GRAS NOTICE FOR 2'-FUCOSYLLACTOSE

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

PREPARED BY:
Inbiose N.V.
Technologiepark 82– bus 41
B-9052 Zwijnaarde
Belgium

DATE:
03 March 2022

GRAS Notice for 2'-Fucosyllactose

TABLE OF CONTENTS

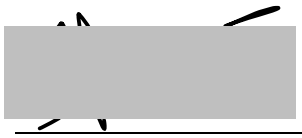
List of Figures and Tables

GRAS Notice for 2'-Fucosyllactose

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, Inbiose N.V. (Inbiose) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of 2'-fucosyllactose (2'-FL), as manufactured by Inbiose, in non-exempt term infant formula and various conventional food and beverage products as described in Section 1.3 below, are not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Inbiose's view that these notified uses of 2'-FL are Generally Recognized as Safe (GRAS). To the best of our knowledge, the data and information presented in this Notice represents a complete and balanced submission that is representative of the generally available literature. Inbiose considered all unfavorable as well as favorable information that is publicly available and/or known to Inbiose and that is pertinent to the evaluation of the safety and GRAS status of 2'-FL as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products, as described herein.

Signed,



March 03, 2022

Joeri Beauprez
CSO
joeri.beauprez@inbiose.com

Date

1.1 Name and Address of Notifier

Inbiose N.V.
Technologiepark 82 – bus 41
B-9052 Zwijnaarde
Belgium
Tel: +32 9 241 57 10

1.2 Common Name of Notified Substance

2'-Fucosyllactose; 2'-FL

1.3 Conditions of Use

Inbiose's 2'-FL is intended for use as an ingredient in non-exempt term infant formula at a use level of 2.4 g/L, and in the same food categories and use levels as those described in previous GRAS Notices (Glycosyn, LLC and Friesland Campina Domo B.V., 2017; DuPont Nutrition & Health, 2017, 2019; U.S. FDA, 2018a,b, 2020).

A summary of the proposed food categories and use levels is presented in Table 1.3-1 below. The intended conditions of use for Inbiose's 2'-FL will be fully substitutional to those described in GRAS Notice (GRN) 735 and 897 (GRN 735 – U.S. FDA, 2018a; GRN 897 – U.S. FDA, 2020).

Table 1.3-1 Summary of the Individual Uses and Maximum Use Levels for 2'-FL Previously Determined to be GRAS in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021a)	Proposed Food Use	Maximum Use Levels (g/kg or g/L)[a]		
		Inbiose	GRN 735	GRN 897
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	5	-	5
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	1.2	0.8	1.2
Infant and Toddler Foods	Non-exempt Term Infant Formulas	2.4	2.4	2.4 [c]
	Toddler Formulas	2.4	2.4	2.4 [c]
	Other Baby Foods for Infants and Young Children	57	57	12 [c]
	Other Drinks for Young Children	10	10	1.2 [c]
Breakfast Cereals	Hot Cereals	31	4.8	31
	Ready-to-eat Cereals	80	80	40
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30	12	30
	Cereal and Granola Bars	30	12	30
Milk Products	Buttermilk*	1.2	-	1.2
	Flavored Milk	1.2	1.2	1.2
	Milk-Based Meal Replacement Drinks, for Weight Reduction[b]	5	1.2	5
	Yogurt*	12	5.3	12
	Formula intended for pregnant women ("mum" formulas; -9 to 0 months)	60	60	-
Dairy Product Analogs	Imitation Milks	1.2	1.2	1.2
	Non-dairy Yogurt	1.2	-	1.2
Processed Fruits and Fruit Juices	Fruit Juices, Drinks, Nectars	1.2	1.2	1.2
Processed Vegetables and Vegetable Juices	Vegetable Juices	1.2	-	1.2
Foods for Special Dietary Use	Oral Nutritional Food Supplements and Enteral and Oral Tube-feeding Formulas for Patients ≥11 Years	20	20	20

2'-FL = 2'-fucosyllactose; CFR = *Code of Federal Regulations*; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; U.S. = United States.

[a] Proposed maximum use levels are presented as g/kg for solids and as g/L for liquids.

[b] Includes ready-to-drink and powder forms.

[c] These intended uses were incorporated by reference to GRN 749.

* Inbiose's 2'-FL is only intended for use in unstandardized products and not in foods where standards of identity exist that preclude its addition.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2021b), Inbiose has concluded that the intended uses of 2'-FL as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Inbiose N.V.
Technologiepark 82 – bus 41
B-9052 Zwijnaarde
Belgium

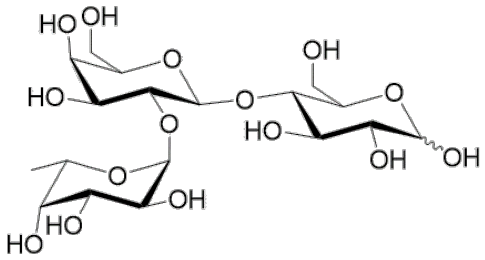
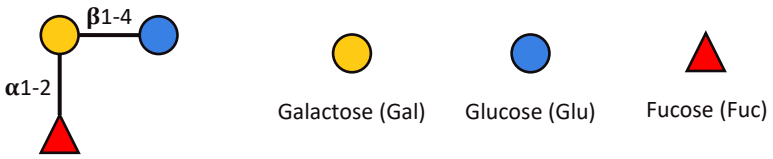
Should the FDA have any questions or additional information requests regarding this Notification, Inbiose will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is Inbiose's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, 5 U.S.C. 552.

Part 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

Common Name:	2'-Fucosyllactose
Abbreviation:	2'-FL
International Union of Pure and Applied Chemistry (IUPAC) Name:	α-L-Fucopyranosyl-(1→2)-β-Dgalactopyranosyl-(1→4)-D-glucopyranose
Chemical Abstracts Service (CAS) Number:	41263-94-9
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.436 g/mol
Chemical Structure:	
Schematic Representation:	

2.1.1 Chemical and Physical Characteristics

2'-FL is an abundant human milk oligosaccharide (HMO), comprised of galactose, glucose, and fucose, and manufactured by Inbiose using fermentation with a genetically modified strain of *Escherichia coli* K-12 MG1655. The final product is a purified white powder containing ≥94% 2'-FL, and small quantities of lactose and other related carbohydrates.

The identity of Inbiose's 2'-FL has been confirmed by nuclear magnetic resonance (NMR), by comparison with a 2'-FL reference standard (Product no: 35/08, IsoSep AB, Sweden) derived from human milk. Based on NMR, the Inbiose 2'-FL is structurally identical to the IsoSep reference. All major signals in the ^{1}H-NMR spectra of 2'-FL were identical among materials isolated from Inbiose's 2'-FL and IsoSep AB reference, and identical to ^{1}H-NMR spectra reported in the literature (Ishizuka *et al.*, 1999; Kjærulff, 2014; van Leeuwen *et al.*, 2014). The typical shifts of the anomeric protons/carbons and those of the methyl group of the fucose moiety further confirm the 2'-FL structure.

2.2 Manufacturing

2.2.1 Production Microorganism

2.2.1.1 Host Organism

The host organism is *Escherichia coli* K-12 strain MG1655, which is the same host organism as described in GRN 749, 897, and 951 (U.S. FDA, 2018b, 2020, 2021c). The taxonomy of the species is as follows:

Bacteria
Proteobacteria
Gammaproteobacteria
Enterobacteriales
Enterobacteriaceae
Escherichia
Escherichia coli
Escherichia coli K-12

The host strain, *E. coli* K-12 strain MG1655, is available from both American Type Culture Collection (ATCC) and the Coli Genetic Stock Center as ATCC#700926 and CGSC#7740, respectively. *E. coli* strains proliferate *via* asexual reproduction. This strain is nonrecombinant, stable, and can easily be maintained as a homogeneous population under the usual laboratory and production conditions. This strain does not produce spores.

E. coli K-12 strain MG1655 is derived from the well-known *E. coli* K-12 strain *via* classical, nonrecombinant genetics and cured of the temperate bacteriophage lambda and F plasmid by means of ultraviolet light and acridine orange, respectively. The genotype of the recipient microorganism is F-lambda-ilvG-rfb-50 rph-1, and the serotype is IRLH48:K- (Blattner *et al*., 1997). Later, additional mutations in commonly used stocks of *E. coli* K-12 strain MG1655 were identified and determined to cause loss of function of the *glpR* and *crl* genes, which are involved in glycerol 3-phosphate and RNA polymerase formation, respectively (Freddolino *et al*., 2012). The complete genome of this strain has been sequenced (GenBank U00096[1]).

The United States Environmental Protection Agency conducted a risk assessment of *E. coli* K-12 under the *Toxic Substances Control Act* (U.S. EPA, 1997). This review concluded that "*the use of E. coli K-12 under contained conditions in fermentation facilities*" will present a low risk of release of this microorganism into the environment and would not pose any significant ecological hazards, based on the following evidence:

- Wild-type *E. coli* is an inhabitant of the human colon;
- Studies have demonstrated that *E. coli* K-12 is a debilitated strain, defective in at least 3 cell wall characteristics that are important for colonization. As a result, *E. coli* K-12 is unable to colonize the human intestinal tract under normal conditions. Even in germ-free mice, *E. coli* K-12 is a poor colonizer;
- Experimental evidence has strongly suggested that indigenous intestinal microorganisms have a large competitive advantage over *E. coli* K-12 strains;
- *E. coli* K-12 lacks the ability to produce toxins that affect humans. There is no record in the literature of *E. coli* K-12 enterotoxin-induced disease in fermentation workers; and
- *E. coli* K-12 has a history of safe commercial use. Its derivative strains are currently used in many industrial applications, including the production of specialty substances L-aspartic, inosinic, and adenylic acids, which the human body produces, and FDA-approved human drugs such as insulin and somatostatin.

Because *E. coli* K-12 is not considered a human or animal pathogen and is not toxicogenic, it falls into Biosafety Level 1 classification and meets the Organisation for Economic Co-operation and Development (OECD) Good Industrial Large-Scale Practice (GILSP) criteria (OECD, 1992). *E. coli* K-12 strain MG1655 has been classified Biosafety Level 1 by the ATCC[2].

2.2.1.2 Production Strain

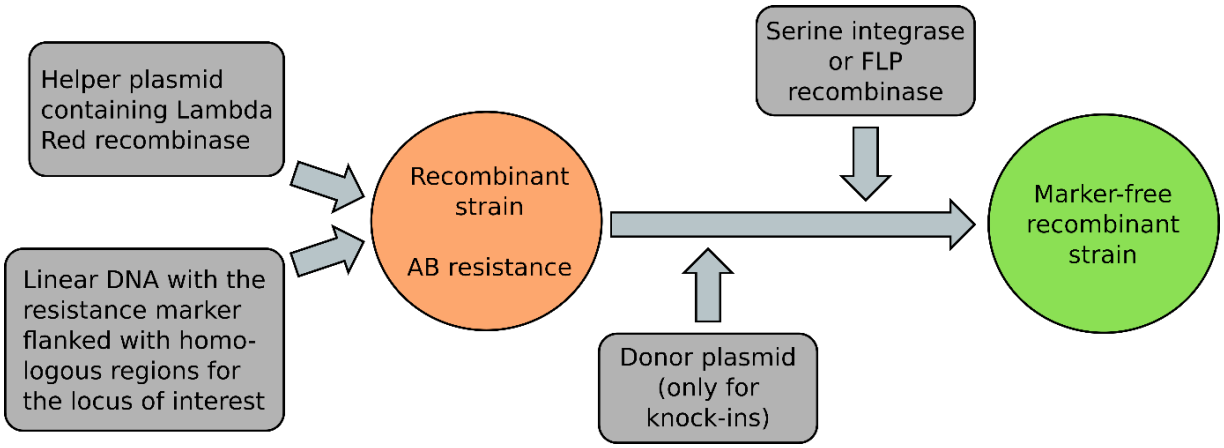
Several modifications, like gene knock-outs, gene insertions, and the addition of a production plasmid, were performed on *E. coli* K-12 strain MG1655 to create a 2'-FL production strain. This *E. coli* production strain is derived from the same parental strain as those that were previously assessed as part of GRNs 749 and 897. A production strain, INB-2FL_03, has been developed, through which the safety was assessed.

[1] https://www.ncbi.nlm.nih.gov/nuccore/545778205/.
[2] https://www.atcc.org/~/ps/47076.ashx.

The general method to introduce genetic modifications like gene deletions and gene knock-ins into the production strain genome is based on the methods described in detail by Datsenko and Wanner (2000) and Snoeck *et al*. (2019). The method is briefly described below in Figure 2.2.1.2-1. In all cases, gene deletions and gene insertions were verified by polymerase chain reaction (PCR), Sanger sequencing, and whole genome sequencing (WGS). As validated through WGS, the final strain does not contain any trace of (i) helper plasmids; (ii) antibiotic markers present on the helper plasmids; or (iii) antibiotic markers inserted into the genome. The removal of the helper plasmid is also validated by (i) PCR and (ii) replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid. In the case of the PCR test, no amplification was observed when the plasmid is not present; in the case of the replica plate, no growth was observed for the strains that do not contain the helper plasmid.

In most cases, DNA scars (att or FRT sites) are left behind, although very small and far apart in the chromosome. Inbiose's host requires an external recombinase to recombine DNA fragments efficiently. The endogenous system requires very large stretches of homology which are not present in the production host, and is very inefficient. After each modification, each of the previous modifications were checked by PCR and Sanger sequencing to ensure no other modifications occurred during the engineering process. No additional modifications or chromosome re-arrangements were observed, which was validated with WGS.

Figure 2.2.1.2-1 General Scheme of the Strain Construction Process*



FLP = flippase; FRT = flippase recognition target.
* At the end after plasmid curing, a complete marker-free recombinant strain is obtained. Helper plasmids used contain a lambda Red recombinase for homologous recombination or a serine integrase recognizing att sites or a FLP recombinase recognizing FRT sites. For genomic knock-ins, an extra donor plasmid containing (heterologous) genes, flanked by att sites, needs to be added.

All heterologous genes introduced into INB-2FL_03 were produced by DNA synthesis and were based on well-known annotated genomes from the respective donor organism. As such, no PCR techniques were used, indicating that there is no risk of undesirable or unintended genes from the donor organism being introduced to the production host. If needed, the heterologous genes were codon-optimized using bio-informatic tools. Additionally, before and after introducing these heterologous genes into the genome of the production host organism, a full Sanger sequencing of the transcription units was performed to ensure their identity.

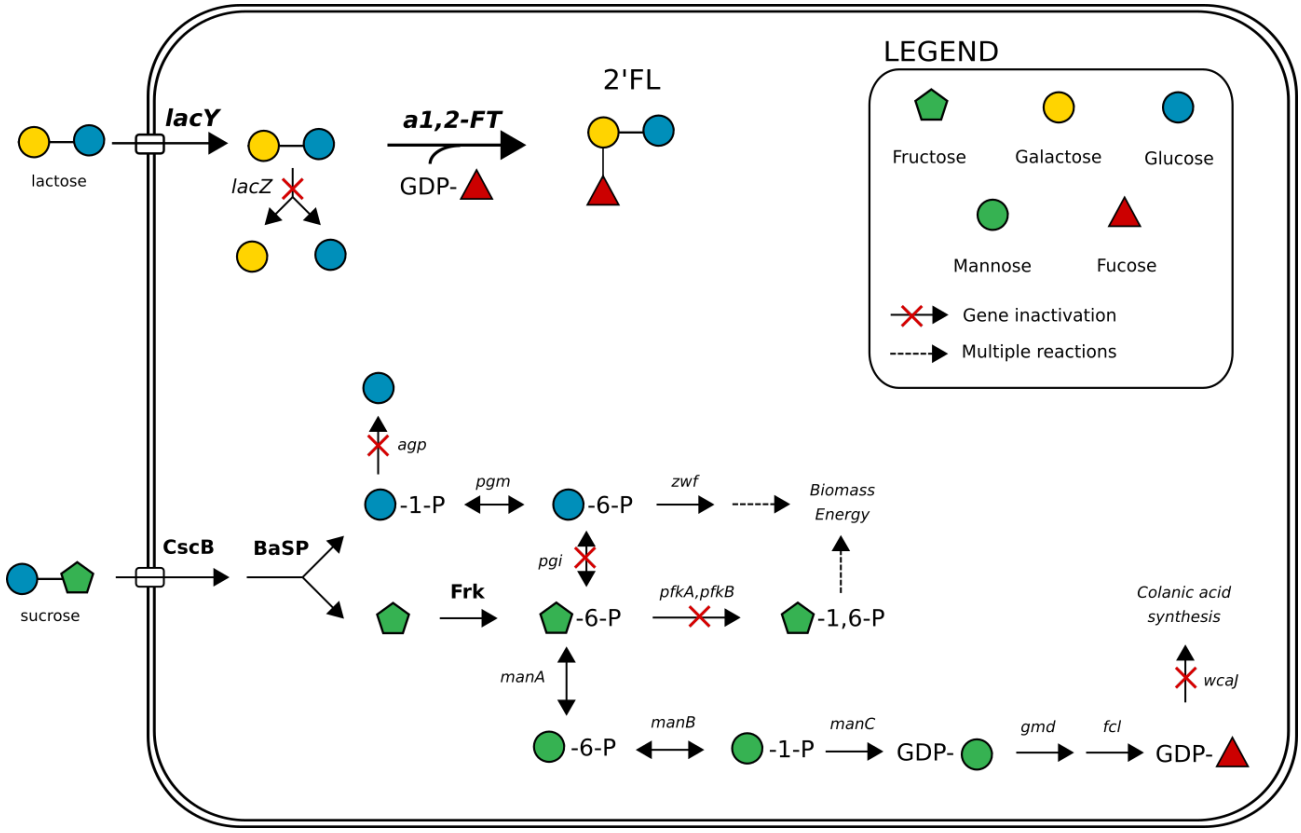
The host organism *E. coli* K-12 strain MG1655 was modified by genomic knock-outs and knock-ins by using the methods, as described above, to obtain efficient biosynthesis of 2'-FL (see Table 2.2.1.2-1 and Figure 2.2.1.2-2).

Table 2.2.1.2-1 Genetic Modification of the Production Organism (Gene Knock-ins)

Origin	Function
Escherichia coli	Lactose permease
Escherichia coli	Sucrose permease
Bifidobacterium adolescentis	Sucrose phosphorylase
Zymomonas mobilis	Fructokinase
Helicobacter sp.	α(1,2)-fucosyltransferase

Knock-outs were performed to avoid breakdown of lactose, improve the flux towards guanosine diphosphate (GDP)-fucose, and avoid the production of unwanted metabolic by-products. This strain was further modified to biosynthesize 2'-FL by the introduction of genes throughout the genome (see Table 2.2.1.2-1). In addition to the chromosomal modifications, a plasmid was also introduced in the production host INB-2FL_03 for overexpression of a fucosyltransferase gene from *Helicobacter sp.* No antibiotic resistance genes were present on the plasmid. The whole vector was synthesized *de novo* and is named pINB-2FL_03. After strain construction, colony PCR, Sanger sequencing, and WGS checks were performed to verify all genetic modifications introduced in the 2'-FL production strain. Production strain INB-2FL_03 does not contain any antibiotic resistant marker on the plasmid or introduced inside its genome.

Figure 2.2.1.2-2 Schematic Overview of the 2'-FL Biosynthetic Pathway in INB-2FL_03 using Sucrose as Carbon Source



2'FL = 2'-Fucosyllactose; a1,2-FT = a1,2-fucosyltransferase; agp = glucose-1-phosphatase; BaSP = sucrose phosphorylase; CscB = sucrose permease; fcl = GDP-L-fucose synthase; Frk = fructokinase; GDP = guanosine diphosphate; gmd = GDP-mannose 4,6-dehydratase; lacY = lactose permease; lacZ = β-galactosidase; manA = mannose-6-phosphate isomerase; manB = phosphomannomutase; manC = mannose-1-phosphate guanylyltransferase; P = phosphate; pfkA = 6-phosphofructokinase 1; pfkB = 6-phosphofructokinase 2; pgi = glucose-6-phosphate isomerase; pgm = phosphoglucomutase; wcaJ = UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase; zwf = NADP+-dependent glucose-6-phosphate dehydrogenase.

Taxonomical verification was performed with FastANI[3]. Assembled contigs of the production strain were compared to *E. coli K-12 MG1655* (U00096.3) reference genome. A whole-genome average nucleotide identity (ANI) of >99.95% was obtained confirming that the production strain is *E. coli* K12 MG1655.

2.2.2 Raw Materials, Processing Aids, and Equipment Specifications

2'-FL is manufactured by Inbiose in compliance with current Good Manufacturing Practice (cGMP) and/or principles of Hazard Analysis and Critical Control Points (HACCP) and/or Food Safety System Certification (FSSC 22000).

[3] https://github.com/ParBLiSS/FastANI.

The manufacture of 2'-FL is largely comparable to the production processes previously evaluated for other HMOs produced by microbial fermentation involving construction of a production organism engineered to synthesize human milk oligosaccharides from lactose, with large-scale fermentation and downstream processing to isolate the human milk oligosaccharide. All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously concluded to be GRAS for their respective uses, or have been the subject of an effective food contact notification.

2.2.3 2'-FL Manufacturing Process

In summary, the manufacturing method for 2'-FL entails a fermentation process with a K-12-based production host (see Section 2.2.1) that produces 2'-FL. This host produces 2'-FL through the utilization of a carbon source (sucrose), combined with lactose in a minimal medium. The product is released into the medium. The remaining intracellular 2'-FL is released after pasteurization. The broth is then subjected to downstream purification and concentration processes to isolate 2'-FL, to remove impurities originating from fermentation (*e.g.*, minerals, substrates, proteins, and other cellular matter) followed by drying (see Figures 2.2.3-1 and 2.2.3-2 below).

In the first step, biomass is removed together with cell components and large molecules (DNA, protein, and lipopolysaccharides). After removal of larger particles, the color is removed using activated charcoal. Subsequently, the salts present in the medium are removed, which are cations (*e.g.*, magnesium, calcium, and ammonium) and anions (*e.g.*, phosphate and sulfate), which are minerals used for growth of the microorganism. Leftover water is removed from the product mainly through evaporation and the product is filtered again to ensure the microbial specification before drying.

Figure 2.2.3-1 Fermentation Process

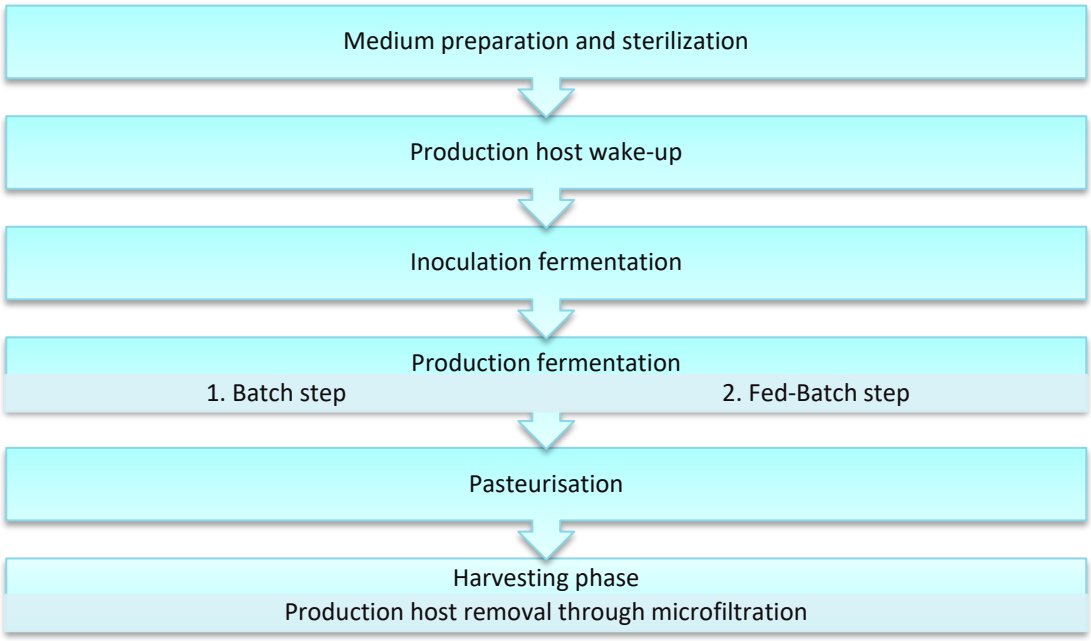
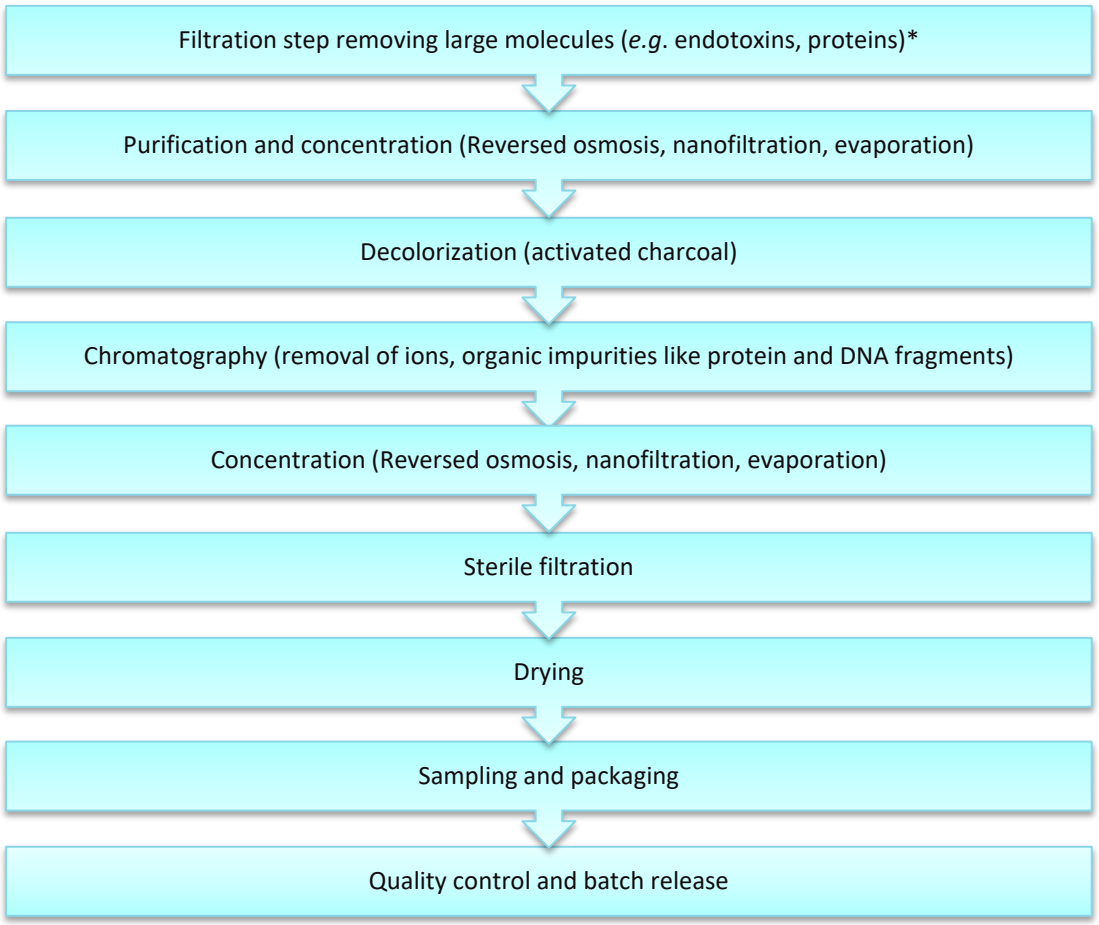


Figure 2.2.3-2 Purification Process



* The filtration steps are done with cut-offs of 0.1 to 5 µm and 1 to 30 kDa.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

To ensure consistent product quality, Inbiose has established a set of specifications for 2'-FL, which includes the acceptability criteria for purity of 2'-FL and the presence of other carbohydrates, chemical parameters, heavy metals, and microbial contaminants, and confirms the absence of the genetically modified production strain and any related endotoxins. The specifications proposed for 2'-FL are presented in Table 2.3.1-1. All parameters are determined using compendial or validated methods.

Table 2.3.1-1 Product Specifications for Inbiose's 2'-FL

Parameter	Specification	Method of Analysis
Identification		
Appearance (Color)	White	Visual
Appearance (Form)	Powder	Visual
Appearance in solution	Clear, colorless to slightly yellow	Visual

Table 2.3.1-1 Product Specifications for Inbiose's 2'-FL

Parameter	Specification	Method of Analysis
Identity (2'-FL)	Conform to reference standard, 2'-FL derived from human milk	NMR
Chemical Specifications		
Moisture	NMT 5.0%	Karl-Fischer, volumetric
pH (20°C, 10% solution)	3.0 to 7.5	Eurofins' internal method, potentiometry
Protein	NMT 100 µg/g	Roti®Nanoquant
Ash	NMT 0.5%	NEN 6810
Endotoxins	NMT 10 E.U./mg	Ph. Eur. 2.6.14
Carbohydrates (% DM)		
2'-FL	NLT 94%	UHPLC-RI
Sum of other carbohydrates[a]	NMT 5.0%	UHPLC-RI
Lactose	NMT 5.0%	UHPLC-RI
Difucosyllactose	NMT 5.0%	UHPLC-RI
Heavy Metals		
Arsenic	NMT 0.2 mg/kg	ICP-MS
Cadmium	NMT 0.05 mg/kg	ICP-MS
Lead	NMT 0.05 mg/kg	ICP-MS
Mercury	NMT 0.5 mg/kg	ICP-MS
Microbiological Contaminants		
Total aerobic mesophilic plate count	NMT 1,000 CFU/g	ISO 4833
Yeast	NMT 100 CFU/g	ISO 7954
Mold	NMT 100 CFU/g	ISO 7954
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella spp.	Absent in 25 g	ISO 6579-1
Cronobacter sakazakii	Absent in 25 g	ISO/TS 22964
Listeria monocytogenes	Absent in 25 g	AFNOR EGS 38/05-03/17
Bacillus cereus	NMT 50 CFU/g	ISO 7932

2'-FL = 2'-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; DM = dry matter; E.U. = endotoxin units; EGS = Eurofins GeneScan; GRN = Generally Recognized as Safe Notice; ICP-MS = inductively coupled plasma mass spectrometry; ISO = International Organization for Standardization; NEN = Royal Netherlands Standardization Institute; NLT = not less than; NMR = nuclear magnetic resonance; NMT = not more than; Ph. Eur. = European Pharmacopoeia; UPLC-RI = ultra-high performance liquid chromatography coupled with refractive index detector.

[a] Sum of other carbohydrates, such as 3-fucosyllactose, 2-fucosyl-D-lactulose, fucosyl-galactose, glucose/galactose, fucose, sorbitol/galactitol, mannitol, and trihexose.

2.3.2 Batch Analysis

Results for the analyses of 3 non-consecutive batches of 2'-FL are summarized in Table 2.3.2-1. The data demonstrate that the production process as described in Section 2.2 results in a consistent product that meets the established product specifications.

Table 2.3.2-1 Analytical Data Obtained from 3 Batches of 2'-FL

Parameter	Specification	Lot Nos.		
		HeraD03	HeraD04	HeraD07
Identification				
Appearance (color)	White	White	White	White
Appearance (form)	Powder	Powder	Powder	Powder
Appearance in solution	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow
pH (20°C, 10% solution)	3.0 to 7.5	6.8	5.5	5.3
Carbohydrates, water free (%DM)				
2'-FL	≥94	97.5	97.3	98.4
Lactose	≤5.0	2.1	2.3	1.0
Difucosyllactose (DFL)	≤5.0	0.13	0.13	0.13
Sum of other carbohydrates[a]	≤5.0	0.30	0.29	0.51
Chemical Analysis				
Water content, volumetric (% w/w)	≤5.0	3.5	4.3	4.2
Protein content (µg/g)	≤100	<25	<25	<25
Total ash (%)	≤0.5	<0.1	<0.1	
Endotoxin (E.U./g)	≤10,000	230	62.2	<50
Heavy Metals				
Arsenic (mg/kg)	≤0.02	<0.01	<0.01	<0.01
Cadmium (mg/kg)	≤0.05	<0.005	<0.005	<0.005
Lead (mg/kg)	≤0.05	<0.01	<0.01	<0.01
Mercury (mg/kg)	≤0.5	<0.01	<0.01	<0.01
Microbiological Contaminants				
Standard plate count (CFU/g)	≤1,000	30	<10	<10
Yeast (CFU/g)	≤100	<10	<10	<10
Mold (CFU/g)	≤100	<10	<10	<10
Coliform / *Enterobacteriaceae*	Absent in 10 g	Absent	Absent	Absent
Salmonella spp.	Absent in 25 g	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii	Absent in 25 g	Absent	Absent	Absent
Listeria monocytogenes	Absent in 25 g	Absent	Absent	Absent
Bacillus cereus (CFU/g)	≤50	<10	<10	<10

2'-FL = 2'-fucosyllactose; CFU = colony forming units; DM = dry matter; E.U. = endotoxin units; GRN = Generally Recognized as Safe Notice

[a] Sum of other carbohydrates, such as 3-fucosyllactose, 2-fucosyl-D-lactulose, fucosyl-galactose, glucose/galactose, fucose, sorbitol/galactitol, mannitol, and trihexose.

2.3.3 Microbiological Endotoxins and Residual Protein Analysis

The content of endotoxins and residual proteins in the 2'-FL product is determined by methods with high sensitivity [Protein content: Roti®Nanoquant method, based on the Bradford assay; and Endotoxins: kinetic-chromogenic test (Method D) described in the European Pharmacopoeia] to ensure the consistency and quality of the 2'-FL product.

The regulatory batches contain only a small quantity of endotoxin and residual proteins, which remain below the proposed specification limits and therefore are not considered a safety concern (see Table 2.3.2-1).

2.3.4 Residual DNA Analysis

To ensure the absence of residual DNA of the production organism, PCR tests were performed on 3 regulatory batches of INB-2FL_03. A short subsequence of the inserted fucosyltransferase gene (derived from *Helicobacter* sp.) on the plasmid and a subsequence of the sucrose phosphorylase gene (derived from *Bifidobacterium adolescentis*) on the genome were targeted to check for residual DNA in the product. For every batch, the analysis was performed in triplicate together with 3 types of positive controls and 1 negative control. The analysis of all regulatory batches of 2'-FL showed no detectable levels of residual DNA in the final product. The limit of detection for the PCR method is 10 ng DNA per gram 2'-FL as recommended in European Food Safety Authority (EFSA) guidelines (EFSA, 2018).

2.4 Stability

The stability of Inbiose's 2'-FL is supported by the real-time and accelerated stability studies summarized in GRNs 546, 735, 749, and 987. The compositional similarities between Inbiose's 2'-FL and other 2'-FL preparations (see Section 6.3) indicate that the stability of the ingredients will be similar. A summary of the real-time and accelerated stability studies, as described in GRNs 546, 735, 749, and 987, is provided below (Glycom A/S, 2014; U.S. FDA, 2015a, 2018a,b, 2021d; DuPont Nutrition & Health, 2017; Glycosyn, LLC and Friesland Campina Domo B.V., 2017; Amyris, Inc., 2020). Additionally, the stability of 2'-FL has been tested in studies conducted under some of the intended conditions of use, which further supports the stability of 2'-FL as an ingredient in food and beverages matrices, which were included in GRN 546 (Glycom A/S, 2014; U.S. FDA, 2015a).

The bulk stability of crystalline, chemically synthesized, Glycom's 2'-FL ingredient was evaluated in a 36-month real-time test and a 6-month accelerated test, as described in Section II.D.1 of GRN 546. In the real-time study [25°C, 60% relative humidity (RH)], no significant change was observed in the 2'-FL content or microbiological parameters of the stored sample and only a minor increase in water content (remaining within acceptable defined product specifications) was measured at the 18-month time point. Results from the 6-month accelerated stability study (40°C, 75% RH) also indicate that 2'-FL does not undergo significant degradation under the described storage conditions. No unknown degradation products were measured following HPLC analysis of the 2'-FL following accelerated storage.

As described in Section 7 and Appendix C of GRN 749, the shelf life of DuPont's 2'-FL was assessed *via* a 6-month accelerated stability study (40°C, 75% RH). The results of this study indicated no significant changes in the evaluated carbohydrate content (*i.e.*, 2'-FL, difucosyllactose, lactose, and other unspecified carbohydrates), moisture content, and microbiological parameters (*i.e.*, standard plate count, yeast and mold, coliform/*Enterobacteriaceae*, *Salmonella* spp., and *Cronobacter sakazakii*) in 3 representative batches following storage for up to 6 months. A minor degree of degradation was reported in the purity of 1 sample and the moisture content slightly increased over the test period due to the hygroscopic nature of the ingredient. No other changes were noted.

As described in Section E.2 of GRN 735, a range of chemical and microbiological specification parameters was tested, along with the overall purity of FrieslandCampina's 2'-FL ingredient, in a 6-month accelerated storage (40°C, 75% RH) and an ongoing 36-month real-time study (25°C, 60% RH). The results from the accelerated stability study indicated no changes in the appearance of the ingredient or the evaluated chemical (*i.e.*, 2'-FL, lactose, allo-lactose, glucose, ash, and water content) and microbiological parameters. The 6-month interim results from the real-time study confirmed that 2'-FL is stable when stored at ambient room temperatures.

Similarly, the stability of Amyris's 2'-FL was assessed in a study conducted under accelerated (40°C, 75% RH) conditions for 13 weeks (Section 2.6; GRN 987). Under these conditions, 2'-FL content remained relatively unchanged along with the other measured parameters (*i.e.*, moisture, 2'-fucosyllactitol, and other minor carbohydrate components).

The stability of 2'-FL has also been assessed under the intended conditions of use. The 2'-FL ingredient produced by Glycom *via* chemical synthesis (compositionally comparable to 2'-FL produced from fermentation) was assessed in powdered infant formula, as described in Section II.D.2 of GRN 546. Glycom's 2'-FL added to a powdered infant formula supplemented with other human-identical milk oligosaccharides (*i.e.*, lacto-*N*-neotetraose), containing a range of other ingredients (*i.e.*, salts, carbohydrates, and proteins), was observed to be stable for up to 18 months of storage at various temperatures (4°C, 20°C, 30°C, and 37°C). The results from additional stability testing of Glycom's 2'-FL in yogurts, ready-to-drink flavored milk, and citrus fruit beverages also indicated that 2'-FL was stable in a range of different products, with no loss in 2'-FL content following typical processing and storage conditions, for up to 28 days post-processing.

Other recently filed GRNs (*e.g.*, GRNs 897 and 929) similarly reflect on stability tests that have been conducted and reported in these earlier filings (DuPont Nutrition & Health, 2019; Jennewein Biotechnologie GmbH, 2020; U.S. FDA, 2021e).

These results demonstrate that 2'-FL is not significantly degraded when stored under the tested conditions and is anticipated to be stable in most food matrices.

Part 3. § 170.235 Dietary Exposure

3.1 Estimated Intake of 2'-FL

As described in Section 3.2 of GRN 897, the estimated intake of 2'-FL as an ingredient in term infant formula (0 to 12 months), toddler formula, and other food and beverage products has been estimated using dietary survey data in the U.S. population. The intake of 2'-FL described in GRN 897 was estimated based on food consumption records collected from the What We Eat in America (WWEIA) component of the National Center for Health Statistics' 2013-2014 National Health and Nutrition Examination Surveys (NHANES) conducted in 2013-2014 and 2015-2016 (CDC, 2015, 2016, 2018; USDA, 2016), incorporating the product uses (*i.e.*, infant and toddler formulas, foods, and drinks) and intake estimates originally presented in GRN 749. Based on the proposed food uses, approximately 81% of the evaluated population groups consisted of eligible 2'-FL consumers. Infants (7 to <12 months) were established to represent the highest mean and 90th percentile all-user consumption of 2'-FL on an absolute basis, at 4.63 and 8.36 g/person/day (*i.e.*, 520.2 and 987.1 mg/kg body weight/day), respectively. Inbiose's 2'-FL ingredient will be fully substitutional to the GRAS infant and toddler food uses described in GRN 897, and this intakes estimate is therefore considered to remain unchanged. The summary of the estimated dietary intake of 2'-FL in the U.S. population, as described in GRN 897, is provided in Table 3.1-1 (U.S. FDA, 2020). Furthermore, due to the overall intended conditions of use for Inbiose's 2'-FL being fully substitutional to those described in both GRN 735 and 897, a summary of estimated dietary intake of 2'-FL, as described in GRN 735, is provided in Table 3.1-2 (U.S. FDA, 2018a).

Table 3.1-1 Summary of the Estimated Daily Intake of 2'-FL[a] from All Proposed Food Uses in the U.S. by Population Group in GRN 897 (2009-2016 NHANES Data)[b]

Population Group	Age Group (Years, Unless Otherwise Specified)	Per User Intakes (g/day)		Per User Intakes (mg/kg bw/day)	
		Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 months	2.93	5.29	449.7	712.4
Infants	7 to 12 months	4.63	8.36	520.2	987.1
Toddlers	13 to 36 months	1.12	1.97	84.9	146.0
Children	3 to 12	1.6	3.5	60	140
Adolescents	13 to 18	1.7	3.9	30	60
Adults	19 to 49	2.2	5.2	30	70
Adults	50 and up	2.5	5.9	30	80
Total Population	3 and up	2.2	5.0	40	100

2'-FL = 2'-fucosyllactose; bw = body weight; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

[a] Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

[b] Table adapted from GRN 897 (U.S. FDA, 2020); full intake assessment reported in GRN 897 GRAS determination.

Table 3.1-2 Summary of the Estimated Daily Intake of 2'-FL[a] from All Proposed Food Uses in the U.S. by Population Group in GRN 735 (2013-2014 NHANES Data)[b]

Population Group	Age Group (Years, Unless Otherwise Specified)	Per User Intakes (g/day)		Per User Intakes (mg/kg bw/day)	
		Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 5 months	1.10	2.75	181	477
Infants	6 to 11 months	2.14	3.86	244	441
Toddlers	12 to 35 months	1.83	2.97	148	243
Children	3 to 11	1.96	3.53	75	147
Female Teenagers	12 to 19	1.47	2.95	24	52
Male Teenagers	12 to 19	1.85	4.16	29	67
Women of Child-Bearing Age	16 to 45	1.22	2.82	18	42
Female Adults	20 years and up	1.32	2.96	19	42
Male Adults	20 years and up	1.59	3.81	19	26
Elderly	65 years and up	1.76	3.74	24	53
Total Population	All ages	1.55	3.41	32	76

2'-FL = 2'-fucosyllactose; bw = body weight; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

[a] Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

[b] Table adapted from GRN 735 (U.S. FDA, 2018a); full intake assessment reported in GRN 735 GRAS determination.

Part 4. § 170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with 2'-FL.

Part 5. § 170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

6.1 Introduction

The first GRAS conclusion notified to the FDA for 2'-FL (produced by chemical synthesis) was submitted by Glycom in 2014 (GRN 546; U.S. FDA, 2015a). A subsequent GRAS notification for 2'-FL produced by Glycom using fermentation technology was submitted to the FDA and filed by the agency without objection in 2016 under GRN 650 (GRN 650; U.S. FDA, 2016). A critical and comprehensive review of the publicly available data and information pertaining to the safety of 2'-FL for use as an ingredient in non-exempt infant formula, and various food and beverage products across multiple categories, was presented in these Notices, and the published information pertinent to safety of 2'-FL presented by Glycom has served as the basis for subsequent GRAS conclusions for similar 2'-FL preparations (Glycom A/S, 2014, 2018; Jennewein Biotechnologie GmbH, 2015, 2020; U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020, 2021e,f; DuPont Nutrition & Health, 2017, 2019; Glycosyn, LLC and Friesland Campina Domo B.V., 2017; BASF SE, 2019). To date, the majority of 2'-FL preparations that have been evaluated under the GRAS procedure have been synthesized by microbial fermentation using genetically modified strains of *E. coli* K-12. Downstream processing using various purification techniques (*e.g.*, chromatography, ion-exchange, nano-filtration, carbon filtration, solvent crystallization) are then applied to produce 2'-FL products that are typically >90% purity. Compositional differences between various 2'-FL preparations are largely limited to differences in the concentrations of residual lactose, and other related sugars; however, these compounds have been demonstrated to be present in human milk or within infant formula and therefore these minor differences in the impurity profiles between various preparations have not been of safety concern. A large number of toxicological evaluations have been conducted using 2'-FL preparations from different manufacturers without evidence of toxicity at the highest doses tested, further supporting the view that residues of these carbohydrate impurities are of low toxicity potential. Within the previous GRAS Notices, data and information supporting the GRAS use of 2'-FL as an ingredient in infant formula and other foods have been critically reviewed by a number of qualified scientific experts, including the FDA, and are publicly available. Additionally, EFSA has issued multiple opinions supporting the safe use of 2'-FL as an ingredient in a variety of foods, including infant and follow-on formula either alone or in combination with other HMOs (EFSA, 2015a,b, 2019).

2'-FL is the most abundant HMO occurring naturally in human breast milk and approximately 85% of the world population is exposed to 2'-FL from human milk (Thurl *et al*., 2017; Menzel *et al*., 2020; Liu *et al*., 2021; Plows *et al*., 2021; Soyyılmaz *et al*., 2021). As reported in Section 3.B of GRN 932, concentrations of 2'-FL in human milk can range from 0.22 to 8.4 g/L, varying depending on the mother's genotype and the lactation stage of the pregnancy (Advanced Protein Technologies, Corp., 2020). These 2'-FL levels in human breast milk have been measured in a wide range of previously-evaluated studies (Grollman and Ginsburg, 1967; Thurl *et al.*, 1996, 2010, 2017; Chaturvedi *et al.*, 1997, 2001; Coppa *et al.*, 1999, 2011; Kunz *et al.*, 1999; Nakhla *et al.*, 1999; Erney *et al.*, 2000, 2001; Sumiyoshi *et al.*, 2003; Morrow *et al.*, 2004; Musumeci *et al.*, 2006; Asakuma *et al.*, 2008, 2011; Leo *et al.*, 2009, 2010; Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013; Castanys-Munoz *et al.*, 2013; Smilowitz *et al.*, 2013; Goehring *et al.*, 2014; Hong *et al.*, 2014; Marx *et al.*, 2014; Balogh *et al.*, 2015; Austin *et al.*, 2016, 2019; Donovan and Comstock, 2016; McGuire *et al.*, 2017; Samuel *et al*., 2019).

Recent studies support the previous data demonstrating that 2'-FL levels in breast milk decrease throughout lactation (Ferreira *et al*., 2020; Lagström *et al*., 2020; Lefebvre *et al*., 2020; Gu *et al*., 2021; Liu *et al*., 2021; Menzel *et al*., 2021; Plows *et al*., 2021; Siziba *et al*., 2021; Soyyılmaz *et al*., 2021; Zhou *et al*., 2021). The results of these studies are summarized in Table 6.1-1.

In a prospective population-based birth cohort study in Turku, Finland performed by Lagström et al. (2020), breast milk samples of 802 mothers were collected at 3 months post-partum and analyzed. The median concentration of 2'-FL in all milk samples was 2.72 g/L [number of samples (N)=802], 2.90 g/L in the secretor group (N=699), and 0.02 g/L in the non-secretor group (N=103) [the original data, which were reported in (nmol/ml), were converted into (g/L) using a molecular weight of 2'-FL of 448.436 g/mol] (Lagström *et al*., 2020). Similar results were observed in the study conducted by Menzel *et al*. (2020), who collected milk samples from German mothers at 3 months after delivery. The levels of 2'-FL in breast milk were 2.04 g/L (median, N=124) in secretor and 0.01 g/L (median, N=21) in non-secretor mothers. A median level of 2'-FL of 1.91 g/L was obtained from secretor and non-secretor samples (N=145). In another study, Gu *et al*. (2021) reported levels of 2'-FL in breast milk collected from 68 mothers in the Nijmegen−Arnhem region in the Netherlands. In total 180 samples were analyzed (N=138 for secretors and N=42 for non-secretors, Lewis +) at 2, 6, and 12 weeks postpartum. 2'-FL levels in secretors decreased from 1.22 g/L at 2 weeks (N=48) to 1.03 g/L at 6 weeks (N=48) and then to 0.91 g/L at 12 weeks (N=42). For non-secretors, the reported 2'-FL concentrations were 0 g/L for all three timepoints.

Soyyılmaz *et al*. (2021) investigated the concentration of diverse HMOs, including 2'-FL, in milk samples from mothers around the world. This meta-analysis data showed that 2'-FL significantly decreases from colostrum (3.18 g/L, 0 to 5 days) to transitional milk (2.07 g/L, 6 to 14 days), and from mature milk (2.28 g/L, 15 to 90 days) to late milk (1.65 g/L days, >90 days). Another systematic review, Thum *et al.* (2021), examined the concentration of HMOs in different geographic locations. Overall, the levels of 2'-FL at the first timepoint (0 to 7 days) ranged from 1.70 to 3.70 g/L, from 0.22 to 3.37 g/L in 5 to 15 days and from 0.98 to 3.02 g/L in >1 month. These results were in line with Soyyılmaz *et al*. (2021).

Ferreira *et al*. (2020) described the variation of 2'-FL concentration up to 4 months postpartum in milk samples of Brazilian mothers who delivered the pre-term babies. The results showed that the median of 2'-FL concentration in analyzed samples of the breast milk was 2.26 g/L in the lactation period of 2 to 50 days and then decreased to 1.91 g/L during the lactational period of 88 to 119 days [the original data, which were reported in (mmol/ml), were converted into (g/L) using a molecular weight of 2'-FL of 448.436 g/mol]. Zhou *et al*. (2021) summarized the concentrations of HMOs in the Chinese population. The concentration of 2'-FL declined gradually during lactation, from 2.93 g/L at 0 to 7 days postpartum to 0.92 g/L at >121 days postpartum. In another recent study of Liu *et al*. (2021), a total of 488 breast milk samples were collected from 335 healthy Chinese mothers at 5 different timepoints. 2'-FL levels remained relatively high in breast milk throughout the full duration of the study. The highest 2'-FL concentrations were reported in the colostrum (2.89 g/L, 0 to 5 days) and then continuously decreased to 1.01 g/L in the late milk (300 to 400 days postpartum).

Plows *et al*. (2021) investigated the change of 2'-FL levels up to 24 months postpartum. The results showed that 2'-FL concentration in breast milk samples collected from Hispanic mothers in a longitudinal cohort study decreased from 3.48 g/L at 1 month postpartum (N=183) to 2.49 g/L at 24 months postpartum (N=26) in samples collected from secretor mothers. For non-secretors, low levels of 2'-FL were reported and remained stable during the study (range of medians: 0.01 to 0.05 g/L). These results are supported by the previous study conducted by Lefebvre *et al.* (2020). In another birth cohort study conducted in south of Germany, Siziba *et al*. (2021) investigated the trajectories of absolute HMO concentrations during lactation. A total of 66 lactating mothers had HMO data available from samples at all three time points. The absolute mean 2'-FL concentration in milk decreased over the first year of lactation; from 2.45 g/L at 6 weeks to 1.65 g/L and 1.43 g/L at 6 months and 12 months of lactation, respectively.

Overall, the 2'-FL levels up to 3.84 g/L were reported in the latest studies (published in 2020 and 2021, Table 6.1-1). As such, the use of 2'-FL as an ingredient in non-exempt term infant formula at levels up to 2.4 g/L is within the reported range that infants are exposed to following the ingestion of human milk.

Table 6.1-1 Levels of 2'-FL in Human Breast Milk Reported in Studies Published Between 2020-2021

Reference Study	Quantification Method	Gestation	Secretor Status	Lewis	Lactation Periods	No. of Samples	Mean Concentration ± SD (g 2'-FL/L)	Median of 2'-FL Concentration (Q1, Q3) (g/L)	Site
Thum *et al*. (2021)	UHPLC-FL, HPLC-MRM-MS, HPAEC-PAD, CE-LIF, HPLC-FL, NMR, UHPLC/QqQ-MS, LC-MS, HPLC-UV, CE-UV	Term	N/A*	Se+ and Se-	0–7 days	N/A	Range: 1.70±1.11 to 3.70±1.94	N/A	Japan, China, Malaysia, Singapore, Spain, Germany, France, Italy, Norway, Portugal, Romania, Sweden, Finland, Nederland, USA, Mexico, Malawi, South Africa, Samoa,
					5–15 days	N/A	Range: 0.22±0.37 to 3.37	N/A	
					11–30 days	N/A	Range: 1.37±1.12 to 3.02	N/A	
					2 months	N/A	Range: 1.18±1.02 to 2.82	N/A	
					3 months	N/A	Range: 0.98±0.89 to 2.21±0.71	N/A	
Siziba *et al*. (2021)	LC-MS	Term	Se+	N/A	6 weeks	66	2.45±1.32	N/A	Germany
					6 months		1.65±1.08		
					12 months		1.43±0.85		
Soyyılmaz *et al*. (2021)	HPLC-FL, HPAEC-PAD, LC-MS/MRM, CE, PC, NMR, nano-LC-chip-TOF (time of flight)	Term	Se+ and Se-	N/A	0–5 days	1,101	3.18	N/A	Europe, Asia, North America, Latin America, Asia Pacific, Middle East
					6–14 days	789	2.07		
					15–90 days	4,048	2.28		
					>90 days	1951	1.65		
Liu *et al*. (2021)	HPAEC-PAD	Term	Se+ and Se-	N/A	0–5 days	96	N/A	2.89(1.71, 4.34)	China
					10–15 days	96		2.16(1.67, 2.81)	
					40–45 days	104		2.06(1.38, 2.69)	
					200–400 days	100		1.03(0.60, 1.52)	
					300–400 days	92		1.01(0.60, 1.48)	
Menzel *et al*. (2021)	LC-FL	Term	Se+ and Se-	N/A	3 months	145	N/A	1.93(1.31, 2.41)	Germany
			Se+			124		2.04 (1.53, 2.72)	
			Se-			21		0.007 (0.006, 0.010)	

Table 6.1-1 Levels of 2'-FL in Human Breast Milk Reported in Studies Published Between 2020-2021

Reference Study	Quantification Method	Gestation	Secretor Status	Lewis	Lactation Periods	No. of Samples	Mean Concentration ± SD (g 2'-FL/L)	Median of 2'-FL Concentration (Q1, Q3) (g/L)	Site
Plows *et al*. (2021)	HPLC–FL	Term	Se+	N/A	1 month	183	N/A	3.48(2.42, 4.34)	Hispanic, the U.S.
			Se+		6 months	104		3.84(2.80, 4.95)	
			Se+		12 months	76		3.35(2.67, 4.14)	
			Se+		18 months	54		3.1(1.95, 3.85)	
			Se+		24 months	26		2.49(1.66, 3.73)	
			Se-		1 month	24		0.02(0.01, 0.05)	
			Se-		6 months	15		0.05(0.02, 0.06)	
			Se-		12 months	7		0.03(0.02, 0.06)	
			Se-		18 months	5		0.03(0.02, 0.03)	
			Se-		24 months	2		0.01(0.01, 0.02)	
Gu *et al*. (2021)	PGC-LC-MS	Term	Se+	Le+	2 weeks	48	1.22±0.36	N/A	Netherlands
			Se+		6 weeks	48	1.03±0.3		
			Se+		12 weeks	42	0.91±0.26		
			Se-		2 weeks	16	0±0		
			Se-		6 weeks	13	0±0		
			Se-		12 weeks	13	0±0		
Zhou *et al*. (2021)	UHPLC, HPLC, HPLC-MS, UHPLC-QqQ-MS, UHPLC-FL, UHPLC-MS-MS	N/A	Se+ and Se-	N/A	1–7 days	N/A	2.93±1.08	N/A	Meta-analysis, China
					8–14 days		1.83±0.57		
					15–60 days		1.71±0.45		
					61–120 days		1.19±0.26		
					>121 days		0.92±0.19		
Lagström *et al*. (2020)	HPLC-MS	Term	Se+ and Se-	N/A	3 months	802	N/A	2.72(1.98, 3.53)	Finland
			Se+			699		2.89(2.23, 3.69)	
			Se-			103		0.02(0.01, 0.05)	

Table 6.1-1 Levels of 2'-FL in Human Breast Milk Reported in Studies Published Between 2020-2021

Reference Study	Quantification Method	Gestation	Secretor Status	Lewis	Lactation Periods	No. of Samples	Mean Concentration ± SD (g 2'-FL/L)	Median of 2'-FL Concentration (Q1, Q3) (g/L)	Site
Lefebvre *et al*. (2020)	UHPLC-FL	N/A	Se-	Le+	3 months	N/A	0.01	0.01	Germany
			Se-	Le+	6 months		0.00	0.00	
			Se-	Le+	12 months		0.02	0.00	
			Se+	Le-	3 months		3.32	3.37	
			Se+	Le-	6 months		3.05	3.23	
			Se+	Le-	12 months		3.47	3.80	
			Se+	Le+	3 months		2.12	2.00	
			Se+	Le+	6 months		1.80	1.69	
			Se+	Le+	12 months		1.56	1.43	
Ferreira *et al*. (2020)	HPLC-FL	Preterm	Se+ and Se-	N/A	2–8 days	52	N/A	2.26(1.18, 2.91)	Brazil
					28–50 days	75		2.26(1.48, 2.62)	
					88–119 days	46		1.91(1.33, 2.76)	

2'-FL = 2'-fucosyllactose; CE-LIF = capillary electrophoresis with laser-induced fluorescence detection; CE-UV = capillary electrophoresis with ultraviolet detection, paper chromatography; HPAEC-PAD = high-performance anion-exchange chromatography with pulsed amperometry detection; HPLC-FL = high performance liquid chromatography with fluorescence detection; HPLC-MRM-MS = high performance liquid chromatography-multiple reaction monitoring-mass spectrometry; HPLC-MS = high performance liquid chromatography with mass spectrometry; HPLC-UV = high performance liquid chromatography with ultraviolet detection; LC-FL = liquid chromatography with fluorescence detection; LC-MS = liquid chromatography with mass spectrometry; Le+ = Lewis positive; Le- = Lewis negative; MRM = multiple reaction monitoring; N/A = not available; NMR = nuclear magnetic resonance, or nano-LC-chip-TOF (time of flight); PC = paper chromatography; PGC-LC-MS = porous graphitized carbon–liquid chromatography–mass spectrometry; SD = standard deviation; Se+ = secretor; Se- = non-secretor; UHPLC-FL = ultra-high-performance liquid chromatography with fluorescence detection; UHPLC-MS-MS = ultra-high-performance liquid chromatography with tandem mass spectrometry; UHPLC/QqQ-MS = ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry; U.S. = United States.

Based on the equivalence of Inbiose's 2'-FL to other 2'-FL preparations with GRAS status, publicly available data and information establishing the GRAS status of 2'-FL are therefore incorporated by reference to previous GRAS evaluations in the sections below (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020, 2021e,f). Since the most recent GRAS conclusion for which the FDA has issued a letter of "no questions" was notified to the FDA in March 2020, an updated comprehensive search of the publicly available scientific literature was conducted to identify new information relevant to the safety of 2'-FL published through 14 January 2022. The following databases were accessed: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine, BIOSIS Toxicology, BIOSIS Previews, CAB ABSTRACTS, Embase, Foodline: SCIENCE, FSTA, MEDLINE, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile. A summary of the historical basis for the GRAS determination of 2'-FL and any newly identified studies relevant to the safety of Inbiose's 2'-FL are provided below.

6.2 Absorption, Distribution, Metabolism and Excretion

As discussed previously, 2'-FL produced by microbial fermentation is structurally identical to the 2'-FL found in human milk and will be physiologically equivalent in terms of absorption, distribution, metabolism, and excretion. Therefore, the metabolism of this HMO, when added to infant formula, is expected to be identical to those of other HMOs naturally present in human breast milk.

The metabolism of HMOs, including 2'-FL, has been previously described in detail (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020, 2021e,f). Briefly, HMOs are resistant to enzymatic hydrolysis and are therefore not significantly digested in the upper gastrointestinal tract (Brand-Miller *et al*., 1998; Engfer *et al*., 2000; Rudloff and Kunz, 2012; EFSA, 2019).

Only minor structural changes of the HMOs were observed after *in vitro* digestion of HMOs using artificial gastric fluid (porcine intestinal brush border membranes within the physiologic range of incubation time, pH, and enzyme activity) (Gnoth *et al.*, 2000). As a result, intact 2'-FL can reach the large intestine, where it is partially metabolized by microbiota into short-chain fatty acids (Salli *et al*., 2019; Van den Abbeele *et al*., 2021). The effects of HMOs on gastrointestinal bacterial growth are bacterial strain- and HMO structure-dependent. Different growth patterns were observed for different bacteria strains when exposed to the same HMOs *in vitro* (Cheng *et al.*, 2021; Van den Abbeele *et al*., 2021). *Bifidobacteriaceae* were shown to be a major group of bacteria involved in the fermentation of 2'-FL (Bunesova *et al*., 2016; Van den Abbeele *et al.,* 2021). A large portion of undigested 2'-FL (ranging from 40 to 97%) was shown to be excreted in the feces (Chaturvedi *et al*., 2001; Coppa *et al*., 2001). Only a small fraction of neutral HMOs including 2'-FL was suggested to be transported transcellularly by receptor-mediated transcytosis, and/or by paracellular flux (Gnoth *et al*., 2001). Indeed, less than 1% of ingested 2'-FL was found to be systematically available in breast-fed infants or infants fed with formula supplemented with 2'-FL (Goehring *et al*., 2014; Marriage *et al*., 2015). A small fraction of absorbed HMOs was excreted unchanged or only slightly metabolized in the urine of breast-fed infants, at levels that correlate with their dietary intake from breast milk (Rudloff *et al*., 2012; Goehring *et al*., 2014).

6.3 Toxicological Studies

Pivotal data and information related to the safety of 2'-FL has been discussed previously and is hereby incorporated by reference to Part IV.B.5 of GRN 546, Section 6.3 of GRN 571, Section IV.E of GRN 650, Part 6 Section B.4 of GRN 735, and Section 6.C of GRN 932 (Glycom A/S, 2014, 2016; U.S. FDA, 2015a,b, 2016, 2018a, 2021f; Jennewein Biotechnologie GmbH, 2015; Glycosyn, LLC and Friesland Campina Domo B.V., 2017; Advanced Protein Technologies, Corp., 2020). Analytical data of Inbiose's 2'-FL product establish the ingredient as chemically identical to its 2'-FL counterpart in human breast milk (see Section 2.1.1). For this notification, the specification of Inbiose's 2'-FL product was compared with other high-purity 2'-FL products previously approved as GRAS (*i.e.*, GRN 546, GRN 571, GRN 650, GRN 735, GRN 749, GRN 897, GRN 932). Based on the carbohydrate analytical data, 2'-FL produced by Inbiose is of equal or greater purity when compared to other high-purity 2'-FL preparations that have previously been determined to be GRAS, and thus, the studies characterizing the toxicity and safety of 2'-FL in animal models are considered relevant to the safety assessment of Inbiose's ingredient (see Table 6.3-1).

No evidence of toxicity related to the administration of 2'-FL has been reported in a wide range of previous 2'-FL GRAS Notice submissions (GRN 546, GRN 571, GRN 650, GRN 735, and GRN 932). Several new studies were identified that had been conducted to evaluate the potential toxicological or genotoxic effects of 2'-FL since the most recent 2'-FL GRAS determination to receive a "no questions" letter from the FDA was prepared (*i.e.*, GRN 932); however, no data were identified that would affect the overall conclusion of safety for the 2'-FL ingredient, as established in previous GRNs.

Therefore, in the absence of any safety concerns from previously evaluated and newly identified preclinical safety and genotoxicity studies, it is reasonable to assume that Inbiose's high-purity 2'-FL ingredient (with comparable low levels of carbohydrate impurities to other high-purity 2'-FL products already considered to be GRAS) is safe for use in non-exempt term infant formulas at use levels up to 2.4 g 2'-FL/L and other food and beverage uses within the general population at levels up to 1.2 g/L or 40 g/kg, consistent with uses listed in other GRNs (GRN 546, GRN 571, GRN 650, GRN 735, GRN 749, GRN 897, GRN 932).

The specifications of Glycom A/S's (Glycom's) 2'-FL (GRN 546 and 650), Jennewein Biotechnologie, GmbH's (Jennewein's) 2'-FL (GRN 571), Glycosyn, LLC and Friesland Campina Domo B.V.'s (Glycosyn/FreislandCampina's) 2'-FL (GRN 735), DuPont Nutrition & Health's (DuPont's) 2'-FL (GRN 749 and 897), and Advanced Protein Technologies Corp.'s (APTech's) 2'-FL (GRN 932) are presented in Table 6.3-1 below for comparison (Glycom A/S, 2014, 2016; Jennewein Biotechnologie, GmbH, 2015; U.S. FDA, 2015a,b, 2016, 2018a,b, 2020, 2021f; Glycosyn, LLC and Friesland Campina Domo B.V., 2017; DuPont Nutrition & Health, 2017, 2019; Advanced Protein Technologies, Corp., 2020).

Table 6.3-1 Product Specifications for Inbiose's 2'-FL in Comparison to Other High-Purity 2'-FL Preparations Used in Toxicological Studies Reported in GRNs 546, 571, 650, 735, 749, 897, and 932

Parameter	Proposed Specifications for Inbiose's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain)	Specifications Reported for Other 2'-FL Products						
		Glycom's 2'-FL from Chemical Synthesis (GRN No. 546)	Jennewein's 2'-FL from Microbial Fermentation with *E. coli* BL21 (DE3 strain) (GRN No. 571)	Glycom's 2'-FL from Microbial Fermentation with *E. coli* K12 (DH1 strain) (GRN No. 650)	Glycosyn/FC's 2'-FL from Microbial Fermentation with *E. coli* K12 (GI724 strain) (GRN No. 735)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 749)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 897)	APTech's 2'-FL from Microbial Fermentation with *C. glutamicum* (APC199 strain) (GRN No. 932)
Identification								
Appearance (Color)	White	White to off-white	White to ivory	White to off-white	White	White to off-white/ivory	White/off-white	White to off-white/ivory
Appearance (Form)	Powder	Powder	Powder	Powder or agglomerates	Homogenous powder	Dry powder	Dry powder	Dry powder
Appearance in solution	Clear, colorless to slightly yellow	NS	NS	NS	NS	Clear, colorless to slightly yellow	NMT 300 ICUMSA	Clear, colorless to slightly yellow
Identity (2'-FL)	Conform to reference standard, 2'-FL derived from human milk	Retention time of main component corresponds to ± 3%	Chemical identity confirmed by NMR and LC-MS/MS	Retention time of main component corresponds to ± 3%	Chemical identity confirmed by NMR	Conform to reference standard, 2'-FL derived from human milk	Conform to reference standard, 2'-FL derived from human milk	Conform to reference standard, 2'-FL derived from human milk and synthetic 2'-FL
Chemical Specifications								
Moisture	NMT 5.0%	NMT 9.0%	NMT 9.0%	NMT 5.0%	NMT 5.0%	NMT 9.0%	NMT 5.0%	NMT 9.0%
pH	3.0 to 7.5 (20°C, 10% solution)	3.0 to 7.5 (20°C in 5% solution)	NS	3.2 to 5.0 (20°C in 5% solution)	3.0 to 7.5 (10% solution)	NS	NS	NS
Protein	NMT 100 µg/g	0.1%	NMT 100 µg/g	0.01%	NMT 0.01%	NMT 100 µg/g	NMT 100 µg/g	NMT 100 µg/g
Ash (%)	NMT 0.5	NMT 0.2	NMT 0.5	NMT 1.5	NMT 0.2	NMT 0.5	NMT 0.5	NMT 0.5
Endotoxins (E.U./mg)	NMT 10	NMT 50	NMT 0.3	NS	NMT 10	NMT 0.3	NMT 0.3	NMT 0.1
Carbohydrates (% DM)								
2'-FL	NLT 94%	NLT 95.0%	NLT 90.0%	NLT 94.0%	NLT 90.0%	NLT 82.0%	NLT 96.0%	NLT 94.0%
Sum of carbohydrates[a]	NS	NS	NS	NLT 96.0%[a]	NS	NS	NS	NS
Lactose	NMT 5.0%	NS	NMT 5.0%	NMT 3.0%	NMT 3.0%	NMT 8.0%	NMT 5.0%	NMT 5.0%

Table 6.3-1 Product Specifications for Inbiose's 2'-FL in Comparison to Other High-Purity 2'-FL Preparations Used in Toxicological Studies Reported in GRNs 546, 571, 650, 735, 749, 897, and 932

Parameter	Proposed Specifications for Inbiose's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain)	Specifications Reported for Other 2'-FL Products						
		Glycom's 2'-FL from Chemical Synthesis (GRN No. 546)	Jennewein's 2'-FL from Microbial Fermentation with *E. coli* BL21 (DE3 strain) (GRN No. 571)	Glycom's 2'-FL from Microbial Fermentation with *E. coli* K12 (DH1 strain) (GRN No. 650)	Glycosyn/FC's 2'-FL from Microbial Fermentation with *E. coli* K12 (GI724 strain) (GRN No. 735)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 749)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 897)	APTech's 2'-FL from Microbial Fermentation with *C. glutamicum* (APC199 strain) (GRN No. 932)
Fucose	NS	NS	NMT 3.0%	NMT 1.0%	NMT 2.0%	NS	NS	NMT 3.0%
Allo-lactose	NS	NS	NS	NS	NMT 2.0%	NS	NS	NS
Glucose	NS	NS	NMT 3.0%	NS	NMT 2.0%	NS	NS	NMT 3.0%
Galactose	NS	NS	NMT 3.0%	NS	NMT 2.0%	NS	NS	NMT 3.0%
Difucosyllactose (DFL)	NMT 5.0%	NS	NMT 5.0%	NMT 1.0%	NS	NMT 7.0%	NMT 5.0%	NMT 5.0%
2'-Fucosyl-D-lactulose	NS	NS	NS	NMT 1.0%	NS	NS	NS	NS
3-fucosyllactose	NS	NS	NMT 5.0%	NS	NS	NS	NS	NMT 5.0%
Fucosyl-galactose	NS	NS	NMT 3.0%	NS	NS	NS	NS	NMT 3.0%
Sum of other carbohydrates[b]	NMT 5.0%[b]	NS	NS	NS	NS	NMT 6.0%[b]	NMT 5.0%[b]	NS
Heavy Metals								
Arsenic (mg/kg)	NMT 0.2	NS	NMT 0.2	NS	NMT 0.1	NMT 0.2	NMT 0.2	NMT 0.1
Cadmium (mg/kg)	NMT 0.05	NS	NMT 0.1	NS	NMT 0.01	NMT 0.05	NMT 0.05	NMT 0.01
Aluminum (mg/kg)	NS	NS	NS	NS	NMT 4.8	NS	NS	NS
Lead (mg/kg)	NMT 0.05	NMT 0.8	NMT 0.02	NMT 0.1	NMT 0.05	NMT 0.05	NMT 0.05	NMT 0.02
Mercury (mg/kg)	NMT 0.5	NS	NMT 0.5	NS	NMT 0.05	NMT 0.5	NMT 0.5	NMT 0.05
Microbiological Contaminants								
Total aerobic mesophilic plate count (CFU/g)	NMT 1,000	NMT 500	NMT 10,000	NMT 500	NMT 3,000	NMT 1,000	NMT 1,000	NMT 500
Yeast (CFU/g)	NMT 100	NMT 10	NMT 100[c]	NMT 10	NMT 10	NMT 100	NMT 100[c]	NMT 100[c]
Mold (CFU/g)	NMT 100	NMT 10	-	NMT 10	NMT 10	NMT 100	-	-
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	Absent in 11 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	NS
Salmonella spp.	Absent in 25 g	Absent in 25 g	Absent in 100 g	Absent in 25 g	Absent in 25 g	Absent in 100 g	Absent in 750 g	Absent in 25 g

Table 6.3-1 Product Specifications for Inbiose's 2'-FL in Comparison to Other High-Purity 2'-FL Preparations Used in Toxicological Studies Reported in GRNs 546, 571, 650, 735, 749, 897, and 932

Parameter	Proposed Specifications for Inbiose's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain)	Specifications Reported for Other 2'-FL Products						
		Glycom's 2'-FL from Chemical Synthesis (GRN No. 546)	Jennewein's 2'-FL from Microbial Fermentation with *E. coli* BL21 (DE3 strain) (GRN No. 571)	Glycom's 2'-FL from Microbial Fermentation with *E. coli* K12 (DH1 strain) (GRN No. 650)	Glycosyn/FC's 2'-FL from Microbial Fermentation with *E. coli* K12 (Gl724 strain) (GRN No. 735)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 749)	DuPont's 2'-FL from Microbial Fermentation with *E. coli* K-12 (MG1655 strain) (GRN No. 897)	APTech's 2'-FL from Microbial Fermentation with *C. glutamicum* (APC199 strain) (GRN No. 932)
Cronobacter sakazakii	Absent in 25 g	Absent in 10 g	Absent in 100 g	Absent in 10 g	Absent in 25 g	Absent in 100 g	Absent in 300 g	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	NS	Absent in 25 g	NS	Absent in 25	Absent in 25	NS
Bacillus cereus (CFU/g)	NMT 50	NMT 50	NS	NMT 50	NMT 100	NMT 10	NMT 10	NS

2'-FL = 2'-fucosyllactose; CFU = colony forming units; E.U. = endotoxin units; GRN = Generally Recognized as Safe Notice; ICUMSA = International Commission for Uniform Methods of Sugar Analysis; LC-MS/MS = liquid chromatography with tandem mass spectrometry; NLT = not less than; NMR = nuclear magnetic resonance; NMT = not more than; NS = not specified.

[a] Sum of carbohydrates (also referred to as sum of human-identical milk saccharides in GRN 650) is defined as the sum of 2'-FL, lactose, difucosyllactose, and fucose.

[b] Sum of other carbohydrates, such as 3-fucosyllactose, 2-fucosyl-D-lactulose, fucosyl-galactose, glucose/galactose, fucose, sorbitol/galactitol, mannitol, and trihexose.

[c] Specification for yeast and mold is combined.

6.3.1 Genotoxicity Studies Conducted with Other 2'-FL Preparations

As described in Section 6.3 above, pivotal genotoxicity data related to 2'-FL has been previously evaluated in many GRNs. The product specifications defined for Inbiose's 2'-FL ingredient are considered to be comparable to other high-purity 2'-FL products previously approved as GRAS (see Table 6.3-1); thus, studies characterizing the genotoxicity of these other preparations are considered relevant to the safety assessment of Inbiose's ingredient. No evidence of genotoxicity related to 2'-FL has been reported in any previous 2'-FL GRN submission (*i.e.*, GRN 546, GRN 571, GRN 650, GRN 735, GRN 749, GRN 897, GRN 932). Studies conducted with a 2'-FL mixture with difucosyllactose (DFL) have also been included in the summary Table 6.3.1-1, below, as they were presented in GRN 815 (Glycom A/S, 2018; U.S. FDA, 2019a).

Table 6.3.1-1 Summary of Genotoxicity Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain or Cell Type	Length of Study	2'-FL Concentration	Result	Reference
Studies conducted with Glycom's 2'-FL (purity 99%) produced by chemical synthesis (GRN 546)					
Bacterial reverse mutation test according to OECD TG 471 (OECD, 1997a)	*Salmonella* Typhimurium TA98, TA100, TA102, TA1535, and TA1537)	Plate incorporation (± S9) and pre-incubation methods (± S9)	2'-FL dissolved in water up to 5,000 µg/plate (± S9)	2'-FL was non-mutagenic under the conditions of this test.	Coulet *et al.* (2013)
In vitro mammalian cell gene mutation test according to OECD TG 476 (OECD, 1997b)	L5178Y tk +/- mouse lymphoma cells	Short-term treatment: 4 hours (± S9); Long-term treatment: 24 hours (- S9)	Short-term treatment: 492 to 5,000 µg/mL for 4 hours (± S9); Long-term treatment: 1.7 to 5,000 µg/mL for 24 hours (-S9)	2'-FL was non-mutagenic in L5178Y tk +/- mouse lymphoma cells.	
Studies conducted with Jennewein´s 2'-FL (purity 92.4%) produced by microbial fermentation (GRN 571)					
Bacterial reverse mutation assay according to OECD TG 471 (OECD, 1997a)	*S.* Typhimurium TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation assay (± S9) and pre-incubation method (± S9)	2'-FL dissolved in DMSO within the concentration range of 31.6 to 5,000 µg/plate (± S9)	2'-FL is not cytotoxic or mutagenic under the conditions of this study.	Lauenstein, 2014a [unpublished]; Report No. 30512 (in: Appendix M2 of GRN 571)
In vivo bone marrow micronucleus test in rat, according to OECD TG 474 (OECD, 1997c)	Groups of 5 male and 5 female (Crl:CD(SD)) rats	24 hours (vehicle and 3 dose groups) and 48 hours (vehicle control and high-dose-treated animals)	2'-FL dissolved in vehicle (0.8% aqueous HPMC) at 500, 1,000 or 2,000 mg/kg bw, positive control (cyclophosphamide (27 mg/kg bw i.p.)	2'-FL did not produce signs of systemic acute toxicity and was mutagenic nor genotoxic under the conditions of this study.	Lauenstein, 2014b [unpublished]; Report No. 30513 (in: Appendix M1 of GRN 571)

Table 6.3.1-1 Summary of Genotoxicity Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain or Cell Type	Length of Study	2'-FL Concentration	Result	Reference
Studies conducted with Glycom´s 2'-FL (purity 97.6%) produced by microbial fermentation (GRN 650)					
Bacterial reverse mutation assay according to OECD TG 471 (OECD, 1997a)	*S.* Typhimurium TA98, TA100, TA1535, and TA1537; *Escherichia coli* WP2uvrA (± S9)	Plate incorporation (± S9) and pre-incubation methods (± S9)	2'-FL dissolved in water at concentration range of 52-5,000 µg/plate (plate incorporation) and 492-5,000 µg/plate (pre-incubation method)	2'-FL was non-mutagenic under the conditions of this test.	Verspeek-Rip (2015) [unpublished], cited in: GRN 650
In vitro micronucleus assay according to OECD TG 487 (OECD, 2016)	Peripheral human lymphocytes	Short-term treatment: 3 hours (± S9); Long-term treatment: 24 hours (- S9)	2'-FL dissolved in water at concentrations of 512, 1,600, or 2,000 µg/mL in short- and long-term treatment	2'-FL was non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.	Verbaan (2015) [unpublished], cited in: GRN 650
Studies conducted with Friesland Campina´s 2'-FL (purity 94%) produced by microbial fermentation (GRN 735)					
Bacterial reverse mutation assay according to OECD TG 471 (OECD, 1997a)	*S.* Typhimurium TA1535, TA1537, TA98, TA100; *E. coli* WP2 uvrA (± S9)	Plate incorporation method	2'-FL dissolved in PBS tested at the concentration range of 62 to 5,000 µg/plate	2'-FL was non-mutagenic under the conditions of this test.	van Berlo *et al*. (2018)
In vitro micronucleus assay according to OECD TG 487 (OECD, 2016)	Peripheral human lymphocytes	Long-term treatment: 24 hours (- S9); Short-term treatment: 4 hours (± S9)	Long-term (24 hours - S9) and short-term treatment (4 hours ± S9): 2'-FL dissolved in culture medium (RPMI1640) at concentration range of 500, 1,000, and 2,000 µg/ml	2'-FL was non-genotoxic under the conditions of this test.	
Studies conducted with APTech's 2'-FL (purity ≥94%) produced by microbial fermentation (GRN 932)					
Bacterial reverse mutation assay according to OECD TG 471 (OECD, 1997a)	*S.* Typhimurium TA1535, TA1537, TA98, TA100; *E. coli* WP2 uvrA (± S9)	1st and 2nd main studies (± S9) – methods not specified	2'-FL (vehicle type not specified) tested at the concentration range of 313-5,000 µg/plate	2'-FL was non-mutagenic under the conditions of this test.	Hong, 2019a [unpublished] Report No. B18674; cited in: Appendix J of GRN 932
In vitro Mammalian Chromosomal Aberration Test	Chinese hamster lung (CHL/IU) cells (± S9)	Long-term treatment: 24 hours (- S9); Short-term treatment: 6 hours (± S9)	2'-FL dissolved in DMSO at concentrations of 1,250, 2,500, and 5,000 ug/mL	2'-FL was non-clastogenic under the conditions of this study.	Hong, 2019b [unpublished] Report No. B18675; cited in: Appendix J of GRN 932

Table 6.3.1-1 Summary of Genotoxicity Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain or Cell Type	Length of Study	2'-FL Concentration	Result	Reference
In vivo Micronucleus Test in ICR mice	Groups of 5 CrlOri:CD1(ICR), SPF male mice	Twice at 24- hour intervals	2'-FL dissolved in saline tested at 0, 2,500 mg/kg, 5,000 and 7,500 mg/kg	All doses were well-tolerated, and no clinical signs were observed. 2'-FL did not induce micronuclei in the bone marrow cells of mice under the conditions of this study.	Hong, 2019c [unpublished] Report No. B18676; cited in: Appendix J of GRN 932
Studies Conducted with Glycom's 2'-FL/DFL mixture (containing 75% 2'-FL) mixture produced by microbial fermentation (GRN 815)					
Bacterial reverse mutation assay according to OECD TG 471 (OECD, 1997a)	*S.* Typhimurium TA1535, TA1537, TA98, TA100; *E. coli* WP2 uvrA (± S9)	Plate incorporation (± S9) and pre-incubation methods (± S9)	2'-FL/DFL mixture up to 5,000 µg/plate for both methods (Test article contained only 75% 2'-FL by weight)	2'-FL/DFL test article was non-mutagenic under the conditions of this test.	Phipps *et al.* (2018)
In vitro micronucleus assay according to OECD TG 487 (OECD, 2016)	Human lymphocytes	Long-term treatment: 24 hours (- S9); Short-term treatment: 3 hours (± S9)	Long-term (24 hours - S9) and short-term treatment (4 hours ± S9): 2'-FL/DFL mixture at concentration range of 500, 1,000, and 2,000 µg/mL (Test article contained only 75% 2'-FL by weight)	2'-FL/DFL test article was non-genotoxic under the conditions of this test.	

+ S9 = with metabolic activation; - S9 = without metabolic activation; 2'-FL = 2'-fucosyllactose; bw = body weight; DFL = difucosyllactose; DMSO = dimethylsulfoxide; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; HPMC = hydroxypropylmethylcellulose; i.p. = intraperitoneally; OECD = Organisation for Economic Co-operation and Development; PBS = phosphate-buffered saline; SPF = specific-pathogen-free; TG = Test Guideline.

In addition, 2 new tests of genotoxicity were identified in the updated search of the scientific literature for studies published after the submission of GRN 932. Both tests were conducted as part of the same study to evaluate the safety of an HMO mixture containing 2'-FL with lacto-*N*-fucopentaose I; however, the results from both tests do not affect the overall conclusion of safety established in previous GRAS Notices.

In the recently conducted bacterial reverse mutation test, the potential mutagenicity of an HMO mixture containing 2'-FL and lacto-*N*-fucopentaose I (LNFP-I) was evaluated *in vitro* with *S.* Typhimurium strains TA98, TA100, TA1535, and TA1537, or *E. coli* strain WP2 *uvrA* (pKM101) (Phipps *et al*., 2021). The evaluated mixture contained 31.5% (w/w) 2'-FL and 59.4% (w/w) LNFP-I and was tested at concentrations of 5.55, 16.65, 55.5, 166.5, 555, 1,665, or 5,550 µg/plate when corrected for LNFP-I and 2'-FL content (approximately 91%) of the test article by weight. No evidence of cytotoxicity or precipitate was observed in plate incorporation or preincubation assays, with or without metabolic activation. Mean revertant colony counts remained consistent across the test article groups and controls at any dose level, with or without metabolic activation. The authors therefore concluded that the test article (containing 31.5% 2'-FL w/w) was not mutagenic.

Additionally, an *in vitro* mammalian cell micronucleus test was conducted using the same HMO mixture (*i.e.*, containing 31.5% and 59.4% 2'-FL and LNFP-I, respectively) with human lymphocytes (Phipps *et al*., 2021). Concentrations ranging from 0.5 to 2,220 µg/plate of the HMO mixture were tested in the absence or presence of metabolic activation for short- (3 hours) and long-term exposures (20 hours). Micronucleus analysis and cytotoxicity were evaluated following the defined exposure periods. No evidence of cytotoxicity and no statistically significant increase in the percentage of micronucleated cells (relative to vehicle controls) were observed at any tested concentration. The authors concluded that the HMO mixture (containing 31.5% 2'-FL w/w) was not clastogenic nor aneugenic at the levels tested.

6.3.2 Toxicological Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

As described in Section 6.3 above, pivotal toxicity data related to the 2'-FL ingredient has been evaluated in many previous GRNs. The product specifications defined for Inbiose's 2'-FL ingredient are considered to be comparable to other high-purity 2'-FL products previously approved as GRAS (see Table 6.3-1); thus, studies characterizing the toxicity of these other preparations are considered relevant to the safety assessment of Inbiose's ingredient. No test article related toxicity has been reported in any previous 2'-FL GRN submission (*i.e.*, GRN 546, GRN 571, GRN 650, GRN 735, GRN 749, GRN 897, and GRN 932). The toxicological studies in GRN 749 and 897 are briefly summarized below in Table 6.3.2-1, and the summaries are presented in the following sections. Studies conducted with a 2'-FL mixture with DFL (as reported in GRN 815) have also been included.

Table 6.3.2-1 Summary of Toxicological Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain	Length of Study	2'-FL Dose and Route of Administration	Result	Reference
Studies conducted with Glycom's 2'-FL (purity 99%) produced by chemical synthesis (GRN 546)					
14-day oral toxicity study	Groups of 5 male and 5 female Wistar Crl:WI (Han) rats	14 days (PND 7 to 20)	0, 2,000, 5,000, or 7,500 mg 2'-FL/kg bw/day, and 7,500 FOS mg/kg bw/day (reference item)	Due to marked clinical signs (transient lower body weight gain and liquid feces) as well as 2 unexplained deaths at 7,500 mg/kg bw/day, 6,000 mg/kg bw/day was the highest recommended dose in the subchronic study.	Coulet *et al.* (2013)
90-day oral toxicity study according to OECD TG 408 (OECD, 1998)	Groups of 10 male and 10 female Wistar Crl:WI (Han) rats	At least 90 days (PND 7 to 97)	0, 2,000, 5,000, or 6,000 mg 2'-FL/kg bw/day 2'-FL), and 6,000 mg/kg bw/day FOS (reference item)	2'-FL was well-tolerated and did not elicit any adverse effects up to 5,000 mg/kg bw/day. The NOAEL of 5,000 mg/kg bw/day was considered.	
Studies conducted with Jennewein's 2'-FL (purity from 94.1% to 97.9%) produced by microbial fermentation (GRN 571)					
7-day dietary toxicity pilot study	Groups of 10 four-week-old female (Crl:CD(SD)) rats	7 days	Oral – dietary; 10% 2'-FL in feed and untreated diet *ad libitum*	2'-FL was well-tolerated. No mortalities were observed and there were no adverse test item-related effects. 10% 2'-FL in diet was recommended for the subchronic treatment	Leuschner, 2014 [unpublished] Report No. 30566; (in: GRN 571: Appendix M3)
90-day oral diet rat study according to OECD TG 408 (OECD, 1998)	Groups of 10 4-week-old male and female rats (Crl:CD(SD)); Additional groups of 3 and 9 animals per sex included in the control (0%) and treatment (10%) groups, respectively, used exclusively for blood sampling	90 days	Oral – dietary; 10% 2'-FL in feed and untreated diet *ad libitum*	NOAEL of 7,660 mg/kg bw/day (corresponding to 10% dietary concentration of 2'-FL) was determined.	Hansen, 2014 [unpublished]; Report No. 30514 (in: Appendix M2 of GRN 571)

Table 6.3.2-1 Summary of Toxicological Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain	Length of Study	2'-FL Dose and Route of Administration	Result	Reference
3-week pre-clinical study of 2'-FL in farm piglets	27 male and 21 female domestic Yorkshire crossbred swine farm piglets	20 days from Day 2 of lactation	Oral – dietary; 0, 200, 500, or 2,000 mg/L corresponding to doses of 0, 29.37, 72.22, or 291.74 mg/kg bw/day in males and 0, 29.30, 74.31, and 298.99 mg/kg bw/day in females, respectively	The dietary exposure to 2'-FL at concentrations up to 2,000 mg/L (up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females) was well-tolerated and supported normal growth patterns in neonatal piglets with no adverse effects.	Hanlon and Thorsrud, 2014 [published]
Studies Conducted with Glycom's 2'-FL (purity 97.6%) produced by microbial fermentation (GRN 650)					
90-day oral toxicity study according OECD TG 408 (OECD, 1998)	Groups of 10 male and 10 female Wistar Crl:WI (Han) rats	Oral gavage from PND 7 till at least PND 97	0 (water), 2,000, 4,000, or 5,000 mg 2'-FL/kg bw/day, or the reference compound, FOS, at a dose concertation of 5,000 mg/kg bw/day	NOAEL of 5,000 mg/kg bw/day, the highest dose tested, was determined.	Penard, 2015 [unpublished] (cited in: GRN 650)
Studies Conducted with Friesland Campina's 2'-FL (purity 94%) produced by microbial fermentation (GRN 735)					
90-day oral diet rat study according to OECD TG 408 (OECD, 1998)	Groups of 10 male and 10 female Crl:WI(Han) rats	90 days	Oral – dietary; 0% (controls), 3%, 6%, and 10% (2'-FL w/w) *ad libitum*	NOAEL of ≥7,250 and ≥7,760 mg 2'-FL /kg bw/day for males and females, respectively (corresponding to 10% dietary concentration of 2'-FL), was determined.	van Berlo *et al.*, 2018
Studies Conducted with APTech's 2'-FL (purity ≥94%) produced by microbial fermentation (GRN 932)					
Single oral dose toxicity study in juvenile rats	Groups of 5 male and female neonatal/juvenile Sprague-Dawley Crl:CD(SD) rats	Oral gavage on PND 7	0 (water) 2,500, 5,000, and 7,500 mg 2'-FL/kg bw	One death at 7,500 mg/kg bw was considered as natural death. The body weight gain was significantly suppressed (males at 7,500 mg/kg). No test item-related gross findings at any dose level. The LD_{50} was concluded to be greater than 7,500 mg/kg bw.	Han, 2019a [unpublished] Report No. B18672 (cited in: Appendix J of GRN 932)

Table 6.3.2-1 Summary of Toxicological Studies Conducted with 2'-FL Preparations Previously Concluded to be GRAS

Type of Study	Species and Strain	Length of Study	2'-FL Dose and Route of Administration	Result	Reference
90-day repeated oral dose toxicity study with a 4-week recovery period in juvenile rats	Groups of 10 male and female neonatal/juvenile Sprague-Dawley Crl:CD(SD) rats; Additional recovery groups of 5 male and female rats received 0 or 7,500 2'-FL mg/kg bw/day	Oral gavage from PND 7 for 90 days	0 (water), 2,500, 5,000, or 7,500 mg 2'-FL/kg bw/day	NOAEL of 7,500 mg/kg bw/day, the highest dose tested, was determined.	Han, 2019b [unpublished] Report No. B18673 (cited in: Appendix J of GRN 932)
Studies conducted with Glycom's 2'-FL/DFL mixture (containing 75% 2'-FL) produced by microbial fermentation (GRN 815)					
14-day repeated oral dose toxicity study in neonatal rats	Groups of 8 male and female neonatal rats [strain NR]	Oral gavage for 14 days	0 (water), 4,000, or 5,000 mg/kg bw/day (Test article contained only 75% 2'-FL by weight)	No test item related deaths. One death at 5,000 mg/kg bw/day was considered incidental. NOAEL of 5,000 mg/kg bw/day, the highest dose tested, was determined.	Flaxmer, 2017 Report No. RW47RS (cited in: Section 6.3.4 of GRN 815)
90-day repeated oral dose toxicity study with a 4-week recovery period in juvenile rats	Groups of 10 male and female neonatal/juvenile Sprague-Dawley Crl:CD(SD) rats; Additional recovery groups of 5 male and female rats received 0 or 5,000 mg test article/kg bw/day	Oral gavage from PND 7 for 90 days	0 (water), 1,000, 3,000, or 5,000 mg/kg bw/day (Test article contained only 75% 2'-FL by weight)	NOAEL of 5,000 mg/kg bw/day, the highest dose tested, was determined.	Phipps *et al*. (2018)

2'-FL = 2'-fucosyllactose; bw = body weight; DFL = difucosyllactose; FOS = fructo-oligosaccharide; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; LD_{50} = median lethal dose; NOAEL = no-observed-adverse-effect level; NR = not reported; OECD = Organisation for Economic Co-operation and Development; PND = Postnatal Day; TG = Test Guideline.

In addition to the previously evaluated studies, 2 new toxicological studies conducted with 2'-FL were identified in the updated search of the scientific literature for studies published after the submission of GRN 932. No toxicologically relevant findings were reported in the newly identified literature and therefore these studies do not affect the overall conclusion of safety established in previous GRAS Notices. The 2 new studies are briefly summarized below.

In a 21-day study conducted with 2-day old neonatal piglets, an HMO mixture [containing 2'-FL, 3-FL, LNT, 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL)] was administered at dose levels of either 5.75 or 8.0 g/L and the following safety endpoints were measured: mortality, clinical observations, body weight, food consumption, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), gross necropsy findings, organ weights, and histopathologic examinations (Hanlon, 2020). The evaluated HMO mixture contained 49.1% 2'-FL by weight. All animals aside from 1 male piglet survived to the end of the dosing period. No toxicologically-relevant effects were noted in any piglets who received the liquid diet containing the HMO test article, relative to the control animals, and the author therefore concluded that the study supports the safety of this HMO mixture (containing 2'-FL) added to infant formula products.

In a subchronic toxicity assessment of a novel HMO mixture containing 2'-FL and LNFP-I (31.5% and 59.4% 2'-FL and LNFP-I, respectively), Sprague-Dawley rats were administered the test substance at dose levels of 1,000, 3,000, or 5,000 mg/kg body weight/day *via* gavage for a 90-day test period (Phipps *et al*., 2021). The low- and mid-dose groups contained 10 males and 10 females per group, whereas the high-dose, vehicle control, and reference control groups contained 15 males and 15 females each. General clinical observations of morbidity and mortality were conducted twice daily and ophthalmic observations were made during the final dosing week. Body weight and food consumption were monitored throughout the dosing period, and physiological development was assessed using a functional observational battery test that included grip strength, auditory function, visual function, and spatial learning during the 12th week of dosing. Hematological, coagulation, and clinical biochemistry parameters were measured in blood samples collected at the end of the dosing period. Similarly, urine samples were collected for urinalysis at the end of the dosing and recovery periods. The rats were then weighed, killed, and necropsied, and individual organs were weighed before histopathological analysis. Nine deaths occurred throughout the study; however, as the majority of these deaths occurred in the reference control group (5), followed by the 2 that occurred in the mid-dose group, the 1 in the high-dose group, and the 1 that was attributed to a dosing error, the deaths were not considered to be related to the administration of the test article. No biologically relevant changes were observed in body weight, food consumption, or physiological development. No test-article related adverse effects were observed at any dose level, and therefore the authors concluded that the no-observed-adverse-effect level for the 2'-FL and LNFP-I mixture is 5,000 mg/kg body weight/day.

6.4 Human Studies

6.4.1 Previously Reviewed Clinical Studies of 2'-FL

Pivotal safety data and information provided from studies of the addition of 2'-FL to infant formula have been discussed previously and are hereby incorporated primarily by reference to Section VIII Part 6.B of GRN 749, Section 6.3 of GRN 897, and Section 6.C.5 of GRN 932 (U.S. FDA, 2018b, 2020, 2021f). Based on analytical data presented demonstrating that 2'-FL produced by Inbiose is of equal or greater purity to other 2'-FL preparations previously determined to be GRAS, studies characterizing the safety of these 2'-FL preparations in humans are considered relevant to the safety assessment of Inbiose's ingredient.

The results confirm that 2'-FL is well-tolerated in infants when provided at concentrations within the normal range measured in human milk. 2'-FL also is well-tolerated in health adult subjects. Adverse effects of high intakes of 2'-FL are similar to those observed with other sources of dietary fiber (*e.g*., gastrointestinal discomfort) and is self-limiting.

The clinical studies previously included in GRNs 546, 571, 650, 735, 749, 815, 852, 897, 929, and 932 are briefly summarized below in Table 6.4.1-1.

Table 6.4.1-1 Summary of Human Studies to Support the Safety of Inbiose's 2'-FL

Type of Study	Population	Length of Study	Dose	Result	Reference
Prospective, controlled, growth and tolerance Study, R	254 term infants enrolled by Day of Life 5, and exclusively fed with formula or human milk since birth	4 months (enrolled by Day of Life 5 until 119)	Formula containing: • 2.4 g /L GOS (n=68) (control formula) • 0.2 g/L 2'-FL and 2.2 g/L GOS (n=62) • 1.0 g/ L 2'-FL and 1.4 g/L of GOS (n=59) Breastfed reference group (n=65)	No significant difference between groups in the percentage of study participants that experienced adverse events. The formulas were well-tolerated and that there was comparable average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomiting.	Marriage *et al.* (2015)
DB, R, PC, PD	100 healthy adult volunteers	2 weeks	Single daily doses of 2'-FL, LNnT, or a combination of 2'-FL and LNnT at a ratio of 2:1 at 5, 10, or 20 g/day Single daily dose of 2'-FL or LNnT alone (at 5, 10, or 20 g/day), or a combination of 2'-FL and LNnT (5, 10, or 20 g/day at a ratio of 2:1) Placebo: Glucose	No clinically relevant changes in hematological or biochemical parameters were observed. 2'-FL was well-tolerated, and no changes in bowel habits *versus* control were noted. The results support that 2'-FL is safe and well-tolerated in healthy adults.	Elison *et al.* (2016)
Blinded, controlled, R, MC, PD	175 healthy, full-term infants (0 to 6 months of age)	Time of enrollment until 12 months of age	Standard infant formula supplemented with 2'-FL (at a target concentration of 1.0 g/L reconstituted formula) in combination with LNnT (at a target concentration of 0.5 g LNnT/L reconstituted formula)	The results indicated that formula containing 2'-FL and LNnT was safe and well-tolerated, and supported age-appropriate growth.	Puccio *et al.* (2017)
Prospective, DB, R, PC, MC	131 healthy term infants	5 weeks	Infant formula containing either 0 (formula control), 0.2 g 2'-FL/L and 0.2 g scFOS/L, or human breast milk (control)	The results indicated that infant formula containing 2'-FL was safe and well-tolerated and bared no significant difference from the human breast milk group.	Kajzer *et al.* (2016); Reverri *et al.* (2018)

Table 6.4.1-1 Summary of Human Studies to Support the Safety of Inbiose's 2'-FL

Type of Study	Population	Length of Study	Dose	Result	Reference
Cross-over DB, PC, FC	67 infants (2 months to 4 years of age) with documented cow milk protein allergy	1 week (7 to 9 days)	A formula supplemented with 1.0 g/L 2'-FL and 0.5 g/L LNnT; minimum of 240 mL daily Control: A formula (hypoallergenic, whey-based, extensively hydrolyzed formula without HMOs)	The results indicated that 2'-FL does not provoke allergic responses in cow milk protein allergy infants.	Nowak-Wegrzyn *et al.* (2019)
DB, R, PC, MC	63 infants (14 days of age)	6 weeks	A formula of partially-hydrolyzed, 100% whey protein with 0 or 0.25 g 2'-FL/L per day	The results indicated that formula containing 2'-FL was safe and well-tolerated.	Storm *et al.* (2019)

2'-FL = 2'-fucosyllactose; DB = double-blind; FC = food challenge; GOS = galacto oligosaccharides; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; MC = multi-center; PC = placebo-controlled; PD = parallel design; R = randomized; scFOS = short-chain fructo-oligosaccharides.

6.4.2 Newly Identified Clinical Studies of 2'-FL

Eleven new clinical studies of 2'-FL, or HMO mixtures containing 2'-FL, were identified in the updated search of the scientific literature for studies published after the submission of GRN 932. No evidence of toxicity related to the oral consumption of 2'-FL was reported in the additional identified studies. Therefore, none of the identified studies summarized in the sections below are expected to affect the overall conclusion of safety established in previous GRNs.

6.4.2.1 Studies Conducted with 2'-FL

In a non-randomized, single-group, multicenter study conducted in infants (0 to 60 days of age) with suspected food protein allergy or persistent feeding intolerance, participants were provided a hypoallergenic casein-based powdered extensively hydrolyzed formula containing 0.2 g 2'-FL/L for 2 months (Ramirez-Farias *et al*., 2021). Thirty-six infants completed the study and changes in primary outcomes were compared to measured values at enrollment (baseline). The primary outcomes of growth (weight, length, and head circumference), formula intake, tolerance measures, and clinical symptoms were assessed. A statistical improvement in weight-for-age z-score was observed over the course of the study. Persistent clinical symptoms either improved, resolved, or remained the same as they were at test initiation following 60 days of 2'-FL administration. Adverse events were observed in 15 infants throughout the study; however, these were generally mild and were not deemed related to the test article by the study investigators. The study authors concluded that 2'-FL, when added to extensively hydrolyzed infant formula, was safe and well-tolerated.

The safety of 2'-FL when used in young children formulas was evaluated as part of a study conducted by Leung *et al.* (2020) to investigate the effects of 3 different formulations on respiratory and gastrointestinal infections in toddlers. In this randomized, controlled, double-blind, parallel-group clinical trial, 2 independent groups of subjects (labelled YCF-A and YCF-C; n=114) received 2 different young children formulae containing 3.0 g 2'-FL/L for a test duration of 6 months. While the primary outcome measured as part of this study were upper respiratory and gastrointestinal tract infections, anthropometric data (*i.e.*, weight-for-age and height-for-age z-scores) for common growth metrics were also measured. No changes in anthropometric data were observed in toddlers who received young children formulae supplemented with 3.0 g 2'-FL/L. Additionally, adverse event occurrence was similar for each young children formulae relative to the reference formula group. The study authors concluded that the young children formulae tested, including those that contained 3.0 g 2'-FL/L, are safe and well-tolerated for use in toddlers.

In a pilot clinical trial included as part of a larger study on the effect of 2'-FL on gut microbiota composition, 20 adult participants (12 completed the study), who had previously been diagnosed with irritable bowel syndrome (IBS), ulcerative colitis, Crohn's disease, or celiac disease, were provided with 40 g of a nutritional formula (containing 2 g 2'-FL) to be consumed twice daily in a reconstituted beverage for 6 weeks for a dose of 4.0 g 2'-FL/day (Ryan *et al*., 2021). Participants were asked to complete questionnaires to characterize their individual gastrointestinal quality of life, inflammatory bowel disease, and digestive system frequency at baseline and at the end of the 6-week test period. Following the test administration period, participants reported an overall improved gastrointestinal quality of life (exceeding the minimal clinically important difference; 15.1 units on the Gastrointestinal Quality of Life index total score) and no serious test-article related adverse events. The presented data indicate that 2'-FL is safe for use in adult populations with chronic gastrointestinal conditions.

6.4.2.2 Studies Conducted with HMO Mixtures Containing 2'-FL

In an open-label prospective study, healthy term infants (7 days to 2 months of age) were separated into 3 groups and were exclusively breastfed (n=45), fed commercial 100% whey infant formula containing both 1.0 g/L 2'-FL (n=63) and 0.5 g/L lacto-*N*-neotetraose (LNnT) and *Lactobacillus reuteri* (DSM 17938), or fed a mixture of trial formula and breast milk (n=48) for 8 weeks (Román Riechmann *et al*., 2020). Primarily, measures of growth (*i.e*., weight, length, and head circumference) and gastrointestinal tolerance (assessed *via* an Infant Gastrointestinal Symptom Questionnaire) were monitored. Any adverse events that occurred throughout the study were also recorded and assessed for duration, intensity, and frequency. No significant differences were observed in anthropometric growth measures, gastrointestinal tolerance, or adverse events at the end of the 8-week test period between the 3 infant groups. Infant formula containing 1.0 g 2'-FL/L (and 0.5 g LNnT/L) was reported to be well-tolerated by the study authors.

In a prospective, open-label, single-arm clinical trial study of treatment options for IBS, 245 adults with a clinical IBS diagnosis who fulfilled the Rome IV criteria[4] for IBS were provided with 5 g of Glycom's 2'-FL/LNnT mixture (97% purity; 4:1 mass ratio) daily over a 12-week intervention period (Palsson *et al*., 2020). Abnormal bowel movements (as measured by the Bristol Stool Form Scale) were measured as the primary outcome of the study. Severity and/or frequency of abdominal pain, bloating, gastrointestinal symptoms, or IBS-related quality of life was compared to baseline levels for each participant. Any adverse events were also noted throughout the course of the study. Frequency of abnormal stool consistency was significantly decreased from baseline following the 12-week intervention. The severity of IBS symptoms experienced by participants who received the HMO mixture was also significantly reduced relative to

[4] https://theromefoundation.org/rome-iv/rome-iv-criteria/

baseline levels. Common side effects of mild gastrointestinal symptoms such as flatulence, abdominal pain and discomfort, and distension were reported throughout the study; however, no serious adverse events were considered to be related to the intervention. The study participants reported that the intervention was well-tolerated, and no safety concerns were identified with its use.

In the subsequent study, the effect of human milk oligosaccharide (Glycom's 2'-FL/LNnT blend) supplementation on adults with IBS was investigated in a parallel, randomized, double-blind, and placebo-controlled study (Iribarren *et al*., 2020). Sixty adult patients (male and female) with IBS symptoms of at least moderate severity were provided placebo (powdered glucose), 5 g 2'-FL/LNnT, or 10 g 2'-FL/LNnT for daily consumption over a 4-week treatment period. The HMO mixture contained 2'-FL and LNnT in a 4:1 ratio. Measurements were taken of body weight and height at baseline and upon test completion, and any adverse events or changes in medication and diet were recorded. Patients were also required to complete a questionnaire to assess severity of gastrointestinal and psychological symptoms. No significant differences were observed between treatment groups related to the severity of gastrointestinal symptoms or their occurrence. No significant adverse events were reported in the study. Thus, daily intake of 5 and 10 g 2'-FL/LNnT was concluded by the study authors to be well-tolerated by adults with IBS.

The effects of HMO supplementation on feeding tolerance, growth, and safety in preterm infants was investigated in a double-blind, placebo-controlled trial conducted with a liquid HMO supplement comprising of 2'-FL and LNnT in a 10:1 ratio (Hascoët *et al*., 2021 [abstract only]). Groups of 43 preterm infants (23 to 33 weeks' gestation) received either 0.374 g/kg body weight/day of the HMO supplement or 0.140 g/kg body weight/day of a glucose placebo. The primary measured outcome of this study was feeding tolerance, which was measured *via* days to reach full enteral feeding from birth. Secondarily, Fenton growth standards were used to calculate anthropometric z-scores, and gastrointestinal tolerance parameters (including adverse events) were also measured. There was no significant difference between the two groups in any of the measured parameters, aside from increased length-for-age and head circumference-for-age z-scores in the HMO test groups. No statistical difference between the groups was noted for gastrointestinal tolerance measures or adverse events, and the researchers concluded that HMO supplementation is safe and well-tolerated.

As a component of a prospective, randomized, double-blinded, placebo-controlled clinical trial to evaluate the effect of HMOs on intestinal bacteria, Fonvig *et al.* (2021) evaluated the safety of daily HMO intake in young overweight children (age 6 to 12) over an 8-week test period. Groups of 25 children at test initiation were provided with 4.5 g/day of either a placebo, 2'-FL/day, or a 4:1 2'-FL/LNnT mixture. Safety was assessed *via* the measurements of a range of biochemical markers (*i.e.*, of inflammatory blood markers, gut barrier integrity proteins, metabolic biomarkers) and occurrence of adverse events or gastrointestinal symptoms. There were no significant differences measured in any of the biochemical parameters and the occurrence of adverse events. Neither test article was observed to induce digestive tolerance issues as assessed by the Gastrointestinal Symptoms Rating Scale. Minor differences in bloating were noted between groups at the trial's midway point, which were considered by the authors to be clinically irrelevant. The authors therefore concluded that 2'-FL administered either on its own or in a mixture containing LNnT is safe and well-tolerated in young children.

In a double-blind, randomized, controlled, multi-country trial of infant formula supplemented with 2'-FL and 3'-galactosyllactose (3'-GL), Vandenplas *et al.* (2020) provided 215 formula-fed infants (≤14 days of age) with either a partially fermented infant formula (control) or the control formula containing 100 mg 2'-FL/100 mL and 15 mg 3'-GL/100 mL until they reached 17 weeks of age. A group of fully breastfed infants were included as an additional reference group. Weight gain was the primary parameter of interest in this study; however, other growth parameters (*i.e.*, length, head circumference, and anthropometric z-scores), gastrointestinal tolerance, and safety outcomes (*i.e.*, adverse events) were also measured. No significant difference was noted between the infants from either test group in any of the measured parameters, and the study authors concluded that 2'-FL and 3'-GL are safe and well-tolerated in healthy term infants.

In a multicenter study conducted to evaluate the safety of a diverse blend of 5 HMOs in term infant formula, 686 healthy formula-fed infants (ranging from ≥7 to ≤21 days old) were provided with infant formula containing 0, 1.5, or 2.5 g HMOs/L until the participants were 6 months of age. The 5-HMO blend contained 2'-FL, 2',3-difucosyllactose, lacto-neotetraose (LNT), 3'-SL, and 6'-SL at unspecified levels (Bauer *et al.*, 2021 [abstract only]). Ninety-six breastfed infants were included as an additional reference group. Measured parameters included weight gain, anthropometric measures, stooling pattern, gastrointestinal tolerance, and adverse events. Weight gain was considered non-inferior (*i.e.*, lower bound of the 95% confidence interval was above the non-inferiority margin of -3 g/day) in the test groups relative to the control group. All other measured parameters were comparable between the test, control, and breastfed reference groups. The blend of 5 HMOs was concluded to be safe and well-tolerated as well as supportive of age-appropriate growth.

The tolerability, safety, and effect on growth of an HMO mixture was recently evaluated in a randomized, multicenter, controlled, parallel nutritional noninferiority study conducted with term infants (Parshat *et al*., 2021). Two hundred and twenty-five healthy term infants were randomly assigned to groups who received infant formula either without (control) or with a mixture containing 5 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). An additional group of breastfed infants was included as a reference group. Measured growth parameters included weight, length, and head circumference. Safety was measured *via* occurrence of adverse events, while tolerability and behavioral parameters were measured *via* stool frequency and consistency, gurgitation, vomiting, and flatulence, and fussiness, crying, and awakening at night. No significant difference was observed in any of the tolerability, safety, or growth parameters, aside from softer stool and increased stool frequency in infants who received the HMO mix. The authors concluded that the HMO mixture is safe and well-tolerated by healthy term infants.

In a very recently published study conducted by Vandenplas *et al.* (2022), full-term infants aged 0 to 6 months with physician-diagnosed cow's milk protein allergy (CMPA) received 100% whey-based extensively hydrolyzed formula (EHF) supplemented with 2'-FL at a concentration of 1.0 g/L and LNnT at 0.5 g/L. This study was designed as a controlled, double-blind, randomized, multicenter, interventional clinical trial. The control formula group received EHF without the addition of HMOs. Body weight, length, and head circumference were measured monthly for 4 months (primary study endpoint), after 6 months, and at the age of 12 months. HMO-supplemented formula was observed to support normal growth in infants with CMPA. No significant group differences in anthropometric parameters were noted, and therefore, both formulas were considered safe and well-tolerated by the study authors.

6.5 Allergenicity

To determine if proteins from the newly introduced recombinant protein sequences in production host INB-2FL_03 could be excreted into the extracellular space during 2'-FL production, bioinformatic analysis of the introduced protein sequences was conducted to check for potential signal peptide (SignalP) sequences targeting for protein excretion (SignalP 5.0; Armenteros *et al*., 2019). Since no SignalP sequences were identified in the introduced sequences, none of the resultant recombinant proteins could be identified as an excreted protein. In addition, the complete cell debris is separated from the final 2'-FL product during the various purification steps included in 2'-FL manufacturing.

The potential allergenic activity of the newly introduced recombinant proteins in *E. coli K-12 MG1655*, introduced to obtain production host INB-2FL_03, was assessed by using the Allergen Online Tool (V21, released on 14 February 2021) of the University of Nebraska – Lincoln (FARRP, 2021). The most current version of the database contained 2233 putative allergen sequences. Potential allergenicity was evaluated by scanning each possible 80-amino acid segment of the recombinant protein (sliding window) to the database, and therefore looking for matches of at least 35% identity. No sequence alerts from potential allergens were identified for the recombinant proteins in INB-2FL_03.

Since lactose is used as substrate in the 2'-FL production process and small amounts of residual lactose are present in the final product, the label “contains milk”, in accordance with the requirements of the *Food Allergy, Labelling and Consumer Protection Act of 2004*, must be added (U.S. FDA, 2004).

6.6 General Recognition

As discussed, the use of 2'-FL as an ingredient in non-exempt term infant formula at levels up to 2.4 g/L and in various conventional food products has been evaluated by multiple experts, qualified through scientific training and experience, in the safety evaluation of food and infant formula ingredients (GRNs 546, 571, 650, 735, 749, and 897). The use of 2'-FL in infant formula at concentrations up to 1.2 g/L (alone or in combination with other HMOs) and various food products also has been the subject of comprehensive evaluations by multiple authoritative bodies, including EFSA (EFSA, 2015a,b, 2019). As Inbiose has demonstrated that 2'-FL manufactured by the company is qualitatively and quantitatively highly similar to 2'-FL ingredients that have been the subject of previous GRAS evaluations and global novel food approvals, and is intended for use in the same foods and at the same use levels as those concluded to be GRAS, conclusions on the safety of 2'-FL for these uses issued by various experts and scientific bodies forms a basis for general recognition of Inbiose’s GRAS conclusion. Convening of a GRAS Panel was therefore not considered necessary to support a GRAS conclusion on the basis that this HMO ingredient has been evaluated by multiple GRAS Panels and authoritative bodies, including the FDA and EFSA.

6.7 Conclusion

Based on the above data and information presented herein, Inbiose has concluded that 2'-FL is GRAS, on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products as described in Section 1.3.

2'-FL therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. § 170.255 List of Supporting Data and Information

Advanced Protein Technologies, Corp. (2020). *Determination of the Generally Recognized as Safe (GRAS) Status of 2'-Fucosyllactose as a Food Ingredient [GRAS Notification]: Parts 1 & 2*. (GRN No. 932). Prepared by Clarksville (MD): NutraSource, Inc. Prepared for Hwasung City, Gyeonggi-do, Republic of Korea: Advanced Protein Technologies, Corp. (APTech). Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=932 [Feb. 18, 2021 - FDA response - no questions].

Amyris, Inc. (2020). *Safety Assessment and Generally Recognized as Safe (GRAS) Notification of 2'-Fucosyllactose for Use as an Ingredient in Foods*. (GRN No. 987). Prepared by Emeryville (CA): Amyris, Inc. Submitted to: College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=987 [pending as of: June 16, 2021].

Armenteros JJA, Tsirigos K, Sønderby C, Petersen TN, Winther O, Brunak S, et al. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37(4):420-423. DOI:10.1038/s41587-019-0036-z.

Asakuma S, Urashima T, Akahori M, Obayashi H, Nakamura T, Kimura K, et al. (2008). Variation of major neutral oligosaccharides levels in human colostrum. Eur J Clin Nutr 62(4):488-494. DOI:10.1038/sj.ejcn.1602738. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Asakuma S, Hatakeyama E, Urashima T, Yoshida E, Katayama T, Yamamoto K, et al. (2011). Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J Biol Chem 286(40):34583-34592 [plus supplementary data]. DOI:10.1074/jbc.M111.248138. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Austin S, De Castro CA, Benet T, Hou Y, Sun H, Thakkar SK, et al. (2016). Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. Nutrients 8(6):346 [22pp]. DOI:10.3390/nu8060346. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Austin S, De Castro CA, Sprenger N, Binia A, Affolter M, Garcia-Rodenas CL, et al. (2019). Human milk oligosaccharides in the milk of mothers delivering term versus preterm infants. Nutrients 11(6):1282 [17pp, plus supplementary data]. DOI:10.3390/nu11061282.

Balogh R, Szarka S, Béni S (2015). Determination and quantification of 2'-O-fucosyllactose and 3-O-fucosyllactose in human milk by GC-MS as O-trimethylsilyl-oxime derivatives. J Pharm Biomed Anal 115:450-456. DOI:10.1016/j.jpba.2015.07.043. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Bao Y, Chen C, Newburg DS (2013). Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. Anal Biochem 433(1):28-35. DOI:10.1016/j.ab.2012.10.003. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

BASF SE (2019). *Generally Recognized as Safe (GRAS) Notice for 2 '-Fucosyllactose*. (GRN No. 852). Submitted by Florham Park (NJ): BASF SE. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=852 [Nov. 15, 2019 - FDA response - no questions].

Bauer V, Arciszewska M, Tarneva M, Dosev S, Sirma D, Olga N, et al. (2021). Term infant formula containing a diverse blend of five human milk oligosaccharides supports age-appropriate growth, is safe and well-tolerated: a double-blind, randomized controlled trial. Cogent Med 8(1):2002558-101 to 2002558-102 [abstract ID: 128]. DOI:10.1080/2331205X.2021.2002558.

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453-1462 [plus supplementary data]. DOI:10.1126/science.277.5331.1453.

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. DOI:10.1016/S0022-3476(98)70185-4.

Bunesova V, Lacroix C, Schwab C (2016). Fucosyllactose and L-fucose utilization of infant *Bifidobacterium longum* and *Bifidobacterium kashiwanohense*. BMC Microbiol 16(1):248 [12pp]. DOI:10.1186/s12866-016-0867-4.

Castanys-Muñoz E, Martin MJ, Prieto PA (2013). 2'-Fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71(12):773-789. DOI:10.1111/nure.12079.

CDC (2015). *National Health and Nutrition Examination Survey (NHANES): 2013-2014*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2013 [page last updated: October 30, 2015].

CDC (2016). *National Health and Nutrition Examination Survey (NHANES): 2013-2014 – Dietary Data*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/Search/DataPage.aspx?Component=Dietary&CycleBeginYear=2013 [date published: September 2016].

CDC (2018). *National Health and Nutrition Examination Survey (NHANES): 2015-2016 – Dietary Data*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/Search/DataPage.aspx?Component=Dietary&CycleBeginYear=2015 [date reviewed: October 30, 2018].

Chaturvedi P, Warren CD, Ruiz-Palacios GM, Pickering LK, Newburg DS (1997). Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. Anal Biochem 251(1):89-97. DOI:10.1006/abio.1997.2250. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, et al. (2001). Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11(5):365-372. DOI:10.1093/glycob/11.5.365. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Cheng L, Akkerman R, Kong C, Walvoort MTC, de Vos P (2021). More than sugar in the milk: human milk oligosaccharides as essential bioactive molecules in breast milk and current insight in beneficial effects. Crit Rev Food Sci Nutr 61(7):1184-120. DOI:10.1080/10408398.2020.1754756.

Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O (1999). Oligosaccharides in human milk during different phases of lacation. Acta Paediatr 88(Suppl. 430):89-94. DOI:10.1111/j.1651-2227.1999.tb01307.x. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Coppa GV, Pierani P, Zampini L, Bruni S, Carloni I, Gabrielli O (2001). Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In: Newburg DS, editor. *Bioactive Components of Human Milk: 8th International Conference of the International Society for Research on Human Milk and Lactation*, Oct. 25-29, 1997, Plymouth, Mass. (Advances in Experimental Medicine and Biology, Vol. 501). New York (NY): Kluwer Academic/Plenum Publishers, pp. 307-314.

Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Ficcadenti A, Padella L, et al. (2011). Oligosaccharides in 4 different milk groups, *Bifidobacteria*, and *Ruminococcus obeum*. J Pediatr Gastroenterol Nutr 53(1):80-87. DOI:10.1097/MPG.0b013e3182073103. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Coulet M, Phothirath P, Constable A, Marsden E, Schilter B (2013). Pre-clinical safety assessment of the synthetic human milk, nature-identical, oligosaccharide Lacto-*N*-neotetraose (LNnT). Food Chem Toxicol 62:528-537. DOI:10.1016/j.fct.2013.09.018.

Datsenko KA, Wanner BL (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97(12):6640-6645. DOI:10.1073/pnas.120163297.

Donovan SM, Comstock SS (2016). Human milk oligosaccharides influence neonatal mucosal and systemic immunity. Ann Nutr Metab 69(Suppl. 2):42-51. DOI:10.1159/000452818. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

DuPont Nutrition & Health (2017). *Comprehensive GRAS Assessment of the Proposed Uses of 2'-O-Fucosyllactose in Term Infant Formulas, Toddler Formulas, and Foods Targeted to Toddlers*. (GRN No. 749). Prepared by Rockville (MD): LSRO Solutions. Prepared for Wilmington (DE): Danisco Nutrition and Health, DuPont Nutrition & Health. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=749 [Apr. 23, 2018 - FDA response - no questions].

DuPont Nutrition & Health (2019). G*enerally Recognized as Safe (GRAS) Determination for the Use of 2'-O-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas*. (GRN No. 897). Prepared by Port Royal (VA): JHeimbach LLC. Prepared for Wilmington (DE): DuPont Nutrition and Health. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=897 [Jun. 12, 2020 - FDA response – no questions].

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

EFSA (2015b). Statement on the safety of lacto-*N*-neotetraose and 2'-*O*-fucosyllactose as novel food ingredients in food supplements for children (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no: EFSA-Q-2015-00594, EFSA-Q-2015-00595, adopted: 28 October 2015 by European Food Safety Authority). EFSA J 13(11):4299 [12pp]. DOI:10.2903/j.efsa.2015.4299. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4299.

EFSA (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA Panel on Additives and Products or Substances Used in Animal Feed/FEEDAP) (Question nos: EFSA-Q-2016-00069, EFSA-Q-2017-00211, adopted: 21 February 2018 by European Food Safety Authority). EFSA J 16(3):5206 [24pp]. DOI:10.2903/j.efsa.2018.5206. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5206.

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 Nutrition, Novel Foods and Food Allergens/NDA) (Question no: EFSA-Q-2018-00374, adopted: 15 May 2019 by European Food Safety Authority). EFSA J 17(6):5717 [23pp]. DOI:10.2903/j.efsa.2019.5717. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5717.

Elison E, Vigsnaes LK, Rindom Krogsgaard L, Rasmussen J, Sørensen N, McConnell B, et al. (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 [plus supplementary table]. 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. DOI:10.1093/ajcn/71.6.1589.

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, et al. (2000). Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 30(2):181-192. DOI:10.1097/00005176-200002000-00016. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Erney R, Hilty M, Pickering L, Ruiz-Palacios G, Prieto P (2001). Human milk oligosaccharides: a novel method provides insight into human genetics. In: Newburg DS, editor. *Bioactive Components of Human Milk*. 8th International Conference of the International Society for Research on Human Milk and Lactation, Oct. 25-29, 1997, Plymouth, Mass. (Advances in Experimental Medicine and Biology, Vol. 501). New York (NY): Kluwer Academic/Plenum Publishers, pp. 285-297. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

FARRP (2021). *AllergenOnline Version 21: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org/ [Released: February 14, 2021].

Ferreira AL, Alves R, Figueiredo A, Alves-Santos N, Freitas-Costa N, Batalha M, et al. (2020). Human milk oligosaccharide profile variation throughout postpartum in healthy women in a Brazilian cohort. Nutrients 12(3):790 [21pp, plus supplementary table]. DOI:10.3390/nu12030790.

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: Glycom A/S, 2018 [GRN 815].

Fonvig CE, Amundsen ID, Vigsnæs LK, Sørensen N, Frithioff-Bøjsøe C, Christiansen M, et al. (2021). Human milk oligosaccharides modulate fecal microbiota and are safe for use in children with overweight: An RCT. J Pediatr Gastroenterol Nutr 73(3):408-414. DOI:10.1097/mpg.0000000000003205.

Freddolino PL, Amini S, Tavazoie S (2012). Newly identified genetic variations in common *Escherichia coli* MG1655 stock cultures. J Bacteriol 194(2):303-306. DOI:10.1128/JB.06087-11.

Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila C, et al. (2011). Preterm milk oligosaccharides during the first month of lactation. Pediatrics 128(6):e1520-e1531. DOI:10.1542/peds.2011-1206. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, et al. (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(1):97-104 [plus supplementary Appendix A]. DOI:10.1016/j.ab.2012.07.027. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, et al. (2014). Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. Electrophoresis 35(6):811-818. DOI:10.1002/elps.201300490. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Glycom A/S (2014). *GRAS Exemption Claim for 2'-O-Fucosyllactose*. (GRN No. 546). Submitted by Lyngby, Denmark: Glycom A/S to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=546 [Correction, Sep. 24, 2014; Sep. 16, 2015 - FDA response - no questions].

Glycom A/S (2016). *GRAS Exemption Claim for 2'-O-Fucosyllactose (2'-FL) Produced by Fermentation*. (GRN 650). Submitted by Kongens Lyngby, Denmark, Glycom A/S to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=650 [Nov. 23, 2016 - FDA response - no questions].

Glycom A/S (2018). *GRAS Notice for 2'-Fucosyllactose / Difucosyltactose (2'-Fl / DFL)*. (GRN No. 815). Prepared by Hørsholm, Denmark: Glycom A/S. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=815 [Aug. 20, 2019 - FDA response - no questions].

Glycosyn, LLC and Friesland Campina Domo B.V. (2017). *GRAS Notification of Purified 2'-Fucosyllactose (2'-FL) Food Usage Conditions for General Recognition of Safety*. (GRN No. 735). Prepared by Waltham (MA): Glycosyn, LLC and Friesland Campina Domo B.V. Submitted by Bonita Springs (FL) GRAS Associates, LLC. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=735 [Apr. 6, 2018 - FDA response - no questions].

Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S (2000). Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12):3014-3020. DOI:10.1093/jn/130.12.3014.

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 chromatography-mass spectrometry technique. J Biol Chem 276(37):34363-34370. DOI:10.1074/jbc.M104805200.

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 [11pp]. DOI:101610.101371/journal.pone.0101692. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Grollman EF, Ginsburg V (1967). Correlation between secretor status and the occurrence of 2'-fucosyllactose in human milk. Biochem Biophys Res Commun 28(1):50-53. DOI:10.1016/0006-291X(67)90404-4. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Gu F, Wang S, Beijers R, De Weerth C, Schols HA (2021). Structure-specific and individual-dependent metabolization of human milk oligosaccharides in infants: A longitudinal birth cohort study. J Agric Food Chem 69(22):6186-6199 [plus supplemental figures]. DOI:10.1021/acs.jafc.0c07484.

Han C-T (2019a) [unpublished]. *Single Oral Dose Toxicity Study of 2'-Fucosyllactose in Juvenile Sprague-Dawley Rats*. (Study Number B18672). Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Part 1, Ref. #4, p. 128, cited as: Biotoxtech, 2019d].

Han C-T (2019b) [unpublished]. *Ninety-Day Repeated Oral Dose Toxicity Study with a Four Week Recovery Period of 2'-Fucosyllactose in Juvenile Sprague-Dawley rats*. (Study Number B18673). Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Part 1, Ref. #5, p. 128, cited as: Biotoxtech, 2019e].

Hanlon PR (2020). A safety evaluation of mixed human milk oligosaccharides in neonatal farm piglets. Toxicol Res Appl 4 [8pp]. DOI:10.1177/2397847320971255.

Hanlon PR, Thorsrud BA (2014). A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets. Food Chem Toxicol 74:343-348 [plus supplementary data]. DOI:10.1016/j.fct.2014.10.025.

Hansen B (2014) [unpublished]. [Appendix M4]: Repeated dose 90-day oral toxicity study of 2´-fucosyllactose in rats - Administration via the diet - Limit test. (LPT Report No. 30514). Prepared by ENVIRO/LPT. In: Jennewein Biotechnologie GmbH (2015). *GRAS Exemption Claim for Use of 2'-Fucosyllactose (2'-FL) in Term Infant and Toddler Formulas [Part 2]*. (GRN 571). Prepared by Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted by Phoenix (AZ): ENVIRON International Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety, pp. 483-835. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

Hascoët J-M, Chevallier M, Gire C, Brat R, Rozé J-C, Norbert K, et al. (2021). Effect of a liquid supplement containing 2 human milk oligosaccharides (HMOs) in preterm infants: a multi-centered, double-blind, randomized, controlled trial. Virtually Presented at: *6th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition (WCPGHAN)*, June 2-5, 2021.

Hong S-Y (2019a) [unpublished]. *Bacterial Reverse Mutation Test of 2'-Fucosyllactose*. (Study Number B18674). Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Part 1, Ref. #1, p. 128, cited as: Biotoxtech, 2019a].

Hong S-Y (2019b) [unpublished]. *In Vitro Mammalian Chromosomal Aberration Test of 2'-Fucosyllactose*. (Study Number B18675). Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Part 1, Ref. #2, p. 128, cited as: Biotoxtech, 2019b].

Hong S-Y (2019c) [unpublished]. *In vivo Micronucleus Test of 2'-Fucosyllactose in ICR Mice*. (Study Number B18676). Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Part 1, Ref. #3, p. 128, cited as: Biotoxtech, 2019c].

Hong Q, Ruhaak LR, Totten SM, Smilowitz JT, German JB, Lebrilla CB (2014). Label-free absolute quantitation of oligosaccharides using multiple reaction monitoring. Anal Chem 86(5):2640-2647. DOI:10.1021/ac404006z.

Iribarren C, Törnblom H, Aziz I, Magnusson MK, Sundin J, Vigsnaes LK, et al. (2020). Human milk oligosaccharide supplementation in irritable bowel syndrome patients: a parallel, randomized, double-blind, placebo-controlled study. Neurogastroenterol Motil 32(10):e13920 [12pp, plus supplementary data]. DOI:10.1111/nmo.13920.

Ishizuka Y, Nemoto T, Fujiwara M, Fujita K-I, Nakanishi H (1999). Three-dimensional structure of fucosyllactoses in an aqueous solution. J Carbohydr Chem 18(5):523-533. DOI:10.1080/07328309908544016.

Jennewein Biotechnologie GmbH (2015). *GRAS Exemption Claim for Use of 2'-Fucosyllactose (2'-FL) in Term Infant and Toddler Formulas [Parts 1 & 2]*. (GRN 571). Prepared by Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted by Phoenix (AZ): ENVIRON International Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

Jennewein Biotechnologie GmbH (2020). *GRAS Notification for 2'-fucosyllactose: Exempt Infant Formula and Additional Uses*. (GRN No. 929). Prepared by Arlington (VA): Ramboll Environment and Health Prepared for Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety. Available at: Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=929 [Feb. 26, 2021 - FDA response - no questions].

Kajzer J, Oliver J, Marriage B (2016). Gastrointestinal tolerance of formula supplemented with oligosaccharides. FASEB J 30(1, Suppl.):abstract 671.4. DOI:10.1096/fasebj.30.1_supplement.671.4.

Kjærulff L (2014). *NMR Structural Studies of Oligosaccharides and Other Natural Products* [PhD dissertation]. Lyngby, Denmark: Technical University of Denmark (DTU), Department of Chemistry. Available at: https://orbit.dtu.dk/en/publications/nmr-structural-studies-of-oligosaccharides-and-other-natural-prod.

Kunz C, Rudloff S, Schad W, Braun D (1999). Lactose-derived oligosaccharides in the milk of elephants: Comparison with human milk. Br J Nutr 82(5):391-399. DOI:10.1017/S0007114599001798. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Lagström H, Rautava S, Ollila H, Kaljonen A, Turta O, Makela J, et al. (2020). Associations between human milk oligosaccharides and growth in infancy and early childhood. Am J Clin Nutr 111(4):769-778 [plus supplementary data]. DOI:10.1093/ajcn/nqaa010.

Lauenstein H-D (2014a) [unpublished]. Appendix M2: Mutagenicity study: Mutagenicity study of 2'-fucosyllactose in the *Salmonella typhimurium* reverse mutation assay (*In vitro*). (LPT Report No. 30512). Prepared by ENVIRON/LPT. In: Jennewein Biotechnologie GmbH (2015). *GRAS Exemption Claim for Use of 2'-Fucosyllactose (2'-FL) in Term Infant and Toddler Formulas [Part 1]*. (GRN 571). Prepared by Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted by Phoenix (AZ): ENVIRON International Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety, pp. 412-472. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

Lauenstein H-D (2014b) [unpublished]. Appendix M1: Micronucleus assay: Micronucleus test of the 2-fucosyllactose in bone marrow cells of the CD rat following oral administration. (LPT Report No. 30513). Prepared by ENVIRON/LPT. In: Jennewein Biotechnologie GmbH (2015). *GRAS Exemption Claim for Use of 2'-Fucosyllactose (2'-FL) in Term Infant and Toddler Formulas [Part 1]*. (GRN 571). Prepared by Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted by Phoenix (AZ): ENVIRON International Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety, pp. 356-411. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

Lefebvre G, Shevlyakova M, Charpagne A, Marquis J, Vogel M, Kirsten T, et al. (2020). Time of lactation and maternal fucosyltransferase genetic polymorphisms determine the variability in human milk oligosaccharides. Front Nutr 7:Article 574459 [12pp, plus supplementary data]. DOI:10.3389/fnut.2020.574459.

Leo F, Asakuma S, Nakamura T, Fukuda K, Senda A, Urashima T (2009). Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high-performance liquid chromatography. J Chromatogr A 1216(9):1520-1523. DOI:10.1016/j.chroma.2009.01.015. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Leo F, Asakuma S, Fukuda K, Senda A, Urashima T (2010). Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Biosci Biotechnol Biochem 74(2):298-303. DOI:10.1271/bbb.90614. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Leung TF, Ulfman LH, Chong MKC, Hon KL, Khouw IMSL, Chan PKS, et al. (2020). A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers. Pediatr Allergy Immunol 31(7):745-754 [plus supplementary data]. DOI:10.1111/pai.13276.

Leuschner PJ (2014) [unpublished]. Appendix M3: Jennewein 2'-FL oral toxicity studies in rats: 7-Day pilot study of 2´-fucosyllactose (CAS NO. 41263-94-9) After repeated dietary administration to female CD® rats. (LPT Report No. 30566). Prepared by ENVIRO/LPT. In: Jennewein Biotechnologie GmbH (2015). *GRAS Exemption Claim for Use of 2'-Fucosyllactose (2'-FL) in Term Infant and Toddler Formulas [Part 1]*. (GRN 571). Prepared by Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH. Submitted by Phoenix (AZ): ENVIRON International Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety, pp. 473-482. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

Liu S, Cai X, Wang J, Mao Y, Zou Y, Tian F, et al. (2021). Six oligosaccharides' variation in breast milk: a study in south China from 0 to 400 days postpartum. Nutrients 13(11):4017 [12pp]. DOI:10.3390/nu13114017.

Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA (2015). Infants fed a lower calorie formula with 2'-fucosyllactose (2'FL) show growth and 2'FL uptake like breast-fed infants. J Pediatr Gastroenterol Nutr 61(6):649-658. DOI:10.1097/MPG.0000000000000889.

Marx C, Bridge R, Wolf AK, Rich W, Kim JH, Bode L (2014). Human milk oligosaccharide composition differs between donor milk and mother's own milk in the NICU. J Hum Lact 30(1):54-61. DOI:10.1177/0890334413513923. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, et al. (2017). What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. Am J Clin Nutr 105(5):1086-1100 [plus supplementary data]. DOI:10.3945/ajcn.116.139980. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Menzel P, Vogel M, Austin S, Sprenger N, Grafe N, Hilbert C, et al. (2021). Concentrations of oligosaccharides in human milk and child growth. BMC Pediatr 21(1):481 [11pp, plus supplementary tables]. DOI:10.1186/s12887-021-02953-0.

Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, et al. (2004). Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. J Pediatr 145(3):297-303. DOI:10.1016/j.jpeds.2004.04.054. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

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. DOI:10.1097/01.mpg.0000228125.70971.af. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Nakhla T, Fu D, Zopf D, Brodsky NL, Hurt H (1999). Neutral oligosaccharide content of preterm human milk. Br J Nutr 82(5):361-367. DOI:10.1017/S0007114599001609. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

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 [10pp]. DOI:10.3390/nu11071447.

OECD (1992). *Safety Considerations for Biotechnology 1992*. Paris, France: Organisation for Economic Co-Operation and Development (OECD). Available at: http://www.oecd.org/dataoecd/8/3/2375496.pdf.

OECD (1997a). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [updated & adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/fr/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [latest version updated & adopted: 26 June 2020].

OECD (1997b). Mammalian erythrocyte micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 474) [updated & adopted: 21st July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264071285-en;jsessionid=60s3nuot5c3s1.x-oecd-live-01 [New Versions updated & adopted: 26 September 2014, 29 July 2016].

OECD (1997c). *In vitro* mammalian cell gene mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 476) [updated & adopted: 21st July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-476-in-vitro-mammalian-cell-gene-mutation-tests-using-the-hprt-and-xprt-genes_9789264264809-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02 [latest version updated & adopted: 29 July 2016].

OECD (2016). *In vitro* mammalian cell micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 487) [updated & adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en.

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 [7pp, plus supplementary figures]. DOI:10.14309/ctg.0000000000000276.

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(7):2871 [19pp, plus supplementary data]. DOI:10.3390/nu13082871.

Penard L (2015) [unpublished]. *2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-week Treatment-free Period: Final Version*. (Study No.: AB20757, Sponsor Ref. No.: GSN037). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. Cited In: Glycom, 2016 [GRN 650].

Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, et al. (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, Mikš MH, et al. (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. DOI:10.1002/jat.4071.

Plows JF, Berger PK, Jones RB, Alderete TL, Yonemitsu C, Najera JA, et al. (2021). Longitudinal changes in human milk oligosaccharides (HMOs) over the course of 24 months of lactation. J Nutr 151(4):876-882. DOI:10.1093/jn/nxaa427.

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(4):624-631. DOI:10.1097/MPG.0000000000001520.

Ramirez-Farias C, Baggs GE, Marriage BJ (2021). Growth, tolerance, and compliance of infants fed an extensively hydrolyzed infant formula with added 2'-FL fucosyllactose (2'-FL) human milk oligosaccharide. Nutrients 13(1):186 [7pp]. DOI:10.3390/nu13010186.

Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW (2018). Review of the clinical experiences of feeding infants formula containing the human milk oligosaccharide 2'-fucosyllactose. Nutrients 10(10):1346 [11pp]. DOI:10.3390/nu10101346.

Román Riechmann E, Moreno Villares JM, Domínguez Ortega F, Carmona Martínez A, Picó Sirvent L, Santana Sandoval L, et al. (2020). Real-world study in infants fed an infant formula with two human milk oligosaccharides. Nutr Hosp 37(4):698-706. DOI:10.20960/nh.03084.

Rudloff S, Kunz C (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr 3(3, Suppl.):398S-405S. DOI:10.3945/an.111.001594.

Rudloff S, Pohlentz G, Borsch 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. DOI:10.1017/S0007114511004016.

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 [16pp, plus supplementary figure]. DOI:10.3390/nu13030938.

Salli K, Anglenius H, Hirvonen J, Hibberd AA, Ahonen I, Saarinen MT, et al. (2019). The effect of 2'-fucosyllactose on simulated infant gut microbiome and metabolites; a pilot study in comparison to GOS and lactose. Sci Rep 9(1):13232 [15pp]. DOI:10.1038/s41598-019-49497-z.

Samuel TM, Binia A, de Castro CA, Thakkar SK, Billeaud C, Agosti M, et al. (2019). Impact of maternal characteristics on human milk oligosaccharide composition over the first 4 months of lactation in a cohort of healthy European mothers. Sci Rep 9(1):11767 [10pp, plus supplementary data]. DOI:10.1038/s41598-019-48337-4.

Siziba LP, Mank M, Stahl B, Gonsalves J, Blijenberg B, Rothenbacher D, et al. (2021). Human milk oligosaccharide profiles over 12 months of lactation: The Ulm SPATZ Health Study. Nutrients 13(6):1973 [18pp, plus supplementary tables]. DOI:10.3390/nu13061973.

Smilowitz JT, O'Sullivan A, Barile D, German JB, Lönnerdal B, Slupsky CM (2013). The human milk metabolome reveals diverse oligosaccharide profiles. J Nutr 143(11):1709-1718 [plus supplementary tables]. DOI:10.3945/ jn.113.178772. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Snoeck N, De Mol ML, Van Herpe D, Goormans A, Maryns I, Coussement P, et al. (2019). Serine integrase recombinational engineering (SIRE): A versatile toolbox for genome editing. Biotechnol Bioeng 116(2):364-374. DOI:10.1002/bit.26854.

Soyyılmaz B, Mikš MH, Röhrig CH, Matwiejuk M, Meszaros-Matwiejuk A, Vigsnæs LK (2021). The mean of milk: a review of human milk oligosaccharide concentrations throughout lactation. Nutrients 13(8):2737 [22pp, plus supplementary tables]. DOI:10.3390/nu13082737.

Storm HM, Shepard J, Czerkies LM, Kineman B, Cohen SS, Reichert H, et al. (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 [10pp]. DOI:10.1177/2333794x19833995.

Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura N, et al. (2003). Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. Br J Nutr 89(1):61-69. DOI:10.1079/BJN2002746. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Thum C, Wall CR, Weiss GA, Wang W, Szeto IMY, Day L (2021). Changes in HMO concentrations throughout lactation: influencing factors, health effects and opportunities. Nutrients 13(7):2272 [29pp, plus supplementary tables]. DOI:10.3390/nu13072272.

Thurl S, Müller-Werner B, Sawatzki G (1996). Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. Anal Biochem 235(2):202-206. DOI:10.1006/abio.1996.0113. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, et al. (2010). Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr 104(9):1261-1271. DOI:10.1017/S0007114510002072. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

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. DOI:10.1093/nutrit/nux044.

U.S. EPA (1997). *Escherichia Coli K-12 Final Risk Assessment: Attachment I--Final Risk Assessment of Escherichia Coli K-12 Derivatives*. Washington (DC): U.S. Environmental Protection Agency (U.S. EPA), Biotechnology Program under the Toxic Substances Control Act (TSCA). Available at: https://www.epa.gov/sites/production/files/2015-09/documents/fra004.pdf [last updated on September 27, 2012].

U.S. FDA (2004). Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA). (Public Law 108-282, Title II). Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://www.fda.gov/food/food-allergens-and-gluten-free-guidance-documents-and-regulatory-information/food-allergen-labeling-and-consumer-protection-act-2004-falcpa [content current as of: 07/16/2018].

U.S. FDA (2015a). *Agency Response Letter GRAS Notice No. GRN 546 [2'-O-Fucosyllactose, Lyngby, Denmark: Glycom A/S]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=546 [Correction: Sep. 24, 2014; Sep. 16, 2015 - FDA response - no questions].

U.S. FDA (2015b). *Agency Response Letter GRAS Notice No. GRN 571 [2'-Fucosyllactose, Reinbreitbach, Germany: Jennewein Biotechnologie, GmbH]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571 [Nov. 6, 2015 - FDA response - no questions].

U.S. FDA (2016). *[Agency Response Letter GRAS Notice No. GRN 650 [2'-O-Fucosyllactose, Lyngby, Denmark: Glycom A/S]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=650 [Nov. 23, 2016; Suppl. letters: Sep. 9, 2020; Sep. 11, 2020 - FDA response - no questions].

U.S. FDA (2018a). *Agency Response Letter GRAS Notice No. GRN 735 [2'-Fucosyllactose, Waltham (MA): Glycosyn, LLC and Friesland Campina Domo B.V.]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=735 [Apr. 6, 2018 - FDA response - no questions].

U.S. FDA (2018b). *Agency Response Letter GRAS Notice No. GRN 749 [2'-O-Fucosyllactose, Wilmington (DE): DuPont Nutrition & Health]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=749 [Apr. 23, 2018 - FDA response - no questions].

U.S. FDA (2019a). *Agency Response Letter GRAS Notice No. GRN 815 [2'-Fucosyllactose and diFucosyllactose, Hørsholm, Denmark Glycom A/S]*. (May 7, 2020, Sep. 11, 2020 - 4 additional letters). Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=815 [Aug. 20, 2019; Corrections, Addit. Correspond.: May 7, 2020; Sep. 11 - FDA response - no questions].

U.S. FDA (2019b). *Agency Response Letter GRAS Notice No. GRN 852 [2'-Fucosyllactose, Florham Park (NJ): BASF SE]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=852 [Nov. 15, 2019 - FDA response - no questions].

U.S. FDA (2020). *Agency Response Letter GRAS Notice No. GRN 897 [2'-O-Fucosyllactose, Wilmington (DE): DuPont Nutrition & Health]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=897 [Feb. 24, 2020; Correction: Apr. 13, 2020 - FDA response - no questions].

U.S. FDA (2021a). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/.

U.S. FDA (2021b). Part 170—Food additives. Section §170.30—Eligibility for classification as generally recognized as safe (GRAS). In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs (U.S. Food and Drug Administration)*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/.

U.S. FDA (2021c). *Agency Response Letter GRAS Notice No. GRN 951 [3-Fucosyllactose, Wilmington (DE): DuPont Nutrition and Biosciences]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=951 [Aug. 12, 2021 - FDA response - no questions].

U.S. FDA (2021d). *Agency Response Letter GRAS Notice No. GRN 987 [2'-fucosyllactose, Emeryville (CA): Amyris, Inc.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=987 [pending as of: June 16, 2021].

U.S. FDA (2021e). *Agency Response Letter GRAS Notice No. GRN 929 [2'-Fucosyllactose, Reinbreitbach, Germany: Jennewein Biotechnologie GmbH]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=929 [Feb. 26, 2021 - FDA response - no questions].

U.S. FDA (2021f). *Agency Response Letter GRAS Notice No. GRN 932 [2'-Fucosyllactose, Hwasung City, Gyeonggi-do, Republic of Korea: Advanced Protein Technologies Corp]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=932 [Feb. 18, 2021 - FDA response - no questions].

USDA (2016). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2013-2014.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [last Modified: October 13, 2016].

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. DOI:10.1016/j.fct.2018.04.049.

Van den Abbeele P, Sprenger N, Ghyselinck J, Marsaux B, Marzorati M, Rochat F (2021). A comparison of the in vitro effects of 2'fucosyllactose and lactose on the composition and activity of gut microbiota from infants and toddlers. Nutrients 13(3):726 [22pp, plus supplementary data]. DOI:10.3390/nu13030726.

van Leeuwen SS, Schoemaker RJ, Gerwig GJ, van Leusen-van Kan EJ, Dijkhuizen L, Kamerling JP (2014). Rapid milk group classification by ^{1}H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. Glycobiology 24(8):728-739. DOI:10.1093/glycob/cwu036.

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.

Vandenplas Y, Żołnowska M, Berni Canani R, Ludman S, Tengelyi Z, Moreno-Álvarez A, et al. (2022). Effects of an extensively hydrolyzed formula supplemented with two human milk oligosaccharides on growth, tolerability, safety and infection risk in infants with cow's milk protein allergy: a randomized, multi-center trial (CINNAMON Study Investigator Group). Nutrients 14(3):530 [14pp]. DOI:10.3390/nu14030530.

Verbaan IAJ (2015) [unpublished]. *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. Cited In: Glycom, 2016 [GRN 650, as: Verbaan, 2015b].

Verspeek-Rip CM (2015) [unpublished]. *Evaluation of the Mutagenic Activity of 2'FL in the Salmonella Typhimurium Reverse Mutation Assay and the Escherichia Coli Reverse Mutation Assay*. (Laboratory Project Identification: Project 507432; Substance 206374/B). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. Cited In: Glycom, 2016 [GRN 650].

Zhou Y, Sun H, Li K, Zheng C, Ju M, Lyu Y, et al. (2021). Dynamic changes in human milk oligosaccharides in Chinese population: a systematic review and meta-analysis. Nutrients 13(9):2912 [13pp, plus supplementary data]. DOI:10.3390/nu13092912.