δ 4.06 and δ 1.05 for isolated one, while δ 4.01 and δ 1.04 for biosynthetic ones. These characteristic resonances were basically the same. Therefore, biosynthetic P1~P3 was identified as Fuc α 1-2Ga1 β 1-4 G1c.

Table B-1 Information on the 1H NMR Spectra of Four Samples

1H	Sample_H1		P1		P2		P3	
	β-isomer	α-isomer	β-isomer	α-isomer	β-isomer	α-isomer	β-isomer	α-isomer
a-1	4.33 (m, *overlap*, 1H)	4.90 (t, *J* =4.0 Hz, 1H)	4.33 (m, *overlap*, 1H)	4.90 (t, *J* =4.0 Hz, 1H)	4.33 (m, *overlap*, 1H)	4.90 (t, *J* =4.0 Hz, 1H)	4.33 (m, *overlap*, 1H)	4.90 (t, *J* =4.0 Hz, 1H)
a-2	2.96 (dt, *J* =8.4, 4.7 Hz, 1H)	3.17 (m, *overlap*, 1H)	2.96 (dt, *J* =8.4, 4.7 Hz, 1H)	3.17 (m, *overlap*, 1H)	2.96 (dt, *J* =8.4, 4.7 Hz, 1H)	3.17 (m, *overlap*, 1H)	2.96 (dt, *J* =8.4, 4.7 Hz, 1H)	3.17 (m, *overlap*, 1H)
a-3	3.25 (dt, *J* =8.8, 2.2 Hz, 1H)	3.54 (m, *overlap*, 1H)	3.25 (dt, *J* =8.8, 2.2 Hz, 1H)	3.54 (m, *overlap*, 1H)	3.25 (dt, *J* =8.8, 2.2 Hz, 1H)	3.54 (m, *overlap*, 1H)	3.25 (dt, *J* =8.8, 2.2 Hz, 1H)	3.54 (m, *overlap*, 1H)
a-4	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)
a-5	3.17 (m, 1H)	3.65 (m, 1H)	3.17 (m, 1H)	3.65 (m, 1H)	3.17 (m, 1H)	3.65 (m, 1H)	3.17 (m, 1H)	3.65 (m, 1H)
a-6	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)	3.64 (m, *overlap*, 1H)
b-1	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)
b-2	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)
b-3	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)
b-4	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)	3.65 (m, *overlap*, 1H)
b-5	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)
b-6	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)	3.54 (m, *overlap*, 2H)
c-1	5.01 (d, *J* = 2.2 Hz, 1H)	5.01 (d, *J* = 2.2 Hz, 1H)	5.02 (d, *J* = 2.2 Hz, 1H)	5.02 (d, *J* =2.2 Hz, 1H)	5.02 (d, *J* = 2.2 Hz, 1H)	5.02 (d, *J* =2.2 Hz, 1H)	5.02 (d, *J* = 2.2 Hz, 1H)	5.02 (d, *J* =2.2 Hz, 1H)
c-2	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)
c-3	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)	3.54 (m, *overlap*, 1H)
c-4	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)	3.45 (m, *overlap*, 1H)
c-5	4.06 (p, *J* = 3.3 Hz, 1H)	4.06 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)	4.01 (p, *J* = 3.3 Hz, 1H)
c-6	1.05 (d, *J* = 6.4 Hz, 3H)	1.05 (d, *J* = 6.4 Hz, 3H)	1.05 (d, *J* = 6.4 Hz, 3H)	1.04 (d, *J* = 6.4 Hz, 3H)	1.05 (d, *J* = 6.4 Hz, 3H)	1.04 (d, *J* = 6.4 Hz, 3H)	1.05 (d, *J* = 6.4 Hz, 3H)	1.04 (d, *J* = 6.4 Hz, 3H)
a-1-O*H*	6.67 (d, *J* = 6.4 Hz, 1H)	6.32 (d, *J* = 6.4 Hz, 1H)	6.66 (d, *J* = 6.5 Hz, 1H)	6.31 (d, *J* = 4.7 Hz, 1H)	6.66 (d, *J* = 6.5 Hz, 1H)	6.31 (d, *J* = 4.7 Hz, 1H)	6.66 (d, *J* = 6.5 Hz, 1H)	6.31 (d, *J* = 4.7 Hz, 1H)
a-2-O*H*	4.97 (d, *J* = 5.0 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.95 (d, *J* = 5.0 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.95 (d, *J* = 5.0 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.95 (d, *J* = 5.0 Hz, 1H)	4.64 (m, *overlap*, 1H)
a-3-O*H*	4.25 (d, *J* = 2.0 Hz, 1H)	4.25 (d, *J* = 2.0 Hz, 1H)	4.24 (d, *J* = 2.0 Hz, 1H)	4.05 (d, *J* = 1.6 Hz, 1H)	4.24 (d, *J* = 2.0 Hz, 1H)	4.05 (d, *J* = 1.6 Hz, 1H)	4.24 (d, *J* = 2.0 Hz, 1H)	4.05 (d, *J* = 1.6 Hz, 1H)
a-6-O*H*	4.64 (m, *overlap*, 1H)	4.57 (t, *J* = 5.6 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.56 (t, *J* = 5.6 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.56 (t, *J* = 5.6 Hz, 1H)	4.64 (m, *overlap*, 1H)	4.56 (t, *J* = 5.6 Hz, 1H)
b-3-O*H*	5.23 (d, *J* = 3.6 Hz, 1H)	5.23 (d, *J* = 3.6 Hz, 1H)	5.20 (d, *J* = 3.6 Hz, 1H)	5.23 (d, *J* = 3.6 Hz, 1H)	5.20 (d, *J* = 3.6 Hz, 1H)	5.23 (d, *J* = 3.6 Hz, 1H)	5.20 (d, *J* = 3.6 Hz, 1H)	5.23 (d, *J* = 3.6 Hz, 1H)

b-4-O*H*	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)
b-6-O*H*	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)	4.64 (m, *overlap*, 1H)
c-2-O*H*	4.80 (d, *J* = 5.6 Hz, 1H)	4.76 (d, *J* = 5.6 Hz, 1H)	4.80 (d, *J* = 5.6 Hz, 1H)	4.76 (d, *J* = 5.6 Hz, 1H)	4.80 (d, *J* = 5.6 Hz, 1H)	4.76 (d, *J* = 5.6 Hz, 1H)	4.80 (d, *J* = 5.6 Hz, 1H)	4.76 (d, *J* = 5.6 Hz, 1H)
c-3-O*H*	4.49 (d, *J* = 4.8 Hz, 1H)	4.49 (d, *J* = 4.8 Hz, 1H)	4.46 (d, *J* = 4.8 Hz, 1H)	4.49 (d, *J* = 4.8 Hz, 1H)	4.46 (d, *J* = 4.8 Hz, 1H)	4.49 (d, *J* = 4.8 Hz, 1H)	4.46 (d, *J* = 4.8 Hz, 1H)	4.49 (d, *J* = 4.8 Hz, 1H)
c-4-O*H*	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)	4.32 (m, *overlap*, 1H)

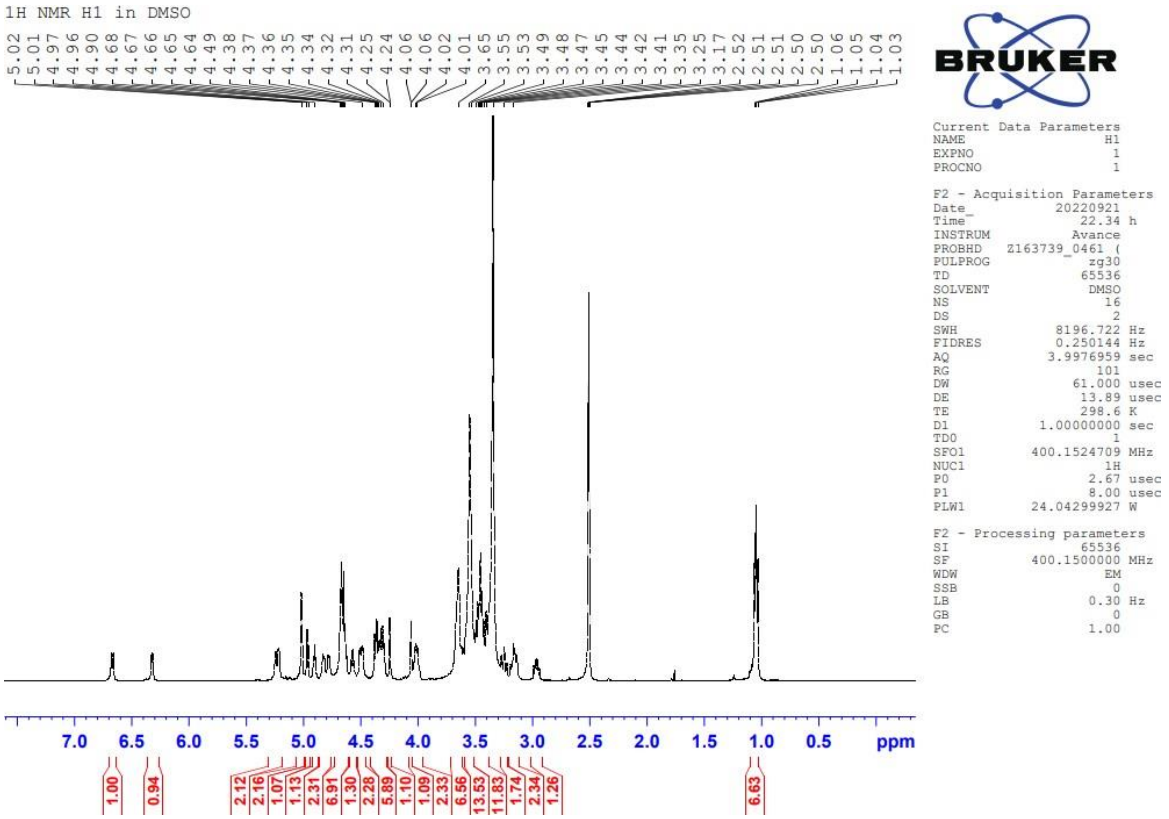

Fig 1 1H-NMR Spectra of H1

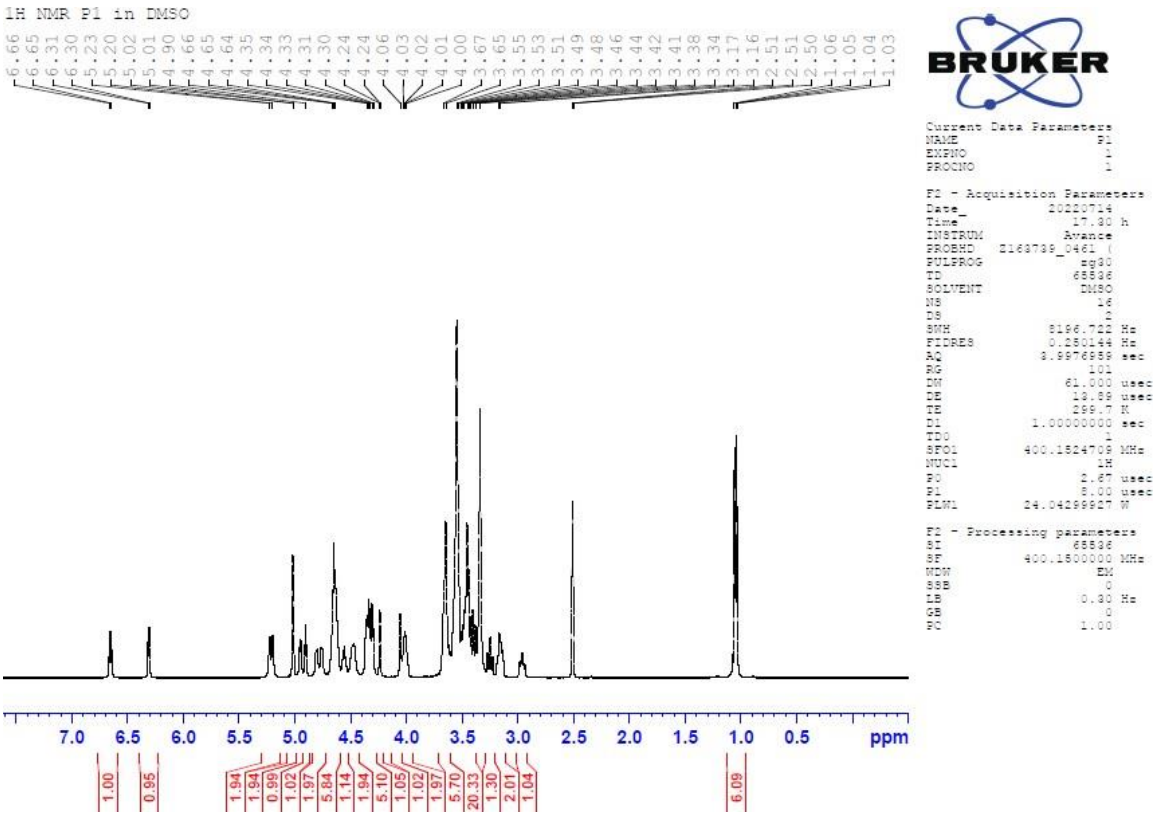

Fig 2 1H-NMR Spectra of P1

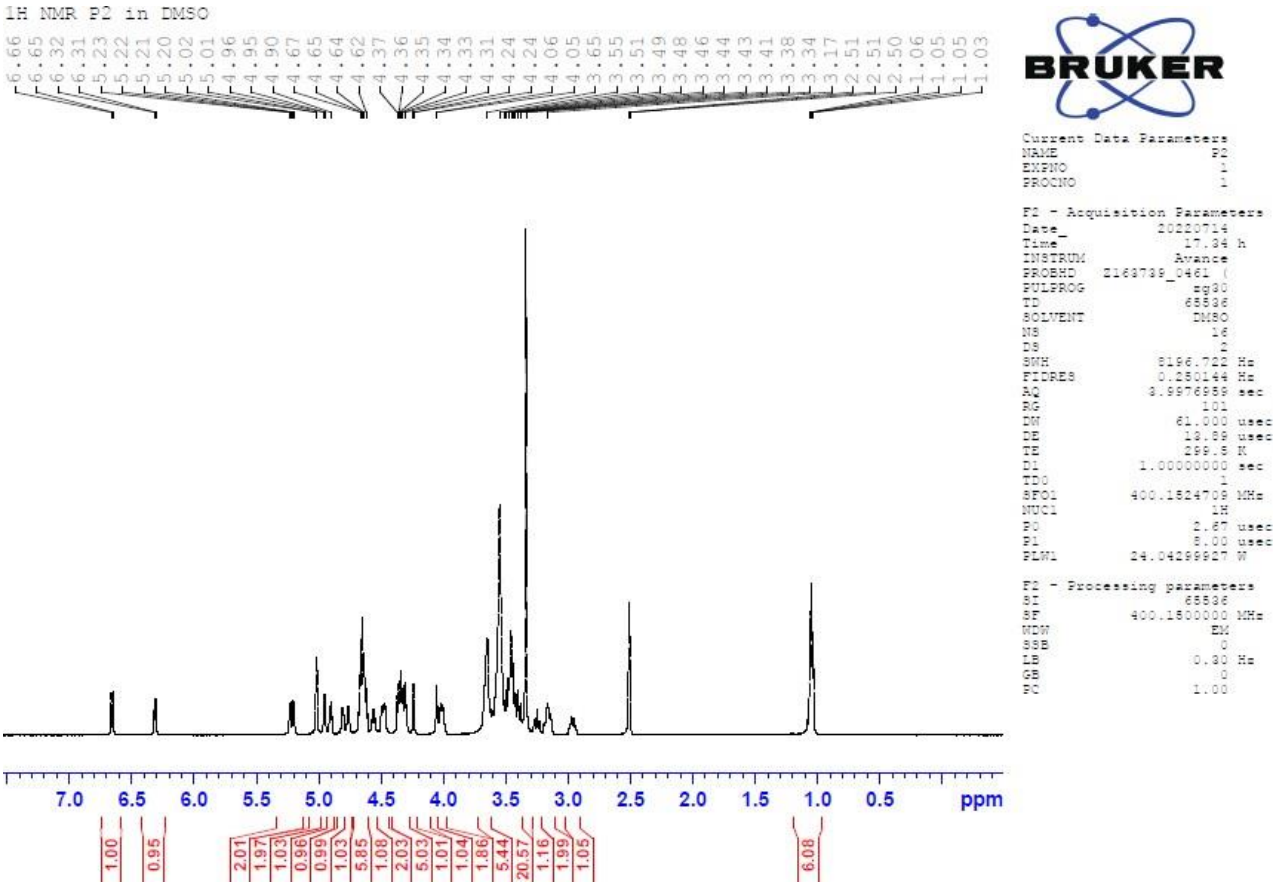

Fig 3 1H-NMR Spectra of P2

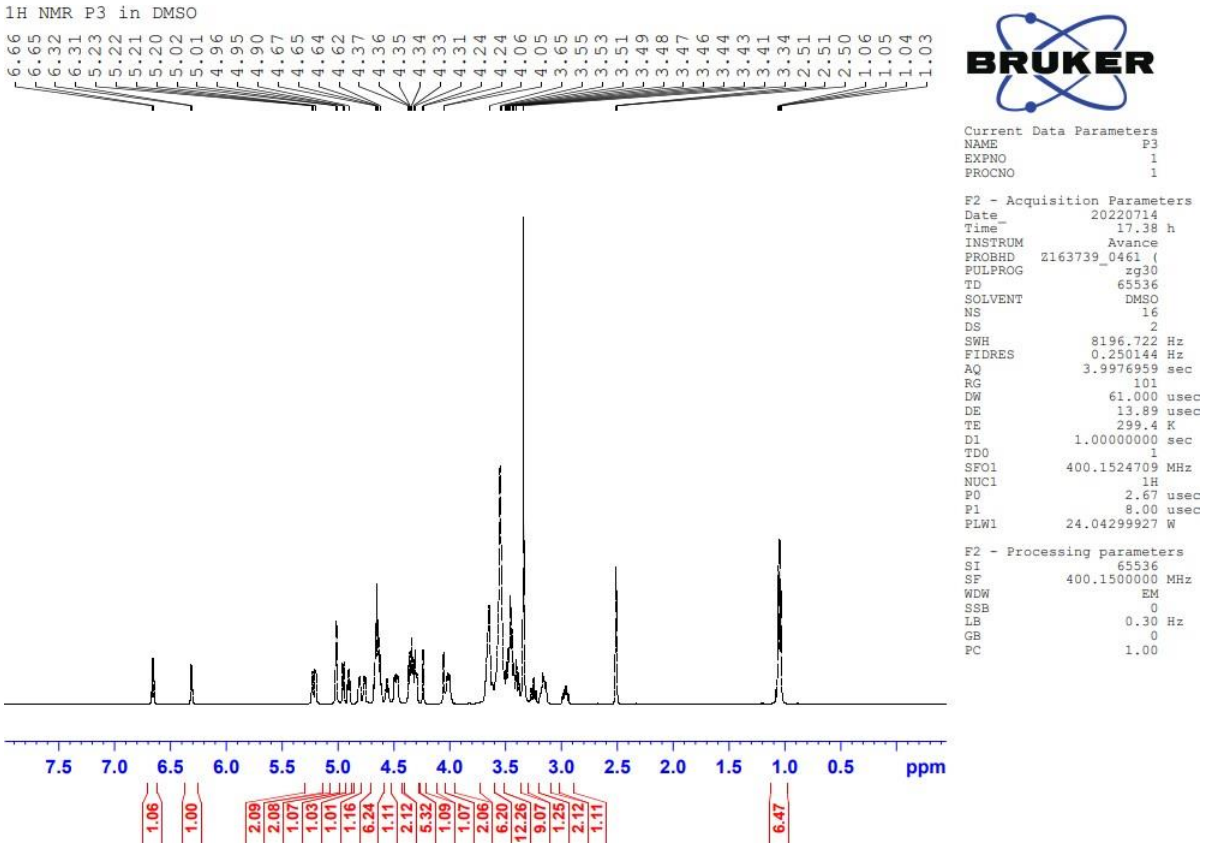

Fig 4 1H-NMR Spectra of P3

From: 于艳艳
To: Anderson, Ellen
Cc: 余瑶盼
Subject: [EXTERNAL] Re:Clarification request for GRN 001157
Date: Monday, July 1, 2024 1:28:00 AM
Attachments: image001.png
Response on clarification request for GRN 001157.pdf

Dear Ellen,

Please kindly find our response in the attached document. Thank you.

All my best regards,

Wing Yu
Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

Email wing.yu@cirs-group.com
Tel +86-0571-89716570
Fax +86-0571-87206533
Add. 11F Building 1, Dongguan Hi-Tech Park, 288 Qiuyi Road, Binjiang District, Hangzhou 310052, China
Web www.cirs-group.com

发件人："Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
发送日期：2024-06-27 10:31:46
收件人："'于艳艳'" <wing.yu@cirs-group.com>
抄送人："余瑶盼" <yyp@cirs-group.com>
主题：Clarification request for GRN 001157

> Dear Wing,

Please see the attached letter that explains our request for clarification.

Sincerely,

Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

From: 于艳艳 <wing.yu@cirs-group.com>
Sent: Wednesday, June 26, 2024 8:52 PM
To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Cc: 余瑶盼 <yyp@cirs-group.com>
Subject: Re:RE: [EXTERNAL] Re:Inquiry on the Status of GRN 001157

Dear Ellen,

Thank you for the update. We will wait for your information.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

Email wing.yu@cirs-group.com
Tel +86-0571-89716570
Fax +86-0571-87206533
Add. 11F Building 1, Dongguan Hi-Tech Park, 288 Qiuyi Road, Binjiang District, Hangzhou 310052, China
Web www.cirs-group.com

发件人："Anderson, Ellen" <Ellen.Anderson@fda.hhs.gov>
发送日期：2024-06-26 23:34:54
收件人："'于艳艳'" <wing.yu@cirs-group.com>
抄送人："余瑶盼" <yyp@cirs-group.com>
主题：RE: [EXTERNAL] Re:Inquiry on the Status of GRN 001157

> Hello Wing,
>
> My apologies for the delayed reply. I've been told the review team may have one more clarifying question to ask about the notice. I will try to get that information to you as soon as possible.

Sincerely,

Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

From: 于艳艳 <wing.yu@cirs-group.com>

Sent: Monday, June 17, 2024 8:00 AM

To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>

Cc: 余瑶盼 <yyp@cirs-group.com>

Subject: [EXTERNAL] Re:Inquiry on the Status of GRN 001157

Dear Ellen,

I hope this message finds you well.

Following up on my previous email, I'm reaching out to kindly request any new information

on the GRN 001157 project. Your brief update would be highly appreciated.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

Email wing.yu@cirs-group.com
Tel +86-0571-89716570
Fax +86-0571-87206533
Add. 11F Building 1, Dongguan Hi-Tech Park, 288 Qiuyi Road, Binjiang District, Hangzhou 310052, China
Web www.cirs-group.com

发件人：**"于艳艳"** <wing.yu@cirs-group.com>
发送日期：2024-06-04 11:40:18
收件人：Ellen.Anderson@fda.hhs.gov
抄送人：**"余瑶盼"** <yyp@cirs-group.com>
主题：Inquiry on the Status of GRN 001157

Dear Ellen,

I hope this message finds you well.

I am writing to kindly inquire about the current status of GRN 001157. Could

we expect the FDA's no question letter in the near future?

We would greatly appreciate any updates you could provide. Thank you in advance for your attention to this matter and looking forward to your prompt response.

All my best regards,

Wing Yu

Food Division | Technical Manager

Hangzhou REACH Technology Group Co, Ltd

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Add. 11F Building 1, Dongguan Hi-Tech Park, 288 Qiuyi Road, Binjiang District, Hangzhou 310052, China
Web www.cirs-group.com

Dear Dr. Anderson,

RE: Clarification request for GRN 001157

This is in response to your letter dated June 26, 2024 in which you "**… request that you provide a clear indication of your intended uses and whether they are 1) consistent with the uses for GRN 000932 summarized in Table 1 below with associated estimates of dietary exposure provided in the GRN 000932 response letter and cited in the original text of GRN 001157 on pages 21 – 22, or 2) consistent with the uses described in GRN 001051 (summarized as cumulative uses in Table 1 below) with associated estimates of dietary exposure presented in GRN 001051 "no questions" response letter.**"

Thank you for seeking this clarification. To clarify, we hereby affirm that our intended uses described in GRN 001157 are consistent with the uses for GRN 000932 summarized in Table 1 in your letter with associated estimates of dietary exposure provided in the GRN 000932 response letter and cited in the original text of GRN 001157 on pages 21-22.

We trust that this satisfies your query.

U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR FOOD SAFETY & APPLIED NUTRITION

June 26, 2024

Fei Yao
Synaura Biotechnology (Shanghai) Co., Ltd.
Floor l-2, Building 2, Lane 500,
Furong Hua Road, Pudong New Area
Shanghai, CHINA

Re: GRAS Notice No. GRN 001157

Dear Fei Yao:

As we prepare our response to Synaura's GRAS Notice GRN 001157 for the intended use of 2′-fucosyllactose (2′-FL), we noted an issue that needs clarification. Our question pertains to the amendment submitted February 14, 2024, specifically Synaura's response to Question 3. In the February 14, 2024 amendment, Synaura cited the December 3, 2020 amendment to GRN 000932 and specifically incorporated into GRN 001157 the foods in Table 2.1 and the revised dietary exposure from that amendment. FDA notes that, in GRN 000932, we asked the notifier (Advanced Protein Technologies Corp. (APTech)) to address the uses in GRN 000932 in the context of the total estimated dietary exposure to 2′-FL. In response, APTech listed four food categories that it noted were not included in GRN 000932 but would be expected to contribute to the overall cumulative dietary exposure to 2′-FL: soft drinks, processed vegetable juices, meal replacement drinks for weight reduction, and tube feeding formulas.[1] Although the notifier for GRN 000932 included these uses in a "cumulative"[2] estimate of dietary exposure, these uses were not included in the intended uses described in GRN 000932 and were not included in the response letter for GRN 000932.

Synaura has stated multiple times in GRN 001157 and in the February 14, 2024, amendment that its intended uses are substitutional for those of GRN 000932. If it is Synaura's intent to include uses that match those in GRN 000932, the use levels and specified food categories for GRN 000932 would not include the additional uses (soft drinks, processed vegetable juices, meal replacement drinks for weight reduction, and tube feeding formulas) noted in the February 14, 2024, amendment to GRN 001157. As shown in Table 1 below, the GRN 000932 uses include many of the uses described in

[1] FDA notes that the use level for 2′-FL cited in GRN 000932 for soft drinks was included in GRN 000815, which is a product that is a combination of 2'-FL and difucosyllactose. The maximum level for 2′-FL in soft drinks is generally given as 1.2 g/L.
[2] We note a few categories (e.g., gluten free bread, certain non-dessert baby foods, coffee and tea, sugar substitutes) were not included in the GRN 000932 "cumulative" estimate. For a discussion of cumulative dietary exposure estimates to 2′-FL that include previous GRAS notices submitted to FDA, we refer you to the response letter for GRN 001051 dated November 21, 2023.

U.S. Food and Drug Administration
Center for Food Safety & Applied Nutrition
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College Park, MD 20740
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GRN 001051, although certain food categories are not included (see footnote 2) and several of the maximum use levels indicated in GRN 000932 are below those of the cumulative uses summarized in GRN 001051.[3] **We request that you provide a clear indication of your intended uses and whether they are 1) consistent with the uses for GRN 000932 summarized in Table 1 below with associated estimates of dietary exposure provided in the GRN 000932 response letter and cited in the original text of GRN 001157 on pages 21-22, or 2) consistent with the uses described in GRN 001051 (summarized as cumulative uses in Table 1 below) with associated estimates of dietary exposure presented in the GRN 001051 "no questions" response letter**.[4]

Table 1: Intended food categories and maximum use levels (g/kg or g/L) for 2′-FL

Food Categories	Use levels[5] GRN 000932 (g/kg or g/L)	Cumulative use levels GRN 001051 (g/kg or g/L)
Breads and baked goods, gluten-free	--	48
Carbonated beverages	--	1.2
Enhanced or fortified waters[6]	0.8	1.2
Sports, isotonic, and "energy" drinks	0.8	6
Hot breakfast cereals, prepared	4.8	31
Ready-to-eat (RTE) cereals, puffed	80	80
RTE cereals, high fiber	30	40
RTE cereals, biscuit-type	20	40
Coffee and tea[7]	--	10
Milk-based coffee drinks	1.2	--
Imitation milks	1.2	1.2
Beverage whiteners (powdered)	--	600
Beverage whiteners (liquid)	--	80
Non-dairy yogurt	--	12
Frozen dairy-based desserts	17	17
Puddings, custards, and mousses	17	17
Fruit pie filling	14.1	14.1
Fruit filling in bars, cookies, yogurt, cakes	30	30

[3]The cumulative uses in GRN 001051 are summarized in the September 23, 2023, and November 15, 2023, amendments to GRN 001051 and in FDA's "no questions" letter responding to GRN 001051.
[4] Estimates of dietary exposure are provided in the September 22, 2023 amendment to GRN 001051.
[5] Synaura states that its intended uses are substitutional for GRNs 000735 and 000932.
[6] GRN 000932 lists fitness waters among the intended uses. Per WWEIA classification for NHANES 2017-2018, products such as Propel "fitness" water fall within the category of "enhanced or fortified waters." Citation: U.S. Department of Agriculture, Agricultural Research Service. 2016. What We Eat in America Food Categories 2013-2014. Available: www.ars.usda.gov/nea/bhnrc/fsrg.
To access the files linking NHANES food codes linked WWEIA food categories on the USDA/ARS Food Surveys Research Group web page, under the "Files" Tab, choose years of interest from: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/dmr-food-categories/.
[7] The category of coffee and tea includes ready-to-drink (e.g., bottled, flavored, pre-sweetened) coffee and tea and powder mixes used to prepare coffee and tea. For estimates of dietary exposure, it is assumed that the intended uses of 2′-FL do not include use in plain brewed coffee or tea.

Food Categories	Use levels[5] GRN 000932 (g/kg or g/L)	Cumulative use levels GRN 001051 (g/kg or g/L)
Non-exempt infant formula for term infants[8]	2.4	2.4
Formula intended for young children (>12 months-3 years)	2.4	2.4
Hot cereals for infants and young children, prepared (from dry instant) and ready-to-serve	10.9	12
Baby foods for infants and young children: yogurt, fruits, vegetables, toddler meals	--	12
Baby food desserts including fruit desserts, cobblers, yogurt/fruit combinations	10.9	12
Drinks for infants and young children: juice and yogurt drinks	10	10
Baby snacks: crackers, pretzels, cookies, and other dry snack items	57	57
Jams, jellies, preserves, and fruit butters	60	60
Cereal bars including snack, granola, and breakfast bars	12	30
Meal replacement bars, general use	12	30
Meal replacement bars, for weight management	12	40
Milk-based meal replacement drinks for general use (including nutritional drinks, smoothies)	1.2	5
Non-milk-based meal replacement drinks for general use (including nutritional drinks, smoothies)	--	5
Milk-based meal replacement drinks for weight management	1.2	12
Non-milk-based meal replacement drinks for weight management	--	12
Milk-based meal replacement beverages for children (e.g., pediatric nutritional drinks)	2.4	12
Unflavored pasteurized and sterilized milk	1.2	1.2
Buttermilk	--	1.2
Flavored and fermented milks	1.2	1.2
Yogurt	5.3	12
Fruit juices and nectars	1.2	1.2
Fruit-flavored drinks and ades	1.2	1.2
Vegetable juices and nectars	--	1.2
Sugar substitutes: table-top sweeteners	--	300
Syrups used to flavor milk beverages	7	7
Nutritional drinks for pregnant women	6	12
Oral and enteral tube feeding formulas for ages ≥11 years	--	20

[8] Although Synaura lists “infant formula (0-6 months)” and “follow-on formula (6-12 months)” among the intended uses, FDA subsumes these uses under one category of non-exempt infant formula for term infants.

Please contact me if you have any questions about this request for clarification.

Sincerely,

Ellen Anderson
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition