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GLYCOM

Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

14 October 2021

Dr. Paulette Gaynor
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

Dear Dr. Gaynor:

Re: GRAS Notice for lacto-*N*-fucopentaose I / 2'-fucosyllactose (LNFP-I / 2'-FL)

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Glycom A/S [Kogle Allé 4, 2970 Hørsholm, Denmark], as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that lacto-*N*-fucopentaose I / 2'-fucosyllactose (LNFP-I / 2'-FL) produced by an *E. coli* K-12 (DH1)-derived strain, is GRAS on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products across multiple categories; these food uses of LNFP-I/2'-FL are therefore not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act. Information setting forth the basis for Glycom's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Christoph H. Röhrig, Ph.D.
Head of HMO Regulatory & Scientific Affairs
Glycom A/S

Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with registered address at Wurmisweg 576, 4303 Kaiseraugst, Switzerland.

GRAS NOTICE FOR LACTO-*N*-FUCOPENTAOSE I / 2'-FUCOSYLLACTOSE (LNFP-I/2'-FL)

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

SUBMITTED BY:
Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

DATE:
14 October 2021

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GRAS Notice for Lacto-*N*-fucopentaose I / 2'-Fucosyllactose (LNFP-I/2'-FL)

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GRAS Notice for Lacto-*N*-fucopentaose I / 2'-Fucosyllactose (LNFP-I/2'-FL)

Part 1. § 170.225 SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Glycom A/S[1] (Glycom) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that lacto-*N*-fucopentaose I (LNFP-I) containing 2'-fucosyllactose (2'-FL) (LNFP-I/2'-FL), as manufactured by Glycom and described herein, is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Glycom's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Glycom, the undersigned hereby certifies that all data and information presented in this Notice represents a complete, representative, and balanced submission, and considered all unfavorable, as well as favorable, information known to Glycom and pertinent to the evaluation of the safety and GRAS status of LNFP-I/2'-FL as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

[redacted]

15 Oct 2021

Christoph Röhrig, Ph.D.
Head of HMO Regulatory Affairs
Glycom A/S
Christoph.roehrig@dsm.com

Date

1.1 Name and Address of Notifier

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark
Tel: +45 8830 9500
Fax: +45 4593 3968

1.2 Common Name of Notified Substance

Lacto-*N*-fucopentaose I / 2'-Fucosyllactose; LNFP-I/2'-FL

[1] Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with registered address at Wurmisweg 576, 4303 Kaiseraugst, Switzerland.

1.3 Conditions of Use

LNFP-I/2'-FL is intended to be added to non-exempt term infant formula[2], foods targeted to young children, as well as uses in specific food and beverage products used by the general population (see Table 1.3-1; use levels are based upon a target quantity of LNFP-I per serving). Use of this ingredient in infant formula (*i.e.*, infants up to 12 months), toddler formulas[3] (*i.e.*, young children older than 12 months), and beverages targeted to young children will provide a use-level of LNFP-I of 1.5 g/L in ready-to-drink and reconstituted products, and up to 8.33 g/kg for products other than beverages for infants and young children (*e.g.*, baby foods). LNFP-I/2'-FL is also intended for use in food and beverages targeted towards the general U.S. population at levels up to 2.0 g/L or 20 g/kg. The maximum use-levels are proposed on the basis of providing similar levels of LNFP-I, on a body weight basis, as those consumed by breastfed infants (see Section 3.1.3). As noted above, use levels are expressed upon the amount of LNFP-I to be added. The corresponding exposure values for 2'-FL consumption are calculated from LNFP-I intakes based on the average values of 2'-FL and LNFP-I across five batches of LNFP-I/2'-FL in Table 2.3.2.2-1 (*i.e.*, 26.5/61.9 = 0.428).

Table 1.3-1 Proposed Food Uses and Use Levels for LNFP-I from LNFP-I/2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	240	0.48	2.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.12	1.5
	Toddler Formulas[e]	100[d]	0.12	1.5
	Other Baby Foods for Infants and Young Children	7 to 170	0.06 to 1.42	8.33
	Other Drinks for Young Children	120	0.18	1.5
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	0.8	20.0
	Cereal and Nutrition Bars	40	0.4	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	240	0.24	1.0
Milk Products	Buttermilk*	240	0.24	1.5
	Flavored Milk	240	0.24	1.5
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.48	2.0
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.10 to 0.25	1.5
	Yogurt*	170	0.34	10.0

[2] Infant formula products to which LNFP-I/2'-FL would be added are most likely to be formula containing partially hydrolyzed cow's milk protein as a protein base.

[3] Formula products targeting young children older than 12 months of age (*e.g.*, Nestlé Boost® Kid Essentials, Nestlé Nutren® Junior, or Pediasure® by Abbott Nutrition).

Table 1.3-1 Proposed Food Uses and Use Levels for LNFP-I from LNFP-I/2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.24	1.0

CFR = *Code of Federal Regulations*; LNFP-I = lacto-*N*-fucopentaose I; RACC = Reference Amounts Customarily Consumed per Eating Occasion; RTE = ready-to-eat; U.S. = United States.

* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2020a). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.

[b] Use level expressed on a LNFP-I basis in the final food, as consumed.

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

[d] RACC not available, 100 mL employed as an approximation.

[e] Formula products targeted toward young children (> 12 months of age)

[f] Portion sizes are based on representative products on the U.S. market.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020a), Glycom has concluded, on the basis of scientific procedures, that LNFP-I/2'-FL is GRAS for addition to non-exempt term infant formula and specified conventional food and beverage products as described in Table 1.3-1.

Part 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

LNFP-I/2'-FL manufactured by Glycom is obtained from microbial fermentation and is isolated from a single fermentation; thus, it is not a blend of separately produced compounds. LNFP-I/2'-FL consists of ≥ 75% LNFP-I and 2'-FL and contains small quantities of D-lactose and L-fucose, and minor amounts of other related and fully characterized carbohydrates originating from the fermentation process, resulting in a total specified saccharide concentration of ≥ 90%.

LNFP-I is a pentasaccharide derived from L-fucose attachment to the 2-position of the non-reducing end of the galactose sugar of lacto-N-tetraose (LNT). LNT in turn consists of D-galactose, D-glucosamine, and D-lactose (which itself consists of D-galactose and D-glucose). 2'-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. LNFP-I and 2'-FL, obtained from microbial fermentation, are chemically and structurally identical to LNFP-I and 2'-FL that are naturally present in human milk, as confirmed by ^{1}H and 2D nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Further description of the structural and chemical identity of LNFP-I/2'-FL is presented below in Table 2.1-1.

Table 2.1-1 Identity of LNFP-I/2'-FL

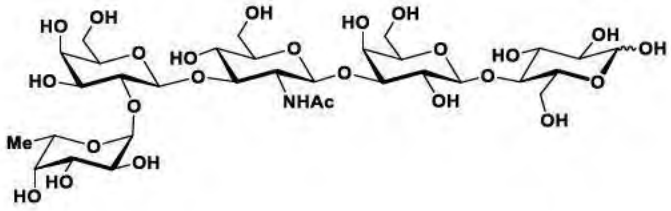
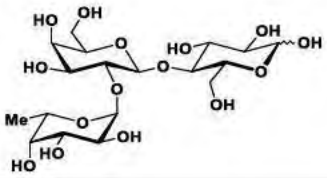
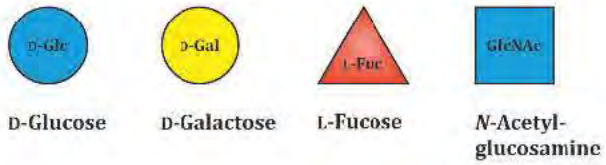
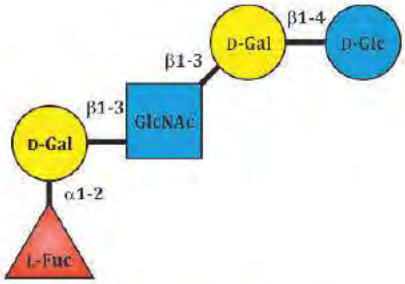
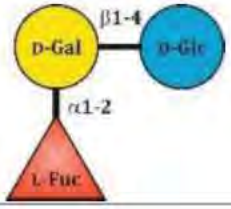
Generic Product Name	Lacto-*N*-fucopentaose I / 2'-fucosyllactose
Common Abbreviations	LNFP-I / 2'-FL (LNFP-I / 2'FL, LNFPI / 2FL mixture, LNF I / 2'-FL, LNF I / 2'FL, LNF I / 2FL)
Trade Name	GlyCare™ LNFP-I / 2FL 8001
Synonyms	LNFP-I: LNF I; 2'-FL: 2'-*O*-Fucosyllactose, 2'-Fucosidolactose, 2-FL
IUPAC Name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose / α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucose
IUPAC Abbreviation (extended)	α-L -Fuc*p*-(1-2)-β-D-Gal*p*-(1-3)-β-D-GlcNAc*p*-(1-3)-β-D-Galp-(1-4)-Glc / α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-D-Glc
IUPAC Abbreviation (condensed)	Fuc-(α1-2)-Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc / Fuc-(α1-2)-Gal-(β1-4)-Glc
Molecular Structure	**LNFP-I**  **2'-FL** 

Table 2.1-1 Identity of LNFP-I/2'-FL

Symbol Nomenclature	 D-Glucose D-Galactose L-Fucose *N*-Acetyl-glucosamine **LNFP-I**  **2'-FL** 
Molecular Formula	$C_{32}H_{55}NO_{25}$ / $C_{18}H_{32}O_{15}$
Molecular Mass	853.77 / 488.44
CAS Number	7578-25-8 / 41263-94-9
CAS Name	*O*-6-Deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-d-glucose / *O*-6-Deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-d-galactopyranosyl-(1→4)-d-glucose mixture

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

Briefly, LNFP-I/2'-FL is produced by a derivative of *Escherichia coli* K-12 DH1 MDO, a platform strain from which other human-identical milk oligosaccharide (HiMO) production strains have been derived (including several GRAS ingredients such as 2'-FL; lacto-*N*-neotetraose (LNnT); 2'-fucosyllactose/difucosyllactose (2'-FL/DFL); lacto-*N*-tetraose (LNT); 6'-sialyllactose (6'-SL) sodium salt; and 3'-sialyllactose (3'-SL) sodium salt). The characteristics of the parental (host) strain and the production strain for LNFP-I/2'-FL are described below.

2.2.1.1 Parental (Host) Strain

The genotypic characteristics of the parental/recipient microorganism, *E. coli* K-12 DH1, are presented in Table 2.2.1.1-1. The genome of *E. coli* K-12 has been sequenced and bioinformatic comparisons of the genomes of *E. coli* K-12 with other safe laboratory strains and various pathogenic isolates have been conducted (Blattner *et al.*, 1997; Lukjancenko *et al.*, 2010). The construction of strain *E. coli* K-12 DH1 has been described in the literature (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). The parental strain, *E. coli* K-12 DH1, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (deposited under DSM No. 4235)[4]. *E. coli* K-12 and its derivatives[5] have been specifically developed and recognized as "safety strains" for molecular biological research in the 1970s (Manning *et al.*, 1977; Smith, 1978) and they are the most widely applied microorganisms in biotechnology research laboratories around the world. In 1997, wild-type *E. coli* K-12 was also among the first organisms in the history of modern sequencing technologies for which the whole genome sequence became available (Blattner *et al.*, 1997). Recent comparison of sequenced *E. coli* genomes shows that K-12 and its closely related "safety strains" possess 10 to 20% fewer genes than their pathogenic cousins (Lukjancenko *et al.*, 2010). *E. coli* K-12-derived strains cannot colonize in the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). *E. coli* K-12 derivatives are currently among the preferred microorganisms for industrial biotechnology with wide application scope (Chen *et al.*, 2013; Theisen and Liao, 2017) and several GRAS ingredients and food enzymes have been authorized in the U.S. that were manufactured from *E. coli* K-12 derivatives [*e.g.*, 2'-FL (U.S. FDA, 2016a, 2018a), LNnT (U.S. FDA, 2015a), 2'-FL/DFL (U.S. FDA, 2019a), LNT (U.S. FDA, 2019b), 6'-SL (U.S. FDA, 2020b), 3'-SL (U.S. FDA, 2020c), alpha-cyclodextrin (U.S. FDA, 2004), chymosin (U.S. FDA, 2020a), L-leucine (U.S. FDA, 2010), and β-galactosidase (U.S. FDA, 2014)].

Table 2.2.1.1-1 Characteristics of the Parental Strain *Escherichia coli* K-12 DH1

Characteristics of *Escherichia coli* K-12 DH1	
Genotype	*F⁻, λ-, gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44.*
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*
Subspecies	Not applicable
Strain	*E. coli* strain K-12 DH1
Culture Collection	The German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen)
Deposition Number	DSM 4235 (ATCC33849)

[4] www.dsmz.de.

[5] Note: In the scientific literature, the term *E. coli* K-12 (or K-12) is only rarely used for the actual wild-type strain. "*E. coli* K-12" is in fact most commonly used collectively for all derivatives of K-12 that have been obtained during the 1970s by non-recombinant methods (*i.e.*, forced random mutagenesis).

2.2.1.2 Production Strain for LNFP-I/2'-FL

The host strain *E. coli* K-12 DH1 (DSMZ, 2015) was optimized for general oligosaccharide expression features (used as a "platform strain") by introduction of seven modifications related to the metabolism of various carbohydrates, thereby, improving the efficiency of the strain. This strain was given the designation "MDO." An overview of the modification events used for construction of strain MDO has been discussed previously and is hereby incorporated by reference to Section II.B.1.2 of GRAS Notice (GRN) 650. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing techniques. This parental strain has served as the host for engineering all of Glycom's production strains that are used to produce other HiMOs that have GRAS status, including LNnT, 2'-FL, 2'-FL/DFL, LNT, 3'-SL, and 6'-SL (U.S. FDA, 2015a, 2016a, 2018a, 2019a,b, 2020b,c).

The MDO strain is further modified to generate the production strain, *E. coli* K-12 DH1 MDO MP2176, to biosynthesize LNFP-I/2'-FL. The production strain is a genomically stable microorganism that provides high titers of LNFP-I/2'-FL. The strain has been deposited in the DSMZ in Braunschweig, Germany.

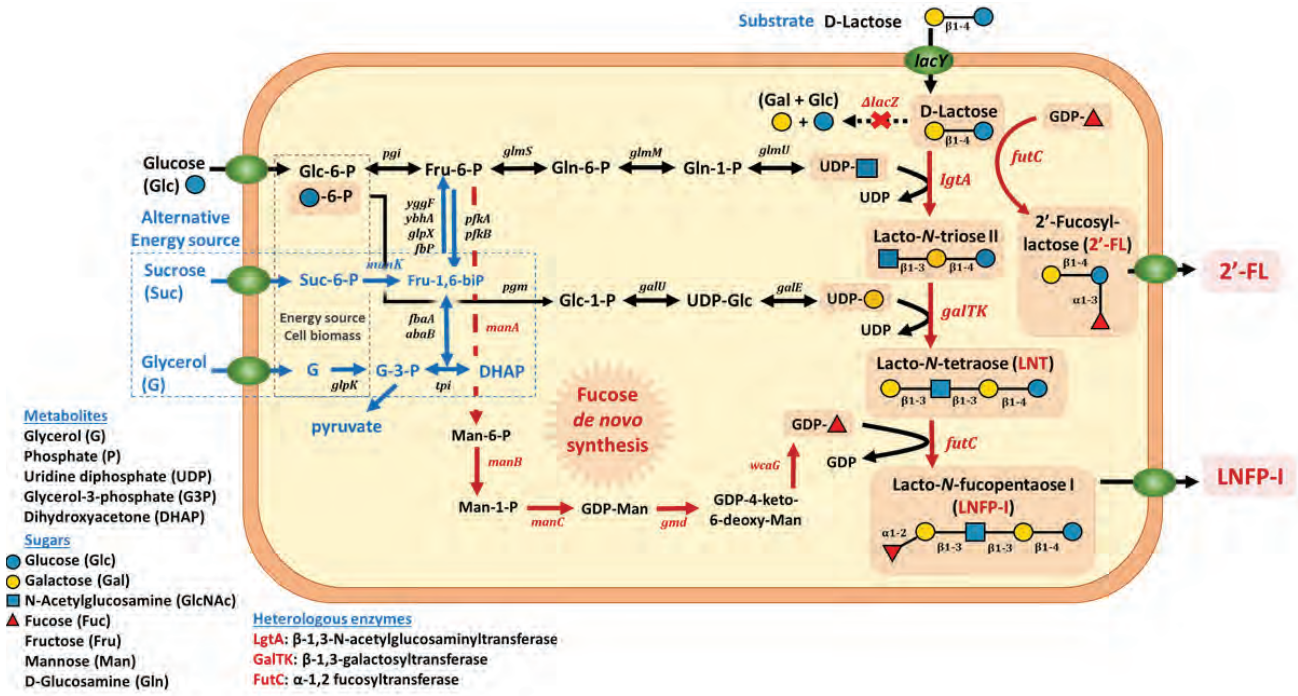
Figure 2.2.1.2-1 shows the biochemical pathway by which the production strain generates LNFP-I and 2'-FL using D-lactose and D-glucose[6] as a substrate and carbon source, respectively. The *lacZ* gene encoding the native *E. coli* β-galactosidase enzyme, which hydrolyzes D-lactose into D-glucose and D-galactose, was knocked-out in the HiMO platform strain to allow D-lactose to act as a substrate of HiMO biosynthesis. The platform strain was then further genetically modified to genomically overexpress the *lgtA* gene. The *lgtA* gene encodes for the enzyme β-1,3-*N*-acetylglucosaminyl-transferase that transfers a GlcNAc unit from the intracellular uridine diphosphate (UDP)-GlcNAc pool to the 3'-position of the galactose residue of lactose in a β-stereospecific manner to form the trisaccharide lacto-*N*-triose II. This sugar is further converted to the tetrasaccharide LNT by the activity of the β-1,3-galactosyl-transferase enzyme that is encoded by the genomically expressed *galTK* gene. Moreover, the strain bears an extra copy of the native colanic acid gene cluster and the heterologous gene *futC* that encodes for the enzyme α-1,2-fucosyl-transferase. Overexpression of the colanic acid gene cluster boosts the guanosine diphosphate (GDP)-fucose pool of the cell, and the FutC enzyme transfers a fucose unit from the activated sugar GDP-fucose to both substrates D-lactose and LNT to form 2'-FL and LNFP-I, respectively. No antibiotic resistance genes are present in the strain and no antibiotics or inducer molecules are used throughout the whole fermentation process.

Defined DNA sequences from the donor microorganisms were identified using genome databanks, codon-optimized by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning, and then generated by DNA synthesis, and are therefore referred to as "synthetic DNA." The gene cassettes used for introduction of the donor genes to the organism are well characterized and have been sequenced to verify their identities. As the introduced genes are produced by DNA synthesis from well characterized annotated genomes and were not cloned directly from the host genome using polymerase chain reaction (PCR)-based methods there is no risk of introducing unintended and undesirable genes from the donor organisms to the production organism.

During manufacture, the production strain remains intact, releases the LNFP-I and 2'-FL molecules to the extracellular space, and is entirely removed through a series of purification steps (as described in Section 2.2.2). Therefore, in this process the production strain is used exclusively as a processing aid.

[6] Alternatively, D-sucrose or glycerol may be used as carbon sources.

Figure 2.2.1.2-1 Pathway for LNFP-I/2'-FL Biosynthesis



2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I.

The inserted enzyme is well characterized and based on its enzymatic function, it was concluded that the introduced gene would not confer toxicogenic or pathogenic properties to the host organism. The genetic modifications made to the production strain result in the expression of proteins that are involved in the normal carbohydrate processing within their donor sources. These proteins are "carbohydrate-active enzymes" ("CAZy"), a panel of enzymes that can degrade, modify, or create glycosidic bonds, and accordingly, are involved in the metabolism of complex carbohydrates. When expressed together in the recipient strains, these proteins work in concert to convert the starting carbohydrates (D-lactose and D-glucose[7]) into oligosaccharides that are identical to those in human milk. In contrast, bacterial protein toxins (exotoxins) are known to mediate their pathogenic effects by disrupting cellular processes through various mechanisms such as proteolysis (*e.g.*, tetanus and botulinum), ADP-ribosylation (*e.g.*, cholera, pertussis, and diphtheria), or membrane disruptions through pore formation (Finlay and Falkow, 1997; Wilson *et al.*, 2002; Popoff, 2018). Bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the LNFP-I/2'-FL production strain, *E. coli* K-12 DH1 MP2176, by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing, colony PCR, and targeted sequencing methods.

[7] Alternatively, D-sucrose, or glycerol.

2.2.2 Description of the Production Process

Glycom's LNFP-I/2'-FL is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacture of LNFP-I/2'-FL is largely comparable to the production processes previously evaluated for other HiMOs with GRAS status (see GRNs 650, 659, 815, 833, 880, and 881 – U.S. FDA, 2016a,b, 2019a,b, 2020b,c). All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, and have been previously determined to be GRAS for their respective uses, or have been the subject of an effective food contact notification. The manufacturing process can be broadly divided into two stages.

In Stage 1 [upstream processing (USP)], D-lactose and D-glucose[8] are converted to LNFP-I/2'-FL by the adapted cellular metabolism of the production microorganism, which uses D-glucose as an energy and carbon source, and D-lactose as a substrate for LNFP-I/2'-FL biosynthesis. The production microorganism is removed from the fermentation medium at the end of the fermentation process.

In Stage 2 [downstream processing (DSP)], a series of purification, isolation, and concentration steps are used to generate the final high-purity LNFP-I/2'-FL product.

A schematic overview of the manufacturing process for LNFP-I/2'-FL is presented in Table 2.2.2-1 below.

Table 2.2.2-1 Overview of the Manufacturing Process for LNFP-I/2'-FL

STAGE 1		**Upstream Processing (USP)**
STEPS	**1**	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase
	4B	Feeding (Fed-Batch) Phase
	4C	Harvest/Storage of Culture Broth
	5	Removal of Microorganism*
STAGE 2		**Downstream Processing (DSP)**
STEPS	**6**	Purification/Concentration 1*
	7	Ion Removal
	8	Decolorization
	9	Purification/Concentration 2*
	10	Drying
	11	Sampling and Packaging
	12	Quality Control and Batch Release

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I.

* After the marked steps additional sterile filtration (microfiltration) is performed to maintain low microbial load during all times of downstream processing and to ensure high microbial quality of the final ingredient. These steps are further reassurance of absence of the production microorganism in final ingredient.

[8] Alternatively, D-sucrose or glycerol.

2.2.3 Quality Control

The manufacture of LNFP-I/2'-FL by microbial fermentation is conducted in accordance with cGMP and HACCP principles. Considering the chemically well-characterized principal raw materials and final products, the whole production process can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by Certificate of Analysis) to allow full control of the production process (refer to Table 2.2.2-1).

Both manufacturing stages (USP and DSP) are controlled by a HACCP plan which includes specifications for equipment, raw materials, product, and packaging materials. Master operating instructions are followed, batch records kept, a number of in-process controls are applied, and the isolated product is controlled by Certificates of Analysis and batch release routines.

The HACCP plan for both manufacturing stages also includes in-process controls to minimize the amount of potential impurities to the lowest level technically possible. Glycom's production process (including all processing aids, raw materials, unit operations, and filter aids) and the food safety management system comply with the Food Safety Systems Certification (FSSC) 22000 and International Organization for Standardization (ISO) 9001.

Incorporation of sterile filtration units throughout the manufacturing process of the HiMOs, ensures high microbiological purity while the presence of the production microorganism is devoid in the final product. The product microorganism is efficiently removed in the ultrafiltration step, which is applied directly following fermentation. In addition, several additional purification steps are carried out in the DSP stage to help achieve a highly purified LNFP-I/2'-FL, which is free from bacterial cells and residual fermentation by-products. The absence of the microorganisms can be measured by analysis for *Enterobacteriaceae* in the final product according to an internationally recognized method (ISO 21528-2). This specification for *Enterobacteriaceae* is set at "≤10 colony-forming units per gram" of test article, which also ensures absence of enumerable production microorganism as *E. coli* belongs to the *Enterobacteriaceae* family.

E. coli K-12 (a Gram-negative bacterium) possesses complex glycolipids of high molecular weight in their cell membrane and are known as lipopolysaccharides (LPS). When LPS enter the blood stream, they are recognized by immune cells and an immune response is elicited, which can result in a serious deleterious systemic reaction if delivered intravenously, such as during infusion therapy and parenteral nutrition. LPS are also referred to as endotoxins; however, this is not to be confused with the protein-type toxins associated with *E. coli*. Following ingestion of LPS, harmless effects are observed, and this is likely due to a combination of deactivation by stomach acid and a low absorption from the gut into the systemic circulation due to their high molecular weight. A strict specification for endotoxin levels is set to control for potential residual endotoxin and thereby, confirm the high purity of the product.

The absence of traces of residual DNA of the production organism in the product following fermentation and purification of LNFP-I/2'-FL is confirmed by three different validated quantitative polymerase chain reaction (qPCR) methods. These qPCR methods target short sub-sequences of the inserted genes *futC*, *IgtA*, and *opt galTK*, as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of representative batches of LNFP-I/2'-FL product demonstrate no detectable levels of residual DNA (limit of quantification of 0.004 mg/kg) present in the final ingredient. The qPCR tests were applied to all analyzed batches and the results were below the limit of quantification (LOQ) in all tested batches (see Section 2.3.3).

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

The specifications for LNFP-I/2'-FL are presented in Table 2.3.1-1. The parameters include LNFP-I/2'-FL, LNFP-I, 2'-FL, 3-fucosyl-lactulose, L-fucose, D-lactose, difucosyl-D-lactose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose. A limit for the sum of other carbohydrates have been established. The determination of these carbohydrates is conducted using high-performance liquid chromatography coupled with corona charged aerosol detection (HPLC-cCAD) and high-performance anion exchange chromatograph coupled with pulsed amperometric detection (HPAEC-PAD) analysis, respectively. Upper limits have also been established for microbiological parameters. All methods of analysis are either internationally recognized or developed internally by Glycom.

Table 2.3.1-1 Specifications for LNFP-I/2'-FL

Description		
LNFP-I/2'-FL (GlyCare™ LNFP-I / 2FL 8001) is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 75% of lacto-*N*-fucopentaose I and 2'-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	White, white to off-white, off-white	ISO 6658
Identification by retention time	RT of main components correspond to RT of standards ± 3%	Glycom method HPLC-13-002
Assay (water-free) – Specified saccharides[a]	≥ 90.0 w/w %	Glycom method HPLC-13-001, HPLC-13-002, HPAEC-HMO-017
Assay (water-free) – LNFP-I and 2'-FL	≥ 75.0 w/w %	Glycom method HPLC-13-002
Assay (water-free) – LNFP-I	≥ 50.0 w/w %	Glycom method HPLC-13-002
Assay (water-free) – 2'-FL	≥ 15.0 w/w %	Glycom method HPLC-13-002
Lacto-*N*-tetraose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-017
3-Fucosyllactose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017
L-Fucose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017
D-Lactose	≤ 10.0 w/w %	Glycom method HPAEC-HMO-017
Difucosyl-D-lactose	≤ 2.0 w/w %	Glycom method HPAEC-HMO-017
LNFP-I fructose isomer	≤ 1.5 w/w %	Glycom method HPLC-13-001
2'-Fucosyl-D-lactulose	≤ 1.0 w/w %	Glycom method HPLC-13-001
Sum of other carbohydrates	≤ 6.0 w/w %	Glycom method HPAEC-HMO-017
pH (20°C, 5% solution)	4.0 to 7.0	Ph. Eur. 2.2.3
Water	≤ 8.0 w/w %	Glycom method KF-001
Ash, sulfated	≤ 0.5 w/w %	Ph. Eur. 2.4.14
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Ph. Eur 2.6.14 (LAL kinetic chromogenic assay)
Lead	≤ 0.1 mg/kg	EN 13805; EPA-6020A

Table 2.3.1-1 Specifications for LNFP-I/2'-FL

Parameter	Specification	Method
Microbiological Specifications		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO-4833-2
Enterobacteriaceae	≤ 10 CFU/g	ISO 21528-2 or NMKL 144
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Molds	≤ 100 CFU/g	ISO 21527-2

2'-FL = 2'-fucosyllactose; AFNOR = Association Francaise de Normalisation; CFU = colony forming units; E.U. = endotoxin units; HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl Fischer; LNFP-I = lacto-*N*-fucopentaose I; NMKL = Nordisk Metodikkomite for Levnedsmidler; Ph. Eur. = European Pharmacopoeia; RT = retention time.

[a] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

2.3.2 Product Analyses

The analytical results of six independent production batches of LNFP-I/2'-FL ([redacted]) are summarized in Table 2.3.2-1 and are discussed in further details in the subsections following. As is demonstrated by the following data, the manufacturing process yields a consistent, high-purity product.

Table 2.3.2-1 Summary of Product Analyses of LNFP-I/2'-FL

Parameters	Specification	Manufacturing Batch No.					
		[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Appearance	Powder, agglomerates, powder with agglomerates	Powder	Powder	Powder	Powder	Powder	Powder with agglomerates
Color	White, white to off-white, off-white	White	White	White	White	White	White
Identification	RT of main components correspond to RT of standards ± 3%	Complies	Complies	Complies	Complies	Complies	Complies
Assay (water-free) – Specified saccharides[a]	≥ 90.0 w/w %	92.65	94.04	93.28	93.82	92.39	96.14
Assay (water-free) – LNFP-I and 2'-FL	≥ 75.0 w/w %	89.46	89.89	88.45	80.84	88.71	93.07
Assay (water-free) – LNFP-I	≥ 50.0 w/w %	57.70	62.91	70.17	59.92	57.40	63.15
Assay (water-free) – 2'-FL	≥ 15.0 w/w %	31.76	26.98	18.29	20.92	31.30	29.92
Lacto-*N*-tetraose	≤ 5.0 w/w %	0.65	1.68	3.37	3.21	1.53	1.08
3-Fucosyllactose	≤ 1.0 w/w %	0.11	< 0.03	< 0.03	0.03	< 0.03	< 0.03
L-Fucose	≤ 1.0 w/w %	< 0.03	< 0.03	< 0.03	0.11	< 0.03	< 0.03
D-Lactose	≤ 10.0 w/w %	0.44	1.42	0.72	8.56	0.89	0.91
Difucosyl-D-lactose	≤ 2.0 w/w %	0.70	0.48	0.28	0.19	0.66	0.68
LNFP-I fructose isomer	≤ 1.5 w/w %	0.67	0.26	0.22	0.18	0.12	0.16
2'-Fucosyl-D-lactulose	≤ 1.0 w/w %	0.60	0.18	0.11	0.18	0.24	0.15
Sum of other carbohydrates	≤ 6.0 w/w %	2.64	1.43	1.51	1.53	2.72	1.51
pH (20°C, in 5% solution)	4.0 to 7.0	4.6	5.9	5.7	5.4	4.4	6.5
Water	≤ 8.0 w/w %	0.78	2.21	2.39	3.96	5.67	2.74
Ash, sulfated	≤ 0.5 w/w %	0.08	< 0.01	< 0.01	< 0.01	0.10	< 0.01
Residual protein by Bradford assay	≤ 0.01 w/w %	< 0.0017	< 0.0017	0.0091	< 0.0017	< 0.0017	< 0.0017
Residual endotoxins	≤ 10 E.U./mg	0.1398	0.0023	0.0357	0.0107	< 0.0025	0.0012

Table 2.3.2-1 Summary of Product Analyses of LNFP-I/2'-FL

Parameters	Specification	Manufacturing Batch No.					
		[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Lead	≤ 0.1 mg/kg	< 0.01	< 0.01	0.01	< 0.01	0.01	< 0.01
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	≤ 10 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella in 25 g	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Yeasts	≤ 100 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10
Molds	≤ 100 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10

2'-FL = 2'-fucosyllactose; CFU = colony forming units; E.U. = endotoxin units; LNFP-I = lacto-*N*-fucopentaose I.

[a] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

2.3.2.1 *Physicochemical Properties*

The physical properties of LNFP-I/2'-FL, as manufactured by Glycom, may be described as white to off-white powder or agglomerates. A summary of the batch analyses corresponding to the selected physicochemical properties of LNFP-I/2'-FL is presented in Table 2.3.2.1-1.

Table 2.3.2.1-1 Batch Results for Selected Physicochemical Properties of LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Appearance	Powder	Powder	Powder	Powder	Powder	Powder with agglomerates
Color	White	White	White	White	White	White
pH (20°C, 5% solution)	4.6	5.9	5.7	5.4	4.4	6.5

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I.

2.3.2.2 *Human-Identical Milk Saccharides and Other Carbohydrates*

LNFP-I/2'-FL also contains levels of D-lactose and other milk saccharide-related substances [L-fucose, 3-fucosyllactose (3-FL), difucosyl-D-lactose, Lacto-*N*-tetraose (LNT), LNFP-I fructose isomer, 2'-fucosyl-D-lactulose]. Results of the HPLC analyses demonstrated that LNFP-I/2'-FL, contributes on average to ~ 88% by weight of dry matter (as presented in Table 2.3.2.2-1). Overall, the sum of specified saccharides fraction of the LNFP-I/2'-FL product adds up to 94% of the final batch weight (on dry matter).

Table 2.3.2.2-1 Batch Results for Fermentation Metabolites and Other Carbohydrate By-Products in LNFP-I/2'-FL

Parameter	Manufacturing Batch No.						
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Assay (water-free) – LNFP-I and 2'-FL (w/w %)	89.46	89.89	88.45	80.84	88.71	93.07	88.4
Assay (water-free) – LNFP-I (w/w %)	57.70	62.91	70.17	59.92	57.40	63.15	61.9
Assay (water-free) – 2'-FL (w/w %)	31.76	26.98	18.29	20.92	31.30	29.92	26.5
L-Fucose (w/w %)	< 0.03	< 0.03	< 0.03	0.11	< 0.03	< 0.03	NA
D-Lactose (w/w %)	0.44	1.42	0.72	8.56	0.89	0.91	2.2
3-Fucosyllactose (w/w %)	0.11	< 0.03	< 0.03	0.03	< 0.03	< 0.03	NA
Difucosyl-D-lactose (w/w %)	0.70	0.48	0.28	0.19	0.66	0.68	0.5
Lacto-*N*-tetraose (w/w %)	0.65	1.68	3.37	3.21	1.53	1.08	1.9

Table 2.3.2.2-1 Batch Results for Fermentation Metabolites and Other Carbohydrate By-Products in LNFP-I/2'-FL

Parameter	Manufacturing Batch No.						
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	Average
LNFP-I fructose isomer (w/w %)	0.67	0.26	0.22	0.18	0.12	0.16	0.3
2'-Fucosyl-D-lactulose (w/w %)	0.60	0.18	0.11	0.18	0.24	0.15	0.2
Assay (water-free) – Specified saccharides[a] (w/w %)	92.65	94.04	93.28	93.82	92.39	96.14	93.7
Sum of other carbohydrates (w/w %)	2.64	1.43	1.51	1.53	2.72	1.51	1.9

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I; NA = not averaged.

[a] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer and 2'-fucosyl-D-lactulose.

LNFP-I and 2'-FL are the main subject matter of this application and are structurally characterized in Section 2.1. The suitability for their use in infant nutrition is supported by their history of consumption in human milk (see Section 3.1.3). Furthermore, 2'-FL and 2'-FL/DFL have GRAS status in the U.S. (U.S. FDA, 2015b,c, 2016a, 2018a,b, 2019a,b, 2020c).

L-Fucose is a monosaccharide naturally occurring in its free form in human milk at concentrations in the range of 20 to 30 mg/L (Choi *et al.*, 2015). As a structural component of LNFP-I and 2'-FL, its levels can increase over storage. In any case, at the low levels detected in the batches of LNFP-I/2'-FL, its exposure is expected to be negligible and not biologically or nutritionally relevant.

D-Lactose is the main solid component of human milk and cow milk-based standard infant formulas, and the resulting exposure from its occurrence as component of the LNFP-I/2'-FL product is insignificant in comparison.

Difucosyl-D-lactose (DFL) is a human milk oligosaccharide (HMO) and has been reviewed previously as part of the GRAS Notice of 2'-FL/DFL. (U.S. FDA, 2019a).

LNT is an HMO that has GRAS status in the U.S. (U.S. FDA, 2019b).

3-FL is also a naturally occurring HMO with GRAS status in the U.S (U.S. FDA, 2021a,b). It is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose.

2'-Fucosyl-D-lactulose is an isomer of 2'-FL arising from the isomerization of the terminal glucose moiety of 2'-FL to fructose. Its safety profile has been reviewed previously as part of the GRAS Notice of 2'-FL/DFL (U.S. FDA, 2019a).

LNFP-I fructose isomer is an isomer of LNFP-I arising from the isomerization of the terminal glucose moiety of LNFP-I to fructose. The safety profiles of similar isomerization products were discussed in the GRAS Notices for 2'-FL, LNnT, 2'-FL/DFL, LNT, 3'-SL, and 6'-SL (U.S. FDA, 2015a, 2016a, 2018a,2019a,b, 2020b,c).

The results presented here demonstrate that LNFP-I/2'-FL complies with the product specification. Other than the content of LNFP-I and 2'-FL, the exposure to other carbohydrate products is expected to be negligible and not biologically or nutritionally relevant.

2.3.2.3 Non-Carbohydrate Residues

Batches of LNFP-I/2'-FL were tested for residual water, sulfated ash, and the presence of macro- and micro-elements. The results indicate only minimal levels of trace elements (see Table 2.3.2.3-1). These levels do not pose any safety issue given the small amount of these minerals relative to allowable quantities in infant formula (see Table 2.3.2.3-2).

Table 2.3.2.3-1 Batch Results for Non-Carbohydrate Residues of LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Water (w/w %)	0.78	2.21	2.39	3.96	5.67	2.74
Sulfated ash (w/w %)	0.08	< 0.01	< 0.01	< 0.01	0.10	< 0.01
Phosphate (w/w %)	< 0.0010	< 0.0005	0.0004	< 0.0001	< 0.006	< 0.0006
Sulfate (w/w %)	0.11	0.0049	0.003	< 0.0044	0.0197	0.00619
Chloride (w/w %)	0.10	0.0435	0.0057	0.0044	0.0206	0.0128
Ammonium (w/w %)	< 0.0050	< 0.001	< 0.001	< 0.001	< 0.005	< 0.001
Potassium (w/w %)	<0.001	< 0.001	0.002	0.001	0.015	< 0.001
Sodium (w/w %)	0.079	0.034	0.007	0.003	0.023	0.023
Magnesium (w/w %)	0.011	< 0.001	0.002	< 0.001	< 0.005	0.001
Calcium (w/w %)	0.005	< 0.001	< 0.001	< 0.001	0.004	< 0.000
Iron (mg/kg)	1	< 0.5	< 1	< 0.1	< 10	< 1
Manganese (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.1	< 1.0	< 0.1
Zinc (mg/kg)	< 0.1	0.2	0.3	0.1	< 0.5	< 0.2
Selenium (mg/kg)	NT*	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Molybdenum (mg/kg)	NT*	< 0.1	< 0.1	< 0.1	< 0.2	< 0.1

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I; NT = not tested.

* These parameters were not tested because they were not used in the production of this batch.

Table 2.3.2.3-2 Summary of Nutrient Allowances in Infant Formula (21 CFR 107.100 – U.S. FDA, 2020a)

Nutrient	Infant Formula Nutrient Specification (21 CFR 107.100)	Maximum Detected Content in LNFP-I/2'-FL		Margin[b]
		As Measured	Equivalent in 100 kcal[a]	
Chloride	55 to 150 mg/100 kcal	0.1%	0.25 mg	**600**
Iron	0.15 to 3.0 mg/100 kcal	1 mg/kg	0.25 µg	**12,000**
Potassium	80 mg to 200 mg/100 kcal	0.015%	0.038 mg	**5,263**
Sodium	20 mg to 60 mg/100 kcal	0.101%	0.25 mg	**240**
Sulfate	Not specified	0.11%	0.28 mg	**Not applicable**

Table 2.3.2.3-2 Summary of Nutrient Allowances in Infant Formula (21 CFR 107.100 – U.S. FDA, 2020a)

Nutrient	Infant Formula Nutrient Specification (21 CFR 107.100)	Maximum Detected Content in LNFP-I/2'-FL		Margin[b]
		As Measured	Equivalent in 100 kcal[a]	
Zinc	Min. 0.5 mg/100 kcal	0.3 mg/kg	0.075 µg	> 6667

2'-FL = 2'-fucosyllactose; CFR = *Code of Federal Regulations*; LNFP-I = lacto-*N*-fucopentaose I.

[a] Assuming an inclusion level of 1.5 g LNFP-I/2'-FL per L and a minimum caloric density of 60 kcal per 100 mL for the infant formula.

[b] Calculated as maximum theoretical level in formula containing LNFP-I/2'-FL divided by the maximum allowable level in infant formula.

2.3.2.4 Microbial Contaminants

The microbiological purity of LNFP-I/2'-FL production batches has been assessed for non-pathogenic microorganisms (bacteria, yeasts, and molds) as general hygiene indicators, as well as for selected food-borne pathogens (see Table 2.3.2.4-1).

Aerobic mesophilic total plate count, *Enterobacteriaceae*, yeasts and molds levels, as well as the presence of *Salmonella* give an indication of a level of total contamination (bioburden) and the absence of the production strain in the ingredient, respectively. The results of these analyses consistently indicate a low bioburden in the finished product and the absence of microbial contaminants. Accordingly, suitable specifications have been established for the ingredient when intended for inclusion at the wet blending stage of infant formula manufacture (*i.e.*, prior to retort) and also for conventional food products (see Table 2.3.1-1). More restrictive release specifications (including additional limits for *Cronobacter* spp., *L. monocytogenes,* and *B. cereus*) are established for ingredients intended for addition at the dry blending stage of infant formula manufacture where subsequent heat-treatment is not applied.

Table 2.3.2.4-1 Batch Results for Microbiological Analysis of LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Aerobic mesophilic total plate count (≤ 1,000 CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae (≤ 10 CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10
Yeasts (≤ 100 CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10
Molds (≤ 100 CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella in 25 g (Absence in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent

2'-FL = 2'-fucosyllactose; CFU = colony forming units; LNFP-I = lacto-*N*-fucopentaose I.

2.3.3 Manufacturing By-Products, Impurities and Contaminants

Carbohydrate-type by-products, as discussed in Section 2.3.2.2, are the main manufacturing impurities present in LNFP-I/2'-FL. These compounds are detectable, and levels are limited by appropriate specifications. Glycom also has established quality control measures for compounds that include microbial endotoxins and residual proteins and precautionary analyses demonstrating the absence of deleterious levels of several other potential residual compounds and trace elements that may originate from

fermentation. These include amino acids and biogenic amines, trace elements, and the presence/absence of genes characteristic for the production microorganism. These by-products, impurities, and contaminants are confirmed to be absent at any safety-relevant levels and as such, are not proposed for addition to the product specifications.

2.3.3.1 Amino Acids and Biogenic Amines

LNFP-I/2'-FL is released into the fermentation broth and the microorganism is removed. Nevertheless, production batches have been analyzed for secondary metabolites and cellular components that may potentially originate from the fermentation medium. Results of analyses of the ingredient for biogenic amines (histamine, tyramine, spermidine, cadaverine, and putrescine), and amino acids and their metabolites (glutamic acid, arginine, histidine, and gamma-aminobutyric acid) did not identify significant detectable levels of these contaminants in any of the manufacturing batches of the finished ingredient (data not shown). Therefore, these compounds do not contribute to the overall compositional data of LNFP-I/2'-FL and do not have to be included in the specification.

2.3.3.2 Microbial Endotoxins and Residual Proteins

The parental strain, *E. coli* K-12, is a Gram-negative bacterium and these bacteria possess complex glycolipids of high molecular weight in their cell walls, called LPS or endotoxins (not to be confused with protein-type toxins). Specifications for endotoxin have been established [≤ 10 endotoxin units (E.U.)/mg] as an additional quality control point to ensure that any microbial endotoxins are efficiently removed and/or not introduced during the production process. The endotoxin content in LNFP-I/2'-FL, was assayed using the *Limulus* amoebocyte lysate kinetic chromogenic assay described in the European Pharmacopoeia.

Similarly, a sensitive residual protein test (based on the Bradford assay) has been applied. Because batch analyses of LNFP-I/2'-FL demonstrated extremely low endotoxin and residual protein concentrations (see Table 2.3.3.2-1), they were not considered as compositional or safety-related data of the LNFP-I/2'-FL final product. However, the presence of residual endotoxins and protein are specified and carefully monitored during routine batch release as an element of HACCP that would allow to identify process deviations in a sensitive manner.

Table 2.3.3.2-1 Batch Results for Microbial Endotoxins and Residual Proteins in LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Residual endotoxins (E.U./mg)	0.1398	0.0023	0.0357	0.0107	< 0.0025	0.0012
Residual protein by Bradford assay (w/w %)	< 0.0017	< 0.0017	0.0091	< 0.0017	< 0.0017	< 0.0017

2'-FL = 2'-fucosyllactose; E.U. = endotoxin units; LNFP-I = lacto-*N*-fucopentaose I.

2.3.3.3 *Absence of Production Organism and its DNA*

The production microorganism is efficiently removed by the ultrafiltration (STEP 5) during USP, which is applied directly after fermentation. Additionally, various sequential filtration and purification processes are applied during DSP (see Table 2.2.2-1) to ensure the final purity of the LNFP-I/2'-FL. The absence of the production microorganisms in the bulk ingredient is demonstrated by the testing of final batches for bacteria from the *Enterobacteriaceae* family according to an internationally recognized method (ISO 21528-2).

Finally, the absence of the production organism in the finished ingredient is also supported by analyses for residual DNA in final production batches. As demonstrated in Table 2.3.3.3-1, the absence of residual DNA from the production organism is confirmed by three different qPCR methods. These qPCR methods target short sub-sequences of the inserted genes *futC*, *lgtA*, and *opt galTK*, as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of representative batches of LNFP-I/2'-FL product demonstrate no detectable levels of residual DNA (limit of quantification of 0.004 mg/kg) present in the final ingredient.

Table 2.3.3.3-1 Levels of Residual DNA in Representative Batches of LNFP-I/2'-FL Produced by Fermentation

Parameter	Manufacturing Batch No.					
futC assay	< LoQ[a]	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
lgtA assay	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
galTK assay	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
23S assay	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

2'-FL = 2'-fucosyllactose; DNA = deoxyribonucleic acid; LNFP-I = lacto-*N*-fucopentaose I; LOQ = limit of quantitation; qPCR = quantitative polymerase chain reaction.
[a] LOQ = 4 µg/kg (parts per billion).

2.3.3.4 *Trace Elements and Heavy Metals*

Trace levels of elements and minerals may be present in LNFP-I/2'-FL as a result of the fermentation process (carry-over from the fermentation medium). These are used as cofactors for different enzymes. Examples of trace elements are iron, manganese, copper, zinc, molybdenum, and selenium added as inorganic salts. The use of trace elements in very low concentrations and sufficient quantities are required for the promotion of bacterial cell growth.

The carry-over of trace elements from the fermentation to the final ingredient is efficiently minimized with nanofiltration and ion-exchange purification techniques. The results of analysis of trace elements and their levels relative to permissible levels in infant formula are presented above in Section 2.3.2.3—these levels do not pose any concern nor meaningful contribution to final levels of these compounds in the finished product.

The results of heavy metal analyses indicate no deleterious concentrations of these contaminants are present in the finished ingredient (see Table 2.3.3.4-1).

Table 2.3.3.4-1 Levels of Heavy Metals in Representative Batches of LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Lead (mg/kg)	< 0.01	< 0.01	0.01	< 0.01	0.01	< 0.01
Copper (mg/kg)	< 0.1	0.2	0.3	0.1	< 0.1	< 0.2
Manganese (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.1	< 1.0	< 0.1
Nickel (mg/kg)	NT*	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Arsenic (total) (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.1	0.170	< 0.100
Cadmium (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.001	< 0.010
Mercury (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.005	< 0.010

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I; NT = not tested.

* These parameters were not tested because they were not used in the production of this batch.

2.4 Stability

Real-time, accelerated, and stressed (forced degradation) stability studies on the pure ("bulk") powdered LNFP-I/2'-FL product have been conducted in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidelines (*Stability Testing of New Drug Substances and Products*) (ICH, 2003) in order to:

1. Test the LNFP-I/2'-FL stability during storage.
2. Define the optimal storage conditions and corresponding re-test dates or best before dates.
3. Investigate degradation pathways when exposed to selected stress factors, to ensure accelerated and real-time studies are meaningful.

For the bulk LNFP-I/2'-FL product, experiments were performed in solid state (in form of amorphous powder) and in liquid form (as aqueous solutions). Stability studies in processed foods included new studies in powdered infant formula and previous studies with 2'-FL and other manufactured HMOs in other foods. Overall, the results indicate that LNFP-I/2'-FL is expected to be stable over its intended shelf life of 5 years and is stable in its intended conditions of use.

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of powdered LNFP-I/2'-FL has been investigated in real-time under both ambient and 25°C/60% relative humidity (RH) conditions. The results for composition, sensory, and microbiological analyses are presented in Table 2.4.1.1-1 below and confirm that the integrity of the product is maintained for at least 3 years. Table 2.4.1.1-2 also provides results from an ongoing controlled study at 25°C and 60% RH.

Table 2.4.1.1-1 Results of the 3-Year Real-Time Stability Study on LNFP-I/2'-FL (Ambient Temperature and Relative Humidity)

Parameter	Sample Time (Months)					
	1		26		36 to 37	
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Appearance	NT*	NT*	Fine powder	Fine powder	Powder	Powder
Color	NT*	NT*	White	White	White	White
Odor	NT*	NT*	Neutral	Neutral	Neutral	Neutral
Flavor	NT*	NT*	Slightly sweet	Slightly sweet	Slightly sweet	Slightly sweet
Assay (water-free) – LNFP-I and 2'-FL (w/w %)	91.8	91.3	91.4	90.9	90.6	89.5
Assay (water-free) – LNFP-I (w/w %)	51.1	58.8	51.7	59.4	50.2	57.8
Assay (water-free) – 2'-FL (w/w %)	40.7	32.4	39.7	31.5	40.4	31.7
L-Fucose (w/w %)	ND	ND	0.03	0.03	< 0.03	< 0.03
D-Lactose (w/w %)	1.66	0.52	1.46	0.42	1.54	0.44
3-Fucosyllactose (w/w %)	0.06	NT	< 0.03	0.10	< 0.03	0.11
Difucosyl-D-lactose (w/w %)	0.68	0.78	0.59	0.61	0.71	0.70
Lacto-*N*-tetraose (w/w %)	1.09	0.75	0.96	0.58	1.02	0.65
LNFP-I fructose isomer (w/w %)	0.23	0.27	0.59	0.69	0.62	0.67
2'-Fucosyl-D-lactulose (w/w %)	NT	NT	0.29	0.41	0.40	0.60
Assay (water-free) – Specified saccharides[a] (w/w %)	95.6	93.7	95.4	93.8	95.0	92.7
Water (w/w %)	0.74	0.74	0.99	1.33	0.98	0.78
Aerobic Mesophilic total plate count (CFU/g)	NT*	NT*	< 10	< 10	< 10	< 10
Enterobacteriaceae (CFU/g)	NT*	NT*	< 10	< 10	< 10	< 10
Yeasts (CFU/g)	NT*	NT*	< 10	< 10	< 10	< 10
Molds (CFU/g)	NT*	NT*	< 10	< 10	< 10	< 10
Salmonella in 25 g	NT*	NT*	Absent	Absent	Absent	Absent

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; CFU = colony forming units; DFL = difucosyl-D-lactose; LNFP-I = lacto-*N*-fucopentaose I; LNT = lacto-*N*-tetraose; ND = not detected; NT = not tested.

* These parameters were not tested because they were not used in the production of this batch.

[a] Specified saccharides includes LNFP-I, 2'-FL, LNT, DFL, 3-FL, D-lactose, L-fucose, LNFP-I fructose isomer and 2'-fucosyl-D-lactulose.

Table 2.4.1.1-2 Interim Results of the 5-Year Real-Time Stability Study on LNFP-I/2'-FL Batch No. [redacted] (25°C, 60% Relative Humidity)

Parameter	Sample Time (Months)		
	0	3	6
Appearance	Powder	Powder with agglomerates	Powder with agglomerates
Color	White	White	White
Assay (water-free) – LNFP-I and 2'-FL (w/w %)	88.71	87.61	88.43
Assay (water-free) – LNFP-I (w/w %)	57.40	57.62	57.19
Assay (water-free) – 2'-FL (w/w %)	31.30	30.89	31.24
L-Fucose (w/w %)	< 0.03	< 0.03	< 0.03
D-Lactose (w/w %)	0.89	0.99	1.04
3-Fucosyllactose (w/w %)	< 0.03	< 0.03	< 0.03
Difucosyl-D-lactose (w/w %)	0.66	0.70	0.68
Lacto-*N*-tetraose (w/w %)	1.53	1.64	1.58
LNFP-I fructose isomer (w/w %)	0.12	0.23	0.18
2'-Fucosyl-D-lactulose (w/w %)	0.24	0.13	0.10
Assay (water-free) – Specified saccharides[a] (w/w %)	92.39	91.48	92.18
Sum of other carbohydrates	2.72	2.86	2.67
Water (w/w %)	5.67	4.76	4.72

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; CFU = colony forming units; DFL = difucosyl-D-lactose; LNFP-I = lacto-*N*-fucopentaose I; LNT = lacto-*N*-tetraose.
[a] Specified saccharides includes LNFP-I, 2'-FL, LNT, DFL, 3-FL, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

2.4.1.2 Accelerated Stability

To support the real-time stability studies reported above, the bulk stability of LNFP-I/2'-FL Batch No. [redacted] is being investigated under accelerated conditions (40°C, 75% RH) for a period of 2 years. The results for up to 6 months are presented in Table 2.4.1.2-1 below. Overall, at this stage, the results indicate that there is no appreciable degradation of LNFP-I/2'-FL and no changes in impurity profile following storage under the defined accelerated conditions.

Table 2.4.1.2-1 Interim Results of the 2-Year Accelerated Stability Study on LNFP-I/2'-FL Batch No. [redacted] (40°C, 75% Relative Humidity)

Parameter	Sample Time (Months)				
	0	1	2	3	6
Appearance	Powder	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates	Powder
Color	White	White	White	White	White
Odor	Neutral	NT*	NT*	NT*	Almost neutral
Flavor	Sweet	NT*	NT*	NT*	Sweet
Assay (water-free) – LNFP-I and 2'-FL (w/w %)	88.71	89.58	88.55	88.49	88.27
Assay (water-free) – LNFP-I (w/w %)	57.40	58.07	57.36	56.90	57.16
Assay (water-free) – 2'-FL (% w/w)	31.30	31.51	31.19	31.59	31.11
L-Fucose (w/w %)	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
D-Lactose (w/w %)	0.89	0.89	0.93	0.99	1.06
3-Fucosyllactose (w/w %)	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
Difucosyl-D-lactose (w/w %)	0.66	0.68	0.66	0.70	0.69
Lacto-*N*-tetraose (w/w %)	1.53	1.63	1.68	1.65	1.61
LNFP-I fructose isomer (w/w %)	0.12	0.12	0.17	0.21	0.17
2'-Fucosyl-D-lactulose (w/w %)	0.24	0.18	0.09	0.13	0.11
Assay (water-free) – Specified saccharides[a] (w/w %)	92.39	93.27	92.26	92.25	92.07
Sum of other carbohydrates	2.72	2.83	2.75	2.91	2.70
Water (w/w %)	5.67	5.33	4.91	4.78	4.73
Aerobic mesophilic total plate count (CFU/g)	< 10	NT*	NT*	NT*	< 10
Enterobacteriaceae (CFU/g)	< 10	NT*	NT*	NT*	< 10
Yeasts (CFU/g)	< 10	NT*	NT*	NT*	< 10
Molds (CFU/g)	< 10	NT*	NT*	NT*	< 10
Salmonella in 25 g	Absent	NT*	NT*	NT*	Absent

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; CFU = colony forming units; DFL = difucosyl-D-lactose; LNFP-I = lacto-*N*-fucopentaose I; LNT = lacto-*N*-tetraose; NT = not tested.

* These parameters were not tested because they were not used in the production of this batch.

[a] Specified saccharides includes LNFP-I, 2'-FL, LNT, DFL, 3-FL, D-lactose, L-fucose, LNFP-I fructose isomer and 2'-fucosyl-D-lactulose.

2.4.1.3 Stress Stability

The stress and forced stability studies described herein, were performed according to a Glycom Test Method which is based on ICH Guidelines (*Stability Testing of New Drug Substances and Products*) and aimed to identify the likely degradation products under harsh, stress conditions.

Forced stability tests of the bulk LNFP-I/2'-FL product in solid state and in aqueous solutions were performed:

1. In the solid state at 80°C for 28 days under dry and wet conditions;
2. 0.1 N hydrochloric acid (HCl) at 60 °C for 1 day;
3. In unbuffered aqueous solution and in aqueous solutions buffered to slightly acidic (pH 5) and to neutral conditions (pH 6.8) at 60°C for 28 days;
4. 0.01 N sodium hydroxide (NaOH) for 1 day;
5. In aqueous solutions under basic (pH 9) and acidic (pH 3) conditions at 60°C or 7 days;
6. In aqueous solution in the presence of 0.1% hydrogen peroxide at room temperature for 1 day; and
7. 4,4'-azobis (4-cyanovaleric acid) (ACVA) 1:0.1 molar ratio at room temperature for 1 day.

The results of these stressed stability studies suggest that the product is more stable if kept in a dry environment under protection against humidity, as the product shows some hygroscopicity. At 80°C, under dry conditions, the product is stable at least for 30 days.

In solution, the product is most stable in water at a pH range between 3 and 5. At 60°C, the unbuffered aqueous solution of the product is stable for at least 4 weeks. At 60°C and pH 3, the solution is stable for 1 day, with the sum of the impurities slowly increasing with longer storage. At 60°C but at pH 5, the solution is stable at least for 2 weeks, with the sum of the impurities very slowly increasing with longer storage. At the same time, the assay is slightly dropping. At pH 6.8 and over, the decomposition of the product starts immediately and is more intense by the increase of pH.

If the results of the study at different pH values are compared to each other, two different ways of decomposition can be supposed under acidic and basic conditions (see Table 2.4.1.3-1).

Table 2.4.1.3-1 Degradation Products of LNFP-I/2'-FL Under Acidic and Basic Conditions

Compound	Acidic Conditions	Basic Conditions
Primary Degradation Products		
LNT	Yes (primary)	No
Fucose	Yes (primary)	No
2-Fucosyl-galactose	Yes	Yes
Lacto-*N*-triose II	Yes	No
2'-Fucosyl-lacto-*N*-triose I	Yes	Yes
Glucose	Yes	No
Lactose	Yes	No
LNFP-I fructose isomer	Yes	Yes (primary)
3-Deoxy-2,3-dehydro-*N*-acetyl-glucosamine (Anhydro-GlcNAc)	No	Yes

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I; LNT = lacto-*N*-tetraose.

2.4.2 Stability Under the Intended Conditions of Use

2.4.2.1 Stability in Infant Formula

The stability of LNFP-I/2'-FL has been assessed in a standard powdered infant formula following long-term storage at various temperatures of 5, 20, 30, and 40°C for 12 months. The interim results are presented in Table 2.4.2.1-1. There is good alignment between expected and observed values suggesting good stability of these compounds after 6-month storage under the tested conditions.

Table 2.4.2.1-1 Results of Stability of LNFP-I/2'-FL in a Commercially Representative Infant Formula Following Storage for up to 3 Months at Various Temperatures

Storage Conditions	Timepoint (Months)	Average LNFP-I Concentration (g/100 g)	Average 2'-FL Concentration (g/100 g)
	T0 - Post Production	0.65	0.33
	T0 - Post Canning	0.66	0.32
5°C	1	0.65	0.33
25°C/60% RH	1	0.64	0.33
30°C/65% RH	1	0.64	0.33
40°C/75% RH	1	0.66	0.34
5°C	3	0.65	0.33
25°C/60% RH	3	0.66	0.33
30°C/65% RH	3	0.66	0.33
40°C/75% RH	3	0.65	0.33
5°C	6	0.67	0.33
25°C/60% RH	6	0.67	0.33
30°C/65% RH	6	0.67	0.33
40°C/75% RH	6	0.67	0.33

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I; RH = relative humidity.

2.4.2.2 Stability in Other Food Matrices

Based on its structure, the stability of LNFP-I is anticipated to be highly similar to 2'-FL, LNT, and LNnT which have GRAS status in the U.S. The stability of a number of other HiMOs have been investigated in other food matrices including yogurts, ready-to-drink flavored milk, and citrus fruit beverages. These have been presented in GRAS notifications for 2'-FL (GRN 546, 650, 735; U.S. FDA, 2015b, 2016a, 2018a), LNnT (GRN 547, 659; U.S. FDA, 2015a, 2016b), and sialic acid (GRN 602; U.S. FDA, 2016c). Briefly, the results of these studies reveal no significant losses of the initial concentrations of these ingredients in these food matrices over the duration of the typical shelf-life of these foods, even when subject to pre-processing, pasteurization, and ultra-high temperature heating.

Part 3. DIETARY EXPOSURE

3.1 History of Use of LNFP-I/2'-FL in Food

3.1.1 LNFP-I

To Glycom's knowledge, LNFP-I has not been "used" as a food ingredient anywhere in the world to date.

3.1.2 2'-FL

2'-FL, either manufactured by chemical synthesis or *via* fermentation technology, is GRAS in the U.S., approved within the European Union (EU) Union list of novel foods, and has now been successfully commercialized in over 70 countries following extensive safety testing, scientific review, and approval by recognized scientific and regulatory bodies (EFSA, 2015; U.S. FDA, 2015a, 2016a; FSAI, 2016). Similarly, 2'-FL/DFL from the same "platform strain" as LNFP-I/2'-FL is GRAS in the U.S. and has been adopted into the EU Union list following scientific review (EFSA, 2019; U.S. FDA, 2019a). Specific approvals for 2'-FL gained around the world by Glycom are summarized in Table 3.1.2-1.

Table 3.1.2-1 Summary of the Regulatory Status of Glycom's 2'-FL in Various Jurisdictions

Jurisdiction	Ingredient	Regulatory Status	Regulatory Citation
U.S.	2'-FL (microbial fermentation - Glycom)	2'-FL is GRAS for its intended uses in infant formula (up to 2.4 g/L) and in various other foods, including but not limited to toddler formula, foods for young children, and milk products.	GRN 650 (U.S. FDA, 2016a)
	2'-FL (chemically synthesized - Glycom)	2'-FL is GRAS for its intended uses in infant formula (up to 2.4 g/L) and in various other foods, including but not limited to toddler formula, foods for young children, and milk products.	GRN 546 (U.S. FDA, 2015b)
EU	2'-FL (microbial fermentation) 2'-FL (chemically synthesized)	Infant formula and follow-on formula: • 2'-FL is permitted at up to 1.2 g/L alone or in combination with up to 0.6 g/L of LNnT at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer. Milk-based drinks and similar products intended for young children: • 2'-FL is permitted at up to 1.2 g/L for "milk-based drinks and similar products added alone or in combination with up to 0.6 g/l LNnT at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer." "Unflavoured pasteurised and sterilised (including UHT) milk-based products": • 2'-FL is permitted at up to 1.2 g/L. 2'-FL is also permitted for addition to a number of other food categories (see EU Union list).	Union List of Authorized Novel Foods – Commission Implementing Regulation (EU) 2017/2470 (EU, 2017)

Table 3.1.2-1 Summary of the Regulatory Status of Glycom's 2'-FL in Various Jurisdictions

Jurisdiction	Ingredient	Regulatory Status	Regulatory Citation
Switzerland	2'-FL (microbial fermentation)	2'-FL is permitted under the same conditions of use as in the EU.	Approval letter dated 05 February 2020 (Swiss Federal Office of Public Health, 2020)[a]
Australia/New Zealand	2'-FL (microbial fermentation)	2'-FL is permitted in infant formula products (0 to 12 months), at a maximum use level of 96 mg/100 kJ (equivalent to 2.4 g/L) for 2'-FL alone, and a total maximum level of 96 mg/100 kJ (equivalent to 2.4 g/L) for 2'-FL and LNnT combined with no more than 24 mg/100 kJ (equivalent to 0.6 g/L) of LNnT.	Australia New Zealand Food Standards Code (FSANZ, 2021)
Brazil	2'-FL (microbial fermentation)	ANVISA has approved the use of Glycom's 2'-FL in infant formula at up to 1.2 g/L in combination with 0.6 g/L LNnT at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer.	OPINION OF PROCESS No. 25351.367590/2017-57/GEARE/GGALI/ANVISA (ANVISA, 2018, 2019)
Singapore	2'-FL (microbial fermentation)	2'-FL may be added to infant formula (0 to 12 months) in an amount not exceeding 120 mg per 100 mL (*i.e.*, 1.2 g/L) of the reconstituted product or ready-to-drink product. AVA has also indicated 2'-FL may be added to growing up milk (for children aged 12 to 36 months), up to a level of 1.2 g/L of the reconstituted product or ready-to-drink product.	Regulation 283(6) of the Food Regulations (SFA, 2020)
Thailand	2'-FL (microbial fermentation)	The Thai FDA has accepted the use of Glycom's 2'-FL in the following food categories: 1. Modified milk for Infants and follow-on modified milk formulas for infants and young children; 2. Infant food and follow-on food formulas for infants and young children; 3. Supplemental food for infants and young children; and 4. Dairy products. The recommended dosage of 2'-FL is as follows: 1. Children aged 0 to 2.9 years can use 2'-FL of not more than 1 g/L in food in ready-to-eat form. 2. Children aged over 3 years can use 2'-FL of not more than 161 mg/kg body weight/day by using the reference weight (updated in 2016) from the National Bureau of Agricultural Commodity and Food Standards.	Opinion letter from the Thai FDA (Thailand FDA, 2019)
Israel	2'-FL (microbial fermentation)	2'-FL can be added to milk-based infant formulas, milk-based follow-on formula, and toddler formulas at maximum concentration of 2 g/L in the final product ready for consumption.	New food directive on: oligosaccharide 2'-*O*-Fucosyllactose Update as of 10 September 2019 (6 in Av 5778) (Israel MOH, 2019)

Table 3.1.2-1 Summary of the Regulatory Status of Glycom's 2'-FL in Various Jurisdictions

Jurisdiction	Ingredient	Regulatory Status	Regulatory Citation
Malaysia	2'-FL (microbial fermentation)	The Malaysian MOH has authorized 2'-FL as optional ingredient in infant formula, follow-up formula, and formulated milk powder for children at a maximum level of 1.2 g/L.	Waiting for official gazettal into the Food Regulations 1985 (Malaysia Government, 2017)

2'-FL = 2'-fucosyllactose; ANVISA = Agência Nacional de Vigilância Sanitária; AVA = Agri-Food & Veterinary Authority of Singapore; EU = European Union; FDA = Food and Drug Administration; FSANZ = Food Standards Australia New Zealand; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; LNnT = lacto-*N*-neotetraose; MOH = Ministry of Health; UHT = ultra-high temperature; U.S. = United States.

[a] https://www.blv.admin.ch/blv/de/home/lebensmittel-und-ernaehrung/rechts-und-vollzugsgrundlagen/bewilligung-und-meldung/gentechnisch-veraenderte-organismen-gvo.html.

[b] https://www.foodstandards.gov.au/code/applications/Pages/A1155%E2%80%932%E2%80%99-FL-and-LNnT-in-infant-formula-and-other-products-.aspx.

3.1.3 History of Consumption of LNFP-I/2'-FL in Human Milk

3.1.3.1 Human Biology Background Relevant to LNFP-I and 2'-FL

LNFP-I and 2'-FL are two important and significant components of the natural HMO fraction of human milk. Human milk contains as its third largest solid component fraction, consisting of a complex family of structurally related oligosaccharides (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). These are known as HMOs because they were first discovered in human milk (reviewed by Malpress and Hytten, 1958) and because they occur in human milk at much higher concentrations than in other mammalian milks (Urashima *et al.*, 2001). More than 140 members of this family have been fully described on a structural basis (Urashima *et al.*, 2011; Chen, 2015; Remoroza *et al.*, 2020), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al.*, 1999; Wu *et al.*, 2010, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012), although high variations are observed on an individual level and in dependency of the lactation period and the genotype of the mother. In contrast, bovine milk contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013), which does not contain fucosylated oligosaccharides at any appreciable level (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Urashima *et al.*, 2012a).

In reviewing the concentrations of HiMOs reported in the following sections, it should be emphasized that the components of infant formula are nutrients that were necessary to sustain the life of humans from an evolutionary perspective and, like all nutrients consumed by humans, are therefore inherently safe when consumed within normal ranges in the diet. From an evolutionary perspective, it also can be surmised that a large margin of safety exists for concentrations of HiMOs in human milk, which is reflected in the wide range of concentrations of LNFP-I and 2'-FL that have been measured across lactational stages and across various demographic population groups. With respect to selection of a target concentration of LNFP-I and 2'-FL for use in infant formula, Glycom has selected a value that falls within the 95th percentile of mean values. This approach ensures that the majority of infants will be provided with concentrations that fall within the population means and is well below the upper ranges of concentrations that have been reported

for human milk samples, therefore, ensuring that the intended uses levels are safe and well tolerated by all infants.

3.1.3.2 Genetic Polymorphisms Shaping Milk Composition: Secretor and Lewis Phenotypes

Nearly 60 to 80% of the total HMO fraction is comprised of neutral fucosylated oligosaccharides that contain the sugar fucose in their chemical structure (Ninonuevo *et al.*, 2006; Bode, 2012). Fucose can principally be added by several different enzymes in three distinct molecular linkages, namely α-(1,2), α-(1,3), and α-(1,4).

Two of these linkages are not found in the milk of all mothers since the genes (*i.e.*, *fut2*, *fut3*) encoding the respective enzymes [*i.e.*, α-(1,2) and α-(1,3/4)-fucosyltransferases FUT2 and FUT3] are subject to genetic polymorphism that reflect events of heredity over evolutionary times, causing partial to total loss of the enzyme function in some proportion of the population. Maintenance of the genetic polymorphism of these traits in the population indicate opposing trends in selective pressures either from environmental (*e.g.*, regional prevalence of infectious agents) or parent-offspring conflicts (Gagneux and Varki, 1999; Bishop and Gagneux, 2007; Varki *et al.*, 2009; Springer and Gagneux, 2013, 2016) .

In consequence, four different milk phenotype groups can be characterized (corresponding to the combination of genotypes *fut2*$^+$/*fut3*$^+$, *fut2*$^-$/*fut3*$^+$, *fut2*$^+$/*fut3*$^-$ and *fut2*$^-$/*fut3*$^-$). One or two functional alleles of a person's *fut2*$^+$ genotype leads to the so-called ***Secretor*** phenotype, which can be detected in their bodily secretions (*e.g.*, milk, blood, saliva, urine) (Grollman and Ginsburg, 1967; Shen *et al.*, 1968). One or two functional allele of the *fut3*$^+$ genotype acts phenotypically *in combination* with the *fut2*$^+$ genotype and leads either to the so-called ***Lewis a*** or ***Lewis b*** phenotypes (Thurl *et al.*, 1997). This is summarized in Table 3.1.3.2-1 below with typical frequencies of the different milk phenotypes and the information which distinct HMO occurs in which milk phenotype group.

2'-FL and LNFP-I are both typical representatives of the Secretor phenotype HMOs.

LNFP-I contains another biologically intriguing structural feature, the *type I linkage* [*i.e.*, Gal-β(1-3)-GlcNAc] which is predominant over type 2 [*i.e.*, Gal-β(1-4)-GlcNAc] derived HMOs in human milk and appears to be a unique feature of humans in contrast to other mammals (Urashima *et al.*, 2012b).

Table 3.1.3.2-1 HMO Groups and Milk Group Secreter Status

Secretor Status	**Secretor**	**Secretor**	**Non-Secretor**	**Non-Secretor**
Milk Group	1	3	2	4
Milk Phenotype	Se+ / Le (a-b+)	Se+ / Le (a-b-)	Se- / Le (a+b-)	Se- / Le (a-b-)
α1,2-fucosylated HMOs (FUT2 enzyme)	+	+	–	–
α1,3-fucosylated HMOs (FUT3, FUT5, FUT6)	+	+	+	+
α1,4-fucosylated HMOs (FUT3 enzyme)	+	–	+	–
Typical frequency	~ 70%	~ 9%	~ 20%	~ 1%
HMOs FUT2+ **&** FUT3+	LNDFH-I, DF-LNH-III, TF-LNH	None	None	None
HMOs FUT2+ **or** FUT3+	**2'-FL**, DFL, **LNFP-I**, F-LNH-I, DF-LNH-I, S-LNFP-I, FS-LNH		LNFP-II, LNDFH-II, F-LNH-II, DF-LNH-II, DF-*para*-LNH, S-LNFP-II	None

Table 3.1.3.2-1 HMO Groups and Milk Group Secreter Status

Secretor Status	Secretor	Secretor	Non-Secretor	Non-Secretor
HMOs contained in all groups	3-FL, 3'-SL, 6'-SL, FSL, LNT, LNnT, LNH, LNnH, LNFP-III, LNFP-V, LNFP-VI, F-LNH-III, F-*para*-LNH-I, DF-*para*-LNnH, LSTa, LSTb, LSTc, DS-LNT, S-LNH, S-LNnH-I, FS-LNnH-I, DS-F-LNH-II			

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; DFL = difucosyllactose; DF-LNH-I = difucosyl-lacto-*N*-hexaose I; DF-LNH-II = difucosyl-lacto-*N*-hexaose II; DF-LNH-III = difucosyl-lacto-*N*-hexaose III; DF-*para*-LNH = difucosyl-*para*-lacto-*N*-hexaose; DF-*para*-LNnH = difucosyl-para-lacto-*N*-neohexaose; DS-F-LNH-II = disialyl-fucosyl-lacto-*N*-hexaose II; DS-LNT = 3',6-disialyllacto-*N*-tetraose; F-LNH-I = fucosyl-lacto-*N*-hexaose I; F-LNH-II = fucosyl-lacto-*N*-hexaose II; F-LNH-III = fucosyl-lacto-*N*-hexaose III; F-*para*-LNH-I = fucosyl-para-lacto-*N*-hexaose I; FSL = 3-fucosyl-3'-sialyllactose; FS-LNH = fucosyl-sialyl-lacto-*N*-hexaose I; FS-LNnH-I = fucosyl-sialyl-lacto-*N*-neohexaose I; HMO = human milk oligosaccharide; LNDFH-II = lacto-*N*-difucohexaose II; LNFDH-I = lacto-*N*-difucohexaose I; LNFP-I = lacto-*N*-fucopentaose I; LNFP-II = lacto-*N*-fucopentaose II; LNFP-III = lacto-*N*-fucopentaose III; LNFP-V = lacto-*N*-fucopentaose V; LNFP-VI = lacto-*N*-fucopentaose VI; LNH = lacto-*N*-hexaose; LNnH = lacto-*N*-neohexaose; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; LSTa = 3'-sialyllacto-*N*-tetraose; LSTb = 6-sialyllacto-*N*-tetraose; LSTc = 6'-sialyllacto-*N*-neotetraose; S-LNFP-I = sialyl-lacto-*N*-fucopentaose I; S-LNFP-II = sialyl-lacto-*N*-fucopentaose II; S-LNH = sialyl-lacto-*N*-hexaose; S-LNnH-I = sialyl-lacto-*N*-neohexaose I; TF-LNH = trifucosyl-lacto-*N*-hexaose.

In summary, LNFP-I is a pentasaccharide HMO derived from the human-characteristic *type I* motif-containing LNT by addition of a fucose in the typical Secretor phenotype HMO linkage (α-1,2-fucose). It therefore combines two biologically intriguing structure motifs in one molecule and occurs at significant concentrations in human milk.

For the biological background of 2'-FL, Glycom refers to previous 2'-FL GRAS Notices from Glycom and others (GRNs 546, 571, 650, 735, 749, 815, 852, and 897 – U.S. FDA, 2015b,c, 2016a, 2018a,b, 2019a,c, 2020d).

3.1.3.3 Quantity of LNFP-I in Human Milk

Please refer to Appendix A for a systematic review of quantitative data for LNFP-I in human milk. Of the 24 publications reporting the concentration of LNFP-I in Secretor milk, the lowest reported mean was 0.18 g LNFP-I/L (Smilowitz *et al.*, 2013) [observed in 52 healthy women enrolled in the Foods for Health Institute Lactation Study at the University of California, Davis (UC-Davis) sampled on Day 90 of lactation] and the highest was 4.47 g LNFP-I/L reported by Elwakiel *et al.* (2018) (observed in Chinese women from the Hohhot region sampled between Day 7 and 140 of lactation). Using the reported standard deviations, values reported by Elwakiel *et al.* (2018) also represented the highest extrapolated 95% confidence limit (CL) at 5.75 g LNFP-I/L. Glycom has selected a 1.5 g LNFP-I/L intended use level in infant formula that falls within the 95th percentile of mean values. This approach ensures that the majority of infants will be provided with concentrations that fall within the population means and is well below the upper ranges of concentrations that have been reported for human milk samples, therefore, ensuring that the intended uses levels are safe and well tolerated by all infants.

3.1.3.4 Quantity of 2'-FL in Human Milk

Please refer to Appendix A for a systematic review of quantitative data for 2'-FL in human milk. Of the 24 publications reporting the concentration of 2'-FL in Secretor milk, the lowest reported mean was 0.68 g 2'-FL/L (Van Niekerk *et al.*, 2014) (observed in mothers participating at a larger clinical trial in Tygerberg Children's Hospital, Cape Town, South Africa, and who gave birth preterm and sampled on Day 28 of lactation; all mother/infant dyads included in this study had been pre-selected for infants that had developed necrotizing enterocolitis) and the highest was 7.23 g 2'-FL/L reported by Gabrielli *et al.* (2011) [observed in samples of women that were investigated by the Polytechnic University of Marche, Ancona, Italy, and who delivered preterm newborns at 25 to 30 weeks of gestation (mean gestational

age: 27.9 weeks) and were sampled on the morning of Day 4 of lactation]. Using the reported standard deviations, values reported by Gabrielli *et al*. (2011) also represented the highest extrapolated 95% CL at 14.01 g 2'-FL/L. As discussed in Section IV.B of GRN 650, a concentration of 2.4 g 2'-FL per liter of infant formula has been concluded to be GRAS, as this concentration represents a use level that was considered representative of mean concentrations that have been reliably measured in human milk samples from full term birth mothers across a variety of demographic groups, Lewis body genotypes, and lactational stages.

3.2 Estimated Intake of LNFP-I/2'-FL from Proposed Uses

The following section describes the estimated intake of the ingredient, expressed on the level of LNFP-I to be added (*i.e.*, water and other carbohydrate content are not included). LNFP-I is not an appreciable component of the background diet outside of human milk (see Section 3.1.3) and thus, was not considered in the exposure modelling herein. Within Section 3.2.2, the corresponding exposure to 2'-FL consumption is calculated from LNFP-I intakes based on the average values of 2'-FL and LNFP-I across five batches of LNFP-I/2'-FL in Table 2.3.2.2-1 (*i.e.*, 26.5/61.9 = 0.428).

3.2.1 Methods

An assessment of the anticipated intake of LNFP-I under the intended conditions of use of LNFP-I/2'-FL (see Table 1.3-1) was conducted using data available in the 2017-2018 cycle of the U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) (CDC, 2021a,b; USDA, 2021a). An abbreviated summary of the survey and methodology employed in the intake assessment of LNFP-I along with the pertinent results is presented herein.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2017 to 2018. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (CDC, 2021a,b; USDA, 2021b). The NHANES data were employed to assess the mean and 90th percentile intake of LNFP-I for each of the following population groups:

- Infants, ages 0 to 6 months;
- Infants, ages 7 months to less than 1 year;
- Toddlers, ages 1 to 2 years;
- Children, ages 3 to 11 years;
- Female teenagers, ages 12 to 19 years;
- Male teenagers, ages 12 to 19 years;
- Female adults of childbearing age, ages 20 to 40;
- Female adults, ages 20 to 64 years;
- Male adults, ages 20 to 64 years;
- Elderly, ages ≥ 65; and
- Total population (ages 2 years and older, and both gender groups combined).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of LNFP-I by the U.S. population[9]. Estimates for the daily intake of LNFP-I represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018; these average amounts comprised the distribution from which mean, and percentile intake estimates were determined. Mean and percentile estimates were generated incorporating survey weights to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of LNFP-I averaged over all individuals surveyed, regardless of whether they consumed food products in which LNFP-I is proposed for use, and therefore, includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing LNFP-I during the two survey days). "Consumer-only" intake refers to the estimated intake of LNFP-I by those individuals who reported consuming food products in which the use of LNFP-I is currently under consideration. Individuals were considered "consumers" if they reported consumption of one or more food products in which LNFP-I is proposed for use on either Day 1 or Day 2 of the survey.

The estimates for the intake of LNFP-I were generated using the maximum use-level indicated for each intended food-use, as presented in Table 1.3-1, together with food consumption data available from the 2017-2018 NHANES datasets. The results of this assessment are presented in Section 3.2.2.

3.2.2 Results

A summary of the estimated daily intake of LNFP-I from proposed food-uses of LNFP-I/2'-FL is provided in Table 3.2.2-1 on an absolute basis (g/person/day) and in Table 3.2.2-2 on a body weight basis (mg/kg body weight/day).

The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 76.2% of the population groups consisted of consumers of food products in which LNFP-I/2'-FL is currently proposed for use (see Table 3.2.2-1). Toddlers had the greatest proportion of consumers at 99%. The consumer-only estimates are more relevant to risk assessments, as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of LNFP-I were determined to be 0.76 and 1.66 g/person/day, respectively. Of the individual population groups, infants aged 7 to < 12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of LNFP-I on an absolute basis, at 2.56 and 4.76 g/person/day, respectively. Female teenagers had the lowest mean and 90th percentile consumer-only intake of 0.57 and 1.23 g/person/day, respectively (see Table 3.2.2-1).

[9] Statistical analysis and data management were conducted in DaDiet Software (Dazult Ltd., 2018). DaDiet Software is a web-based software tool that allows accurate estimate of exposure to nutrients and to 3-FL added to foods, including contaminants, food additives, and novel ingredients. The main input components are concentration (use-level) data and food consumption data. Data sets are combined in the software to provide accurate and efficient exposure assessments.

Table 3.2.2-1 Summary of the Estimated Daily Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	1.14	2.29	76.2	139	1.50	2.88
Infants	7 to < 12 m	2.50	4.76	97.5	122	2.56	4.76
Toddlers	1 to 2 y	0.85	1.58	99.0	300	0.86	1.58
Children	3 to 11 y	0.73	1.47	97.4	970	0.75	1.47
Female Teenagers	12 to 19 y	0.53	1.23	92.3	413	0.57	1.23
Male Teenagers	12 to 19 y	0.74	1.48	94.9	417	0.78	1.48
Female Adults of Childbearing Age	20 to 40 y	0.57	1.24	87.7	610	0.65	1.29
Female Adults	20 to 64 y	0.62	1.43	86.9	1,408	0.72	1.51
Male Adults	20 to 64 y	0.81	1.96	89.0	1,260	0.91	2.02
Elderly	65 y and older	0.58	1.39	87.7	904	0.66	1.46
Total Population	2 y and older	0.69	1.57	89.8	5,523	0.76	1.66

LNFP-I = lacto-*N*-fucopentaose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of LNFP-I were determined to be 13 and 29 mg/kg body weight/day, respectively. Among the individual population groups, infants aged 7 to < 12 months were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 285 and 533 mg/kg body weight/day, respectively. The elderly and female adults of childbearing age had the lowest mean and 90th percentile consumer-only intake of 9 and 18 mg/kg body weight/day, respectively (see Table 3.2.2-2).

Table 3.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	174	361	76.2	139	229	388
Infants	7 to < 12 m	277	533	97.5	122	285	533
Toddlers	1 to 2 y	70	132	98.9	291	71	132
Children	3 to 11 y	29	60	97.4	967	29	60
Female Teenagers	12 to 19 y	9	21	92.4	407	10	24
Male Teenagers	12 to 19 y	12	23	95.2	415	12	23
Female Adults of Childbearing Age	20 to 40 y	8	17	87.7	609	9	18
Female Adults	20 to 64 y	9	20	87.0	1,402	10	22
Male Adults	20 to 64 y	9	22	89.0	1,252	10	22

Table 3.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Elderly	65 y and older	8	18	88.0	891	9	18
Total Population	2 y and older	12	27	89.9	5,478	13	29

LNFP-I = lacto-*N*-fucopentaose; bw = body weight; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

3.2.3 Summary and Conclusions

Consumption data and information pertaining to the individual proposed food-uses of LNFP-I/2'-FL were used to estimate the *per capita* and consumer-only intakes of LNFP-I (estimates performed on a LNFP-I basis not including moisture or other carbohydrates) for specific demographic groups and for the total U.S. population. There were several assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in the exposure assessment that all food products within a food category contain LNFP-I/2'-FL at the maximum specified level of use. In reality, the levels added to specific foods will vary depending on the nature of the food product, and it is unlikely that LNFP-I/2'-FL will have 100% market penetration in all identified food categories.

In summary, on consumer-only basis, the resulting mean and 90th percentile intakes of LNFP-I by the total U.S. population from all proposed food-uses were estimated to be 0.76 g/person/day (13 mg/kg body weight/day) and 1.66 g/person/day (29 mg/kg body weight/day), respectively. Among the individual population groups, infants aged 7 to < 12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of LNFP-I on an absolute basis and body weight basis, at 2.56 and 4.76 g/person/day, respectively, equivalent to 285 and 533 mg/kg body weight/day. The female teenagers had the lowest mean and 90th percentile consumer-only intake of 0.57 and 1.23 g/person/day, respectively.

The corresponding amount of 2'-FL from this ingredient has been determined based on the average values of 2'-FL and LNFP-I across five batches of LNFP-I/2'-FL, *i.e.*, 26.5/61.9 = 0.428 (see Table 2.3.2.2-1). Considering the highest estimated mean and 90th percentile body weight intakes (infants aged 7 to < 12 months) of 285 and 533 mg LNFP-I/kg body weight/day, this is equivalent to 122 and 228 mg/kg body weight/day of 2'-FL[10,11]. Glycom has notified the Agency of the GRAS uses of 2'-FL as an ingredient in non-exempt term infant formula, toddler formulas, and other conventional foods under GRNs 546 and 650. Exposure analyses conducted for all GRAS uses resulted in estimated mean and 90th percentile body weight intakes of 2'-FL in infants aged 7 to < 12 months of 188.9 and 295.8 mg/kg body weight/day, respectively (U.S. FDA, 2016b). See Section 6.7 for considerations of additional dietary intake of HiMOs.

[10] Calculated as 285 mg LNFP-I/kg body weight/day x 0.428 2'-FL/LNFP-I = 122 mg 2'-FL/kg body weight/day.
[11] Calculated as 533 mg LNFP-I/kg body weight/day x 0.428 2'-FL/LNFP-I = 228 mg 2'-FL/kg body weight/day.

Part 4. SELF-LIMITING LEVELS OF USE

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

Part 5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

Part 6. NARRATIVE AND SAFETY INFORMATION

6.1 Introduction

Glycom has conducted a scientific procedures GRAS evaluation of LNFP-I/2'-FL for use as an ingredient in infant formula and specified conventional food applications marketed to the general population. LNFP-I/2'-FL, as manufactured by Glycom, has been demonstrated to be identical in structure to its natural counterpart secreted into human milk and therefore, can be referred to as an HiMO. The ingredient will be added to infant formula at levels that will result in concentrations that are within the 95th percentile range of reported mean concentrations that have been measured in human milk samples obtained from lactating women across all lactational stages, and therefore, the safety of adding LNFP-I/2'-FL to infant formula is supported by pivotal information establishing its history of safe consumption by breastfeeding infants. As infants are a sensitive population group, the safety of dietary ingestion of HiMOs from human milk consumption also can be extended to adults consuming HiMOs at comparable ingestion levels in conventional food products.

Since conclusions on the GRAS use of LNFP-I/2'-FL in infant formula are based on extrapolation of safe levels established from usual concentrations that have been measured in human milk samples, toxicological evaluations in animals, or tolerance studies in neonatal piglets, were not necessary to establish an appropriate margin of safety for dietary intakes of LNFP-I/2'-FL from the intended food uses.

Notwithstanding the above conclusions, LNFP-I/2'-FL has been tested in a comprehensive series of toxicological studies, including a bacterial reverse mutation assay, an *in vitro* mammalian cell micronucleus test in human lymphocytes, and an adapted subchronic (90-day) oral toxicity study in neonatal rats (Phipps *et al.*, 2021). These studies were all conducted with test articles that are representative of the material intended to be commercially marketed by Glycom, and they were performed in accordance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) and appropriate OECD test guidelines (OECD, 1998). Detailed descriptions of these studies are presented in Section 6.4. No genotoxicity was reported, and a no-observed-adverse-effect level (NOAEL) determination of 5,000 mg/kg body weight/day (the highest dose tested) was reported by the authors. Overall, there were no findings to suggest that Glycom's conclusions on extrapolation of safety from the history of safe use would be inappropriate.

The safety of the production organism is based upon the long-history of safe use of *E. coli* K-12 in food production and, to date, eight HiMO ingredients produced by Glycom using the company's platform strain and similar DSP methods have been evaluated in toxicity studies in neonatal rats and in *in vitro* genotoxicity assays without evidence of toxicity or genotoxic potential.

Finally, Glycom evaluated the allergenicity risk of LNFP-I/2'-FL (see Section 6.6). As a purified ingredient, LNFP-I/2'-FL manufactured by Glycom has established strict specification parameters for residual proteins as assayed by a modified Bradford method with a detection limit of 17 ppm. The amino acid sequences of heterologous genes introduced into the production organism were evaluated using the *in-silico* tool Allergen Online (version 21) hosted by the University of Nebraska's Food Allergen Research and Resource Program (FARRP, 2021). No alignments ≥ 35% identity were identified between any of the recombinant proteins and known/putative allergen sequences within the database. LNFP-I/2'-FL manufactured by Glycom was concluded to be of low allergenic risk. As milk derived lactose is used as a substrate during fermentation, LNFP-I/2'-FL would be labeled as "contains milk" in accordance with the requirements of the Food Allergen Labeling and Consumer Protection Act of 2004.

6.2 Literature Search

Glycom considered the totality of publicly available data and information relevant to the safety of LNFP-I and 2'-FL, and performed a literature search for studies relevant to the safety of LNFP-I and 2'-FL. In order to identify studies reporting on relevant safety outcomes for LNFP-I (published any time) and 2'-FL (published since May 2019)[12], a comprehensive and detailed search of the published scientific literature was conducted using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of LNFP-I/2'-FL have considered all publicly available sources of information including favorable and potentially unfavorable information. Based on Glycom's updated search of the literature, the company did not identify published studies to suggest LNFP-I/2'-FL is unsafe for use as a food ingredient. The toxicological studies identified in the scientific literature examining the safety of LNFP-I/2'-FL are summarized in Section 6.4.

6.3 Absorption, Distribution, Metabolism, and Excretion

The manufactured LNFP-I and 2'-FL components of the final ingredient are structurally identical to their naturally occurring counterparts in human milk. The absorption, distribution, metabolism, and excretion of HMOs have been the subject of extensive investigation (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al.*, 2000; Chaturvedi *et al.*, 2001; Rudloff and Kunz, 2012), and it can be concluded that HMOs, including LNFP-I and 2'-FL, do not undergo any significant digestion in the upper gastrointestinal tract. Very small quantities of ingested HMOs have been reported to be absorbed intact (approximately 1 to 2% of the total amount of HMO ingested) and are excreted unchanged in urine. Therefore, the potential for absorption without digestion and excretion intact from consumption of LNFP-I/2'-FL is not a safety concern for infants. Since infants comprise the most sensitive age group, it may be concluded that the absorption of LNFP-I/2'-FL does not pose a safety concern for other age groups.

6.4 Toxicological Studies

The risk assessment approach for LNFP-I/2'-FL follows the same procedures used to support the safety of other HiMOs that have been concluded to be GRAS for use in infant formula by Glycom and others. The pivotal data and information supporting the safety of Glycom's HiMO ingredients are based on qualitative data establishing that HiMOs manufactured by Glycom are chemically and structurally identical to corresponding HMOs present in human milk, and the fact that the intended uses of LNFP-I/2'-FL in infant formula are within ranges of LNFP-I and 2'-FL that have been quantitated in human milk samples across all lactational stages. Since all of Glycom's HiMOs are intended to be used alone or in combination with other HiMOs at levels that are individually and cumulatively within the range that has been reported in human milk samples, the risk assessment does not require derivation of a margin of safety for exposure to the ingredients from infant formula use relative to a NOAEL value from toxicological investigation.

As such, toxicological studies conducted on LNFP-I/2'-FL produced by fermentation are largely corroborative and support that non-HiMO constituents originating from the production process and from the fermentation organism are not present at levels of toxicological concern.

[12] The publication date for the EFSA Opinion on the safety of 2'-FL/DFL as a novel food (EFSA, 2019).

It is noted that, to date, eight toxicity studies in neonatal rats have been conducted with HiMO ingredients manufactured using production strains derived from Glycom's *E. coli* K-12 DH1 MDO lineage (see toxicological studies conducted on 2'-FL, LNnT, 2'-FL/DFL, LNT, 3'-SL, 6'-SL, LNFP-I/2'-FL; Coulet *et al*., 2013, 2014; Phipps *et al*., 2018a,b, 2019a,b, 2021; U.S. FDA, 2015a, 2016a, 2018a, 2019a,b, 2020b,c)[13]. No clear evidence of test article-related toxicity has been reported in any of these studies. These findings support general conclusions that HiMOs are of low inherent toxicity in animals and that Glycom's platform strain is GRAS for its intended use in HiMO production.

6.4.1 90-Day Toxicity Study in the Neonatal Rat (Phipps *et al*., 2021)

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of LNFP-I/2'-FL when administered, by gavage to neonatal rats from Day 7 of age (Phipps *et al.*, 2021). The study was conducted in compliance with the OECD principles of GLP (OECD, 1998) and the most recent version of OECD Test Guideline 408 (OECD, 2018), but was adapted by using neonatal animals (as LNFP-I/2'-FL is primarily intended for use in infant formula).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (vehicle – water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day LNFP-I/2'-FL, by gavage at a dose volume of 10 mL/kg body weight, once daily for 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose LNFP-I/2'-FL group and identify any effects related to the general fiber-like characteristics of the reference material. Doses of LNFP-I/2'-FL and the reference control were corrected to account for "other carbohydrates" within the test article batches (thus, the high dose corresponded to a total carbohydrate amount of 5,550 mg/kg body weight/day). A further 5 males and 5 females in the vehicle control, high-dose LNFP-I/2'-FL and reference control groups were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks to assess the reversibility of any observed effects seen in the dosing period.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of dosing until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control, and high-dose animals were examined in Week 13. Blood samples were collected for hematology, blood chemistry, and thyroid hormone [triiodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH)] analysis during Week 13 and at the end of the recovery period; blood samples were also collected for blood chemistry at the end of the recovery period. Urine samples were collected for urinalysis in Week 13 and at the end of the recovery period.

In Week 11/12, all animals were subjected to a functional observational battery consisting of observations in hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response, and pupil closure response), ulna length, sexual maturation (balano-preputial separation and vaginal opening for males and females, respectively) and estrous cycle monitoring were also recorded for all animals during the dosing period.

[13] 3-FL, also produced by a production organism from the *E. coli* K-12 DH1 MDO lineage, has also been subject to a toxicological battery of studies.

At the end of the dosing and recovery periods, all surviving animals were subjected to a gross macroscopic necropsy, where (for all animals after the dosing period, and for vehicle control, reference control, and high-dose LNFP-I/2'-FL animals after the recovery period) selected organs (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, submandibular and sublingual salivary glands, seminal vesicles, spleen, testes, thymus, thyroid/parathyroid glands, and uterus/cervix) were weighed and fixed. At the end of the treatment period, a full list of tissues [adrenal glands, aorta, brain, cecum, colon, duodenum, epididymides, eyes, femur, Harderian glands, head, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric and left axillary lymph nodes, esophagus, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerves, seminal vesicles, skeletal muscle, skin (with mammary glands), spinal cord, spleen, sternum, stomach, testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix), and vagina] for early decedents and animals in the vehicle control and high dose LNFP-I/2'-FL groups, were examined microscopically. Wet vaginal smears were collected by lavage from all females at necropsy to determine the stage of estrous.

There were no test item-related deaths. Five animals (1 male and 1 female from the mid-dose group, and 1 male and 2 females in the reference control group) were found dead between Days 7 and 9 of dosing. Three animals (2 reference control males and 1 female given 5,000 mg/kg body weight/day) were killed between Days 8 and 11 of dosing for reasons of animal welfare due to body weight loss or general poor clinical condition. The cause of death was undetermined in all cases, as there were no macroscopic or microscopic changes at necropsy. One high-dose male died on Day 15 of dosing, as a direct result of a dosing error. Although cause of death could not be determined in 8 of the cases, as these deaths predominantly occurred in the reference control group (5 animals), followed by the mid-dose group (2 animals), they were considered to be unrelated to administration of LNFP-I/2'-FL.

There were no test item-related clinical signs during the study, nor were there any ocular findings in Week 13 of dosing.

No biologically relevant differences in the age or body weight at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation or vaginal opening for males and females, respectively) were reported. The mean body weights at balano-preputial skinfold separation for males given 3,000 or 5,000 mg/kg body weight/day (235 g for both groups) were statistically significantly higher than for vehicle controls (220 g). However, there was no dose-response relationship and the body weight for reference control males (249 g) was also statistically significantly higher than that of vehicle controls.

There were no statistically significant differences in the age of attainment of the surface and air righting reflexes, performance in the pupil reflex and startle response tests, or mean ulna growth, between LNFP-I/2'-FL groups and vehicle controls. Behavior of the animals during the in-hand and arena observations, as well as Morris maze performance, were similar across all groups. Estrous cycles were unaffected by LNFP-I/2'-FL administration, with most females in all groups showing an estrus smear prior to termination.

Hematological parameters were unaffected by LNFP-I/2'-FL administration. Where statistically significant differences were observed, they were not associated with a dose response (increased neutrophil count for males given 3,000 or 5,000 mg/kg body weight/day, increased monocytes and large unstained cells for mid-dose males, decreased platelets for all male LNFP-I/2'-FL groups and reference controls of both sexes, decreased reticulocytes for low- and mid-dose females, decreased mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration for females given 5,000 mg/kg body weight/day, and shortened prothrombin time for all female LNFP-I/2'-FL groups) and were therefore considered to be unrelated to the test article.

No test article-related adverse effects on clinical biochemistry parameters were reported. Statistically significant differences compared with vehicle controls were either not associated with a dose-response relationship (increased inorganic phosphorus for females at 3,000 or 5,000 mg/kg body weight/day) or the change was in the wrong direction for toxicological relevance (decreased alanine aminotransferase for females given 5,000 mg/kg body weight/day).

Increases in T3 relative to vehicle controls were observed at 5,000 mg/kg body weight/day, both for males (vehicle controls = 558 pg/mL; 5,000 mg/kg body weight/day = 832 pg/mL) and females (vehicle controls = 817 pg/mL; 5,000 mg/kg body weight/day = 1,380 pg/mL); however, there was no clear dose response for either sex, and high-dose values were comparable with those for respective reference controls (reference control males = 1,050 pg/mL; reference control females = 1,090 pg/mL). Increases in TSH for low- and mid-dose males, and for mid-dose females (primarily due to an abnormally high value for Female No 113, with a value of 2,840 pg/mL compared with 506 to 1,390 pg/mL for other females in that group), were also not associated with a dose response, with high-dose values being comparable with those for vehicle controls. T4 values were generally similar across all groups for both sexes.

There were no test article-related changes in urinary parameters. Statistically significant increases in urinary pH (all male LNFP-I/2'-FL groups and females given 3,000 or 5,000 mg/kg body weight/day) and decreased specific gravity (males given 5,000 mg/kg body weight/day) at the end of the dosing period were not associated with a dose response and were, therefore, considered to be unrelated to the test article; the high-dose female pH value and high-dose male specific gravity value were also comparable with those for reference controls. Values for all parameters were generally within historical control ranges for all groups and both sexes. Whilst a few (6 out of 9) individual specific gravity values for males given 5,000 mg/kg body weight/day were below the lower CL of the historical control range, the same was also seen for reference control males (5 out of 7 individual values below the lower CL).

No test article-related differences in organ weights were reported. Salivary gland weight was statistically significantly lower for mid-dose males compared with vehicle controls, but there was no dose-response relationship. Statistically significant reductions in adrenal weight (males given 5,000 mg/kg body weight/day), brain weight (females given 5,000 mg/kg body weight/day) and lungs and bronchi weight (females given 5,000 mg/kg body weight/day) were also observed, but the differences were limited to one gender and mean values for LNFP-I/2'-FL groups were similar to those for respective reference controls.

There were no LNFP-I/2'-FL-related macroscopic or histological abnormalities; the only findings reported were incidental and generally consistent with changes encountered in Sprague Dawley rats of this age kept under laboratory conditions.

The results demonstrate that once daily gavage administration of LNFP-I/2'-FL to neonatal Crl:CD(SD) rats for 90 days (from Day 7 of age) at doses up to 5,000 mg/kg body weight/day (total carbohydrate amount of 5,550 mg/kg body weight/day) was well tolerated and not associated with any test article-related adverse effects.

It was concluded, therefore, that 5,000 mg/kg body weight/day (the highest dose tested) was the NOAEL.

6.4.2 Genotoxicity Studies

6.4.2.1 Bacterial Reverse Mutation Test (Phipps et al., 2021)

The potential mutagenicity of LNFP-I/2'-FL was evaluated in a bacterial reverse mutation test (Ames test), which was performed in compliance with the OECD principles of GLP (OECD, 1998) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[14] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and U.S. Food and Drug Administration (FDA) Redbook IV.C.1.a. (U.S. FDA, 2000) (Phipps *et al.*, 2021).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with LNFP-I at concentrations of up 5,000 µg/plate—the OECD Test Guideline 471 (OECD, 1997) maximum recommended concentration—in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for LNFP-I/2'-FL and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence [2-aminoanthracene and benzo(a)pyrene in the presence of metabolic activation] of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity following exposure to LNFP-I/2'-FL in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that LNFP-I/2'-FL is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) (OECD, 1997).

6.4.2.2 In Vitro Mammalian Cell Micronucleus Test (Phipps et al., 2021)

The clastogenic and aneugenic potential of LNFP-I/2'-FL was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes, in compliance with the OECD principles of GLP (OECD, 1998) and according to OECD Test Guideline 487 (OECD, 2016) (Phipps *et al.*, 2021).

[14] Council Regulation (EC) No 440/2008 of 30 May 2008 implementing test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:32008R0440 (Latest Consolidated Version: 16/10/2019).

An initial preliminary cytotoxicity test was conducted using LNFP-I/2'-FL at concentrations up to 2,000 µg/mL—the OECD Test Guideline 487 (OECD, 2016) maximum recommended concentration—in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation. The first 3-hour treatment in the absence of S9 mix was rejected due to non-acceptance of the positive control. Following an additional 3-hour treatment in the absence of S9 mix, two 3-hour treatments in the presence of S9 mix, and 20-hour treatment in the absence of S9 mix, there were no significant reductions in the cytokinesis-block proliferative index (CBPI) at any LNFP-I/2'-FL concentration tested, compared with vehicle controls.

In the main experiment for micronucleus analysis, human lymphocytes were treated with concentrations of LNFP-I at 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

In the first 3-hour treatment in the presence of S9 mix, all mean micronucleus frequencies were within the laboratory historical 95% CL, except for the mean micronucleus frequency at the low concentration (500 µg/mL), which was slightly above the 95% CL (10.5 *vs*. 9.2). However, this 3-hour treatment was repeated and the mean micronucleus frequencies for all test item treated cultures in this repeat experiment were within the laboratory historical 95% CL. Therefore, there was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that LNFP-I/2'-FL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration) (OECD, 2016).

6.5 Human Intervention Studies

Studies in humans evaluating the consumption of LNFP-I/2'-FL have not been published to date. However, clinical studies have been conducted on 2'-FL, which are incorporated by reference to GRNs 546, 571, 650, 735, 749, 815, 852, and 897 (U.S. FDA, 2015b,c, 2016a, 2018a,b, 2019a,c, 2020d). For a summary of relevant clinical studies evaluating the addition of 2'-FL to infant formula see Section 6.4 of GRN 815. Clinical data from randomized controlled studies indicate that 2'-FL is well tolerated and does not give rise to any safety concerns when orally administered to infants at up to 1.2 g/L in infant formula (Puccio *et al.*, 2017, NCT01715246); in young children (ages 1 to 2.5 years) at 3 g/L in young child formula products (Leung *et al.*, 2020; Netherlands Trial Register NL4627) and adults at up to 20 g/day (Elison *et al.*, 2016; NCT01927900).

6.6 Allergenicity

LNFP-I/2'-FL is a high-purity ingredient and is specified to contain ≤ 0.0017% protein on a w/w basis.

Glycom has assessed the allergenic potential of the recombinant proteins introduced to the *E. coli* K-12 host using the search algorithms provided by the Allergen Online tool (ver. 21) of the University of Nebraska (FARRP, 2021). This database has been updated last on 14 February 2021 and contains sequences of 2,233 known and putative allergens. The online tool allows searches by three different search algorithms each

with its own alert limit for potential allergenicity: (i) Full sequence length (FASTA) comparison with an alert threshold of greater than 50% sequence similarity indicating potential allergenic potential (ii) 80 amino acid sequence segments (sliding window) comparison with an alert threshold of greater than 35% sequence identity (iii) 8 mer sequence segments (sliding window)[15]. No sequence alerts for potential cross-reactivity to known allergens were identified.

6.7 Other Considerations – Additive Dietary Intakes of LNFP-I/2'-FL with Other HiMOs and Resistant Oligosaccharides

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, such as 2'-FL, 2'-FL/DFL, LNT, LNnT, 3'-SL, 6'-SL, and LNFP-I/2'-FL will be used in combination to produce infant formula products that are as compositionally representative of human milk as possible, taking into account their natural variation. Glycom recognizes that there are known gastrointestinal tolerance issues that can develop if consumed levels of indigestible carbohydrates, such as HiMOs, are too high in sensitive populations including infants. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all geographies considering natural variation. As discussed in Section 1.3, a concentration of 1.5 g/L of LNFP-I/2'-FL in infant formula will ensure that consumers of infant formula containing LNFP-I/2'-FL will be provided with nutritional concentrations that remain below the exposure that covers 95% of the population of infants consuming human milk. The use of mean values from milk samples across multiple studies ensures that reference values for appropriate concentrations of HiMOs are not impacted by potential artifacts of a particular analytical method, or by values from individual mothers that may be biological aberrations.

The maximum level of HiMOs used in combination (*i.e.*, an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in samples of mature human milk (Rudloff and Kunz, 2012; Bode, 2013; Xu *et al.,* 2017). In all cases where Glycom's HiMOs will be used in combination with other HiMOs, the total concentrations of HiMOs will fall within conservative means of the general population, thereby ensuring that levels provided will be of nutritional value and be safe and well tolerated. For example, the total quantities of HiMOs that could be added to infant formula based on existing and future GRAS Notifications for HiMOs manufactured by Glycom or others (*e.g.*, 2'-FL, DFL, LNT, LNnT, 3'-SL, 6'-SL, and LNFP-I) would be below 6.22 g/L at their maximum GRAS use levels[16]. This concentration is below the concentrations of total HMOs naturally occurring in human milk samples which are in the region of 12 g for mature milk and as high as 25 g in colostrum (Rudloff and Kunz, 2012; Bode, 2013; Xu *et al.,* 2017). Therefore, considering that Glycom's HiMOs are identical to their natural counterparts in human milk, the total amount of HMOs used singly and in combination in infant formula products are not a safety or tolerability concern in infants. In fact, the most sensitive consumer group, infants in age of 1 to 4 days, is exposed to the highest concentrations of HMOs, as the early milk "colostrum" contains the highest levels of HMOs, and it is therefore, apparent that infants have an inherent high tolerance for these compounds.

[15] As noted on the Allergen Online website, the scientific evidence that an 8 amino acid match would identify possible cross-reactive proteins is limited (*i.e.*, two proteins sharing only a single short identity match do not share IgE binding in the absence of more extensive identity alignments) and the algorithm is provided for regulatory purposes. Glycom has performed this search as an over-conservative measure.

[16] Summation of maximum use levels for HiMOs used in infant formula: 2.4 g/L 2'-FL (GRN 650) + 0.6 g/L LNnT (GRN 659) + 0.32 g/L DFL (GRN 815) + 0.8 g/L LNT (GRN 833) + 0.4 g/L 6'-SL (GRN 880) + 0.2 g/L 3'-SL (GRN 881) + 1.5 g/L LNFP-I.

2'-FL has been Notified to the U.S. FDA as GRAS by Glycom as a single ingredient and as a mixture with DFL (U.S. FDA, 2016b, 2019a). Based on the highest measured content of 2'-FL in LNFP-I/2'-FL batches, the maximum resulting use level of 2'-FL from the intended use of LNFP-I/2'-FL described herein is anticipated to be no more than 0.83 g/L[17], while the maximum use level of 2'-FL described in GRN 650 is 2.4 g/L (U.S. FDA, 2016b). Uses of any 2'-FL-containing ingredients would not provide 2'-FL levels that would exceed the level determined to be GRAS for 2'-FL alone (2.4 g/L).

Glycom also recognized the possibility that the company's HiMOs may be used in combination with other non-digestible carbohydrate sources such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which have GRAS status for use in infant formula. Although Glycom is not a manufacturer of infant formula, and is therefore not in a position to comment on the levels of resistant oligosaccharides such as GOS or FOS that could be used with a HiMO, or even the likelihood that such combinations would be introduced to the market, Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the *Federal Food, Drug, and Cosmetic Act* [21 USC §350(a)]. Specifically, under Section 412(d)(1) of the *Federal Food, Drug, and Cosmetic Act*, a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula (U.S. FDA, 2021c). Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination (*e.g.*, use of LNFP-I/2'-FL with an indigestible oligosaccharide such as GOS) would be well tolerated as part of the Agency's 90-day notification procedure. Section 412, therefore, ensures that any combination of HiMO whether used singularly, or on an additive basis with various HiMOs will be the subject of corroborative safety and tolerance testing in infants.

The intended use level of 1.5 g/L of LNFP-I/2'-FL represents a value that is conservative, as it falls below the highest reported mean values (4.47 LNFP-I/L) and is several-fold below the 95% CL (5.75 g LNFP-I/L) that can be derived from the published literature (see Appendix A). It is Glycom's view that it is not necessary to calculate safe/tolerable intake levels of HiMOs for infants by utilizing mg/kg body weight calculations. Since dietary intakes of HiMOs by infants are exclusively provided by infant formula, reference levels for safe intakes should be conducted by comparing target concentrations in infant formula to concentrations in human milk. Accordingly, Glycom did not calculate a tolerable upper level as no concentration of LNFP-I/2'-FL in human milk has been reported to be deleterious; however, it seems reasonable to conclude that the upper-range of values that have been reliably reported for human milk samples represents an observed safe level or highest observed intake (HOI) value that can be extrapolated to other population groups (Hathcock and Kriengsinyos, 2011)[18]. Glycom recognizes that LNFP-I/2'-FL is proposed for addition to other foods that may substitute for infant formula as the infants age, and therefore, there is the possibility that dietary intakes from infant formula and conventional foods will be at least partially additive on occasion. Glycom notes, however, that target concentrations of LNFP-I/2'-FL in infant formula relative to background concentrations provide a significant margin of safety that would fall within the typical range

[17] The highest ratio of 2'-FL to bulk powder is at 31.76 % observed in batch [redacted] where LNFP-I is at 57.7%. Therefore, at the highest observed ratio of 2'-FL, the GRAS use levels of 1.5 g LNFP-I/L would result in, at most, an equivalent use level of 0.83 g 2'-FL/L.

[18] In situations where no evidence of toxicity has been observed in the clinical dataset and derivation of an upper limit is not possible, use of the HOI has been suggested as an alternative. The HOI represents the "*highest intake with adequate data to show, with acceptable confidence, the absence of adverse effects up to that intake.*" [Hathcock J, Kriengsinyos W (2011). Highest Observed Intake: definition, regulatory uses and provisional values. Regul Toxicol Pharmacol 61(1):115-118].

experienced by breastfed infants and therefore such sporadic occurrences of added consumption form infant formula and other foods are not a safety or tolerance concern.

6.8 GRAS Panel Evaluation

Glycom has concluded that LNFP-I/2'-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of LNFP-I/2'-FL, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Professor Emeritus, Virginia Commonwealth University School of Medicine), Dr. George C. Fahey (Professor Emeritus, University of Illinois), and Dr. Ronald Kleinman (Professor, Harvard Medical School).

The GRAS Panel, convened by Glycom, independently and critically evaluated all data and information presented herein, and also concluded that LNFP-I/2'-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of LNFP-I/2'-FL, is presented in Appendix B.

6.9 Conclusion

Based on the above data and information presented herein, Glycom has concluded that the intended uses of LNFP-I/2'-FL in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, is GRAS based on scientific procedures. General recognition of Glycom's GRAS conclusion is supported by the unanimous consensus rendered by an independent GRAS Panel, qualified by experience and scientific training, to evaluate the use of LNFP-I/2'-FL in infant formula and conventional food, who similarly concluded that the intended use of LNFP-I/2'-FL in infant formula and conventional food as described herein is GRAS.

LNFP-I/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. LIST OF SUPPORTING DATA AND INFORMATION

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Last Amended	Section Title
101—Food labeling	101.12	4-1-20	Reference amounts customarily consumed per eating occasion
107—Infant formula	107.100	4-1-20	Nutrient specifications
170—Food additives	170.3	4-1-19	Definitions
	170.30	4-1-19	Eligibility for classification as generally recognized as safe (GRAS)
184—Direct food substances affirmed as generally recognized as safe	184.1685	4-1-19	Rennet (animal-derived) and chymosin preparation (fermentation-derived)

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APPENDIX A
Comprehensive Report on the Quantitative Data for LNFP-I and 2'-FL in Human Milk

Comprehensive Report on the Quantitative Data for LNFP-I and 2'-FL in Human Milk

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Comprehensive Report on the Quantitative Data for LNFP-I and 2'-FL in Human Milk

1.0 QUANTITATIVE DATA ON THE NATURAL CONCENTRATIONS OF HMOS IN HUMAN MILK

1.1 The Oligosaccharide Fraction of Human Milk

Lacto-*N*-fucopentaose I (LNFP-I) and 2'-fucosyllactose (2'-FL)] are important and significant component of the natural human milk oligosaccharide (HMO) fraction of human milk. Human milk contains, as its third largest solid component, a fraction consisting of a complex family of structurally related oligosaccharides (György *et al.*, 1952; Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). These are known as HMOs because they were first discovered in human milk (reviewed by Malpress and Hytten, 1958) and because they occur in human milk at much higher concentrations than in other mammalian milk (Urashima *et al.*, 2001).

More than 140 members of this family have been fully described on a structural basis (Urashima *et al.*, 2011; Chen, 2015; Remoroza *et al.*, 2020), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al.*, 1999; Wu *et al.*, 2010; Wu *et al.*, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012) with significant variation on individual level and in dependency of the lactation period.

In contrast, bovine milk contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013) that does not contain fucosylated oligosaccharides at any appreciable level (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Messer and Urashima, 2002; Urashima *et al.*, 2012a).

HMOs consist of a lactose core, to which one or several of the four monosaccharides, *N*-acetyl-D-glucose, D-galactose, L-fucose, and/or *N*-acetyl-D-neuraminic acid (“sialic acid”) are attached in specific connectivity and linkages. Although HMOs are diverse in their structure, they can be categorized into three main classes: neutral core HMOs (containing the aminosugar GlcNAc), neutral fucosylated HMOs (containing fucose), and acidic HMOs (containing sialic acid).

1.2 History of Human Milk Phenotype Discovery

In the 1960s, ground-breaking work performed by Victor Ginsburg (Kobata *et al.*, 2004) and Akira Kobata (Endo, 2010) revealed the enzymatic basis for the human blood groups, which are *carbohydrate-based* cell-surface antigens, and their close structural and biosynthetic relationship to the freely occurring milk oligosaccharides (Grollman *et al.*, 1969; Shen *et al.*, 1968; Kobata *et al.*, 1968). In this context it had been initially recognized in 1967 that not all mothers excrete 2'-FL into their milk, because they do not express a specific enzyme that is needed for 2'-FL biosynthesis (Grollman and Ginsburg, 1967). This observation is not limited to the freely occurring 2'-FL in milk, but includes other cell-surface bound “2'-FL” epitopes that are

typically secreted into other bodily fluids like blood and saliva. Therefore, the term “non-secretor” was coined for this phenotype. Based on this initial finding it was established that human milk can be categorized into four different “milk groups” based on the combination of the presence (or absence) of distinct structural features in their oligosaccharide fraction – the Secretor and Lewis phenotype − and that these different milk groups contain a significantly different amount of fucosylated HMOs in general (Thurl *et al.*, 1997).

The categorization of milk group is indeed related and comparable to “blood groups,” but with the important difference that every mother is a “universal donor” of milk, due to the fact that her child’s Secretor and Lewis genotype can differ from its mother due to inheritance of a different allele from the father (heterozygosity). In practical clearness, this can also be concluded from the long and safe tradition of wet nursing that was commonplace before the invention of infant formulas (Stevens *et al.*, 2009; Mason *et al.*, 2013) or from the fact that milk banks serving neonatal intensive care units (NICUs) pool donor milk without reports of adverse events (Ahrabi and Schanler, 2013; Marx *et al.*, 2014). It means that all milk groups are fundamentally safe for an infant; however, there are indeed differences in the nutritional effects of each milk group for the infant which can be investigated in observational studies where mother-child pairs are stratified according to milk phenotypes (and possibly also infant genotypes).

1.3 Genetic Polymorphisms Shaping Milk Composition: Secretor and Lewis Phenotypes

Nearly 60 to 80% of the total HMO fraction is comprised of neutral fucosylated oligosaccharides that contain the sugar fucose in their chemical structure (Ninonuevo *et al.*, 2006; Bode, 2012). Fucose can principally be added by several different enzymes in four distinct molecular linkages, namely α-(1,2) to D-galactose, α-(1,3) to D-glucose and/or D-GlcNAc (*i.e.*, *N*-acetyl-D-glucosamine), and α-(1,4) to D-GlcNAc.

Two of these linkages are not found in the milk of all mothers since the genes (*i.e.*, *fut2*, *fut3*) encoding the respective enzymes [*i.e.*, α-(**1,2**), and α-(**1,3/4**)-fucosyltransferases FUT2 and FUT3] are subject to genetic polymorphism that reflect events of heredity over evolutionary times causing partial to total loss of the enzyme function in some proportion of the population. Maintenance of the genetic polymorphism of these traits in the population indicate opposing trends in selective pressures either from environmental (*e.g.*, regional prevalence of infectious agents) or parent-offspring conflicts (Gagneux and Varki, 1999; Bishop and Gagneux, 2007; Varki *et al.*, 2009; Springer and Gagneux, 2013; Springer and Gagneux, 2016).

In consequence, four different milk phenotype groups can be characterized in the global population (corresponding to the combination of genotypes *fut2*$^{+}$/*fut3*$^{+}$, *fut2*$^{-}$/*fut3*$^{+}$, *fut2*$^{+}$/*fut3*$^{-}$, and *fut2*$^{-}$/*fut3*$^{-}$). A person’s *fut2*$^{+}$ genotype leads to the so-called ***Secretor*** phenotype, which – as briefly mentioned above − has received its name historically from the observation that soluble blood group substances (carrying epitopes A, B, and 0) can be detected in their bodily secretions (*e.g.*, milk, blood, saliva, urine) (Grollman and Ginsburg, 1967; Shen *et al.*, 1968). The *fut3*$^{+}$ genotype acts phenotypically *in combination* with the *fut2*$^{+}$ genotype and leads either to the so-called ***Lewis a*** or ***Lewis b*** phenotypes (Thurl *et al.*, 1997). See Table 1.3-1 for a summary of this information, typical frequencies of the different milk phenotypes and the information which distinct HMO occurs in which milk phenotype group.

Table 1.3-1 Milk Phenotype Groups

Secretor Status	Secretor	Secretor	Non-Secretor	Non-Secretor
Milk Group	**1**	**3**	**2**	**4**
Milk Phenotype	Se**+** / Le (a−b+)	Se**+** / Le (a−b−)	Se**−** / Le (a+b−)	Se**−** / Le (a−b−)
α1,2-fucosylated HMOs (**FUT2** enzyme)	**+**	**+**	**−**	**−**
α1,4-fucosylated HMOs (**FUT3** enzyme)	**+**	**−**	**+**	**−**
α1,3-fucosylated HMOs (FUT3, FUT5, FUT6 enzymes)	**+**	**+**	**+**	**+**
Typical frequency	~ 70%	~ 9%	~ 20%	~ 1%
HMOs FUT2+ **&** FUT3+	LNDFH-I, DF-LNH-III, TF-LNH	None	None	None
HMOs FUT2+ **or** FUT3+	**2'-FL**, DFL, **LNFP-I**, F-LNH-I, DF-LNH-I, S-LNFP-I, FS-LNH		LNFP-II, LNDFH-II, F-LNH-II, DF-LNH-II, DF-*para*-LNH, S-LNFP-II	None
HMOs contained in all groups	3-FL, 3'-SL, 6'-SL, FSL, LNT, LNnT, LNH, LNnH, LNFP-III, LNFP-V, LNFP-VI, F-LNH-III, F-*para*-LNH-I, DF-*para*-LNnH, LSTa, LSTb, LSTc, DS-LNT, S-LNH, S-LNnH-I, FS-LNnH-I, DS-F-LNH-II			

For abbreviations please see **List of Abbreviations**.

The milk phenotype groups are oftentimes simply referred to as "milk groups." The key characteristic of each milk group can be expressed in words as follows (please note the order of Milk Groups 2 and 3 are inverted in Table 1.2-1 to allow for grouping of Secretor and Non-Secretor phenotype HMOs):

- **Milk Group 1** is excreted by mothers who express both enzymes encoded by the *fut2* and *fut3* genes in their mammary glands and thus synthesize both α-1,2-fucosylated HMO and α-1,4-fucosylated HMOs. This is one of the Secretor phenotype groups.

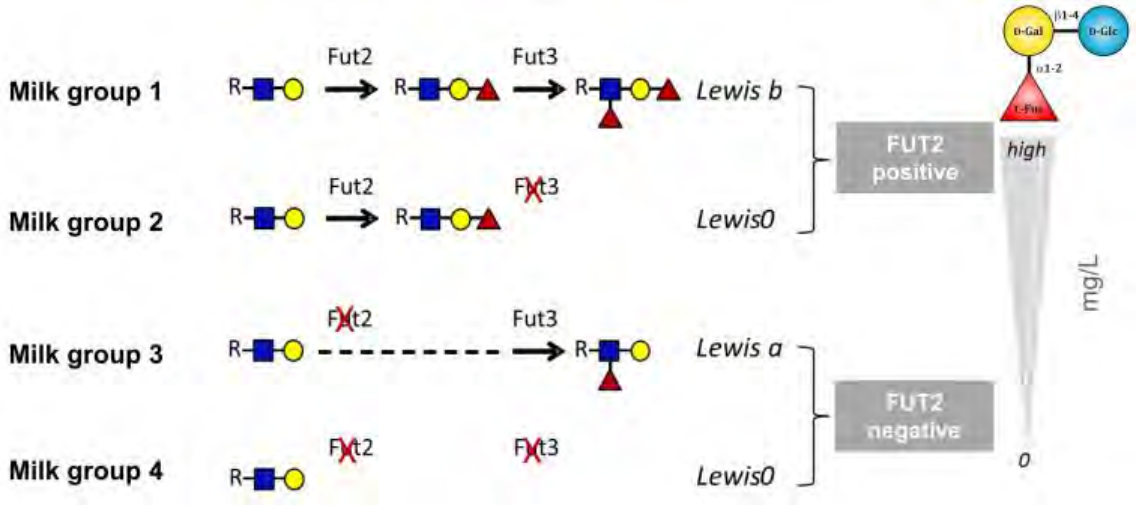
- **Milk Group 2** is excreted by mothers who express only the enzyme encoded by the *fut3* gene but not the enzyme encoded by the *fut2* gene in their mammary glands and thus synthesize α-1,4-fucosylated HMOs but not α-1,2-fucosylated HMOs. This is one of the Non-Secretor phenotype groups.

- **Milk Group 3** is excreted by mothers who express only the enzyme encoded by the *fut2* gene but not the enzyme encoded by the *fut3* gene in their mammary glands and thus synthesize α-1,2-fucosylated HMOs but not α-1,4-fucosylated HMOs. This is one of the Secretor phenotype groups.

- **Milk Group 4** is excreted by mothers who express neither enzyme encoded by the *fut2* nor *fut3* genes in their mammary glands and thus cannot produce α-1,2-fucosylated HMOs nor α-1,4-fucosylated HMOs. This is one of the Non-Secretor phenotype groups.

Generally, the concentration of fucosylated HMOs in human milk is decreasing from Milk Group 1 > 2 ≈ 3 > 4.

Figure 1.3-1 Concentration of 2'-FL Increases from Milk Group 4 < 2 < 3 < 1



The prevalence of these four milk groups varies to some extend globally according to ethnicity or geographical location, but is typically, and in most ethnicities including the Caucasian and Chinese population, around 70% Group 1 (express *FUT2* and *FUT3*), 19% Group 2 (express FUT3 but not FUT2), 10% Group 3 (express FUT2 but not FUT3) and 1% Group 4 (no expression of either *FUT2* or *FUT3*) (Thurl *et al.*, 2010; Castanys-Muñoz *et al.*, 2013; Austin *et al.*, 2016), so that it can be roughly stated that 70to 80% of the global population are Secretors.

These phenotypes (*i.e.*, Secretor and Lewis) can be best understood in the context of general human polymorphisms for the carbohydrate histo-blood group and Lewis antigens and we refer to excellent overview articles for more detail (Oriol *et al.*, 1986; Hod *et al.*, 2009; Ferrer-Admetlla *et al.*, 2009).

Curiously, while more than 200 different HMO structures can be detected in human milk by sensitive analytical techniques, the ten most abundant HMOs alone account typically and on average for more than 75% of the oligosaccharide fraction by mass. Among the five to ten most abundant HMOs are a number of fucosylated HMOs: 2'-fucosyllactose (2'-FL), lacto-*N*-fucopentaose I (LNFP-I), lacto-*N*-difucohexaose I (LNDFH-I), 3-fucosyllactose (3-FL), and difucosyllactose (DFL) (Thurl *et al.*, 2017; Bych *et al.*, 2019; Molnar-Gabor *et al.*, 2019; Hundshammer and Minge, 2020).

Two of these HMOs (LNFP-I and LNDFH-I) also contain – next to the fucose structural element − another biologically intriguing feature of HMO structures, the *type I linkage* [*i.e.*, Gal-β(1-3)-GlcNAc]. The type I linkage is predominant over type 2 [*i.e.*, Gal-β(1-4)-GlcNAc] derived HMOs in human milk, which appears to be a unique feature of humans in contrast to other mammals (Urashima *et al.*, 2012b).

2'-FL is a trisaccharide consisting of L-fucose, D-galactose and D-glucose and is broadly reported as the most abundant HMO among women expressing α-1,2-fucosyltransferase (Secretors) – Milk Phenotype Groups 1 and 3 – but also among pooled Secretor and non-Secretor human milk. There are no other α-(**1,2**)-fucosyltransferases (other than FUT2) known to act in the mammary gland which could compensate for its genetic loss.

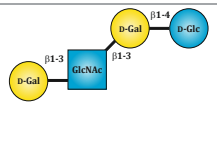
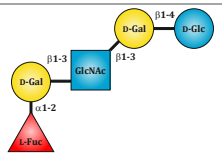
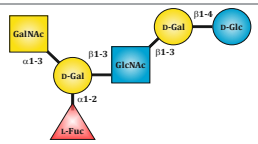
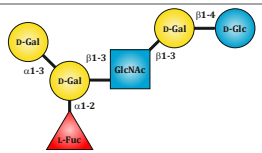
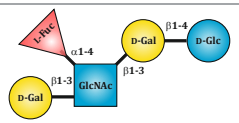
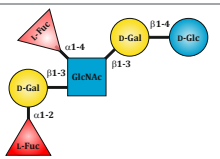
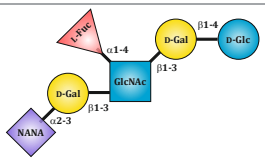
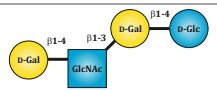
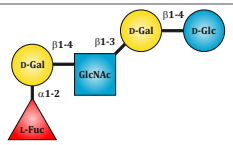
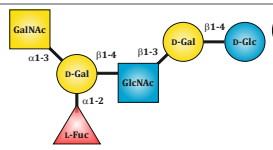
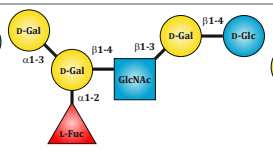
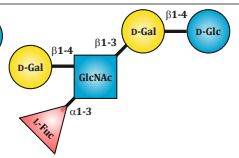
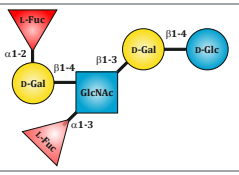
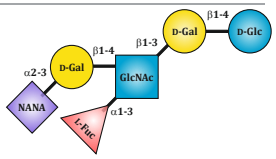
LNFP-I is a pentasaccharide HMO derived from the human-characteristic *type I* motif-containing lacto-*N*-tetraose (LNT) by addition of a fucose in the typical Secretor phenotype HMO linkage (α-1,2-fucose). It therefore combines two biologically interesting structure motifs in one molecule and occurs at large concentrations in breast milk. It is broadly reported as one of the most abundant HMO among women expressing α-1,2-fucosyltransferase (Secretors) – Milk Phenotype Groups 1 and 3 – but also among pooled Secretor and non-Secretor human milk. There are no other α-(**1,2**)-fucosyltransferases (than FUT2) known to act in the mammary gland which could compensate for its genetic loss.

1.4 Evolutionary Rationale for HMOs

All cellular surfaces are covered by a dense carbohydrate layer, the glycocalix (Varki, 2011; Varki, 2017), and presentation of select carbohydrate epitopes differs between species characteristically (Bishop and Gagneux, 2007). Human epithelial surfaces are rich in carbohydrate blood group and Lewis antigen epitopes (Oriol *et al.*, 1986; Ravn and Dabelsteen, 2000; Hod *et al.*, 2009). These human blood group and Lewis antigen epitopes are common binding receptors for pathogens (viral, bacterial, fungal, protozoan, and parasitical), and selective recognition is needed for infection (Zopf and Roth, 1996; Sharon and Ofek, 2000; Marionneau *et al.*, 2005; Le Pendu *et al.*, 2006).

Human milk has evolved under a trade-off optimization process between mother and child during mammalian evolution (Trivers, 1974; German *et al.*, 2002; Petherick, 2010; Oftedal, 2012). HMOs are freely occurring oligosaccharides, but their structures present identical epitopes to human cell surface-linked carbohydrate epitopes (*i.e.*, human blood group and Lewis antigens) (Bode and Jantscher-Krenn, 2012; Newburg and Grave, 2014). The latter are part of the defensive barrier lining the epithelial surfaces (in the form of the glycocalyx and mucin glycans), and were subject to strong selection (evolutionary pressure) due to a “molecular arms race” between infectious agents and humans (Messer and Urashima, 2002; Bishop and Gagneux, 2007; Urashima *et al.*, 2012a; Springer and Gagneux, 2013). The fact that identical carbohydrate epitopes are expressed as free oligosaccharides in the breast and excreted into milk thus proposes that these free oligosaccharides serve as an intriguing defense mechanism of mammals in general and humans in particular (see Table 1.4-1 for an overview of Histo-Blood Group and Lewis Antigens as presented as free epitopes by the Human Milk Oligosaccharides). Human milk contains the largest number and amounts of oligosaccharides of all mammalian species investigated (Newburg *et al.*, 1999; Newburg, 2000; Tao *et al.*, 2011).

Table 1.4-1 Histo-Blood Group and Lewis Antigens and their Connection to HMO Structures

TYPE 1 Antigens[a]							
Name	**Lewis^{C}**	**Lewis^{D}**	**A** *(type 1)*	**B** *(type 1)*	**Lewis^{A}**	**Lewis^{B}**	**Sialyl-Lewis^{A}**
Structure							
HMO[b,c]	LNT	LNFP I, F-LNH I, DF-LNH I, FS-LNH, TF-*iso*-LNO I, Tetra-F-*iso*-LNO, DF-LND I, DF-LND V	**not expressed in human milk**	**not expressed in human milk**	LNFP II, LNDFH II, DF-LNH II, DF-*para*-LNH, TF-*para*-LNH II, DF-LNnO I, DF-*iso*-LNO I, TF-LNnO	LNDFH I, TF-LNH, TF-*para*-LNH I, TetraF-*iso*-LNO, TetraF-*para*-LNO, PentaF-*iso*-LNO, TetraF-LND I	F-LSTa, S-LNFP II, FS-LNH IV, DFS-LNH II, FS-LNO, FS-*iso*-LNO, DFS-*iso*-LNO I, DFS-*iso*-LNO II, DFS-LNO
Name	**i**	**H** (0)	**A** *(type 2)*	**B** *(type 2)*	**Lewis^{X}**	**Lewis^{Y}**	**Sialyl-Lewis^{X}**
Structure							
HMO[b,c]	LNnT	F-LNnD II, 2'-FL[d]	**not expressed in human milk**	**not expressed in human milk**	LNFP III, LNDFH III, F-LNH II, DF-LNH I + II, DF-LNnH, DF-*para*-LNnH, TF-LNH, TF-*para*-LNnH, 3FL[d]	DFS-LNnH, LDFT[d]	SDF-LNH I, SDF-LNH II, FDS-LNnH, FSL[d]

Fuc = Fucose; Gal = Galactose; Glc = Glucose; GlcNAc = *N*-Acetyl-glucosamine; HMO = Human milk oligosaccharide; Neu5Ac = *N*-acetyl-neuraminic acid (sialic acid)

[a] The structure presented by the terminal 3-4 sugars possesses the highest antigenic activity, with the backbone extension merely modulating the immune response.

[b] The list includes HMOs possessing the terminal 3-4 antigenic sugars as part of their structure.

[c] Abbreviations of HMOs are according to Urashima *et al.* (Urashima *et al.*, 2011).

[d] The structure of the simple lactose-derived HMOs (2'FL, 3FL, FSL) mimics the respective antigens (glucose replaces N-acetylglucosamine).

This hypothesis is supported by many preclinical studies that demonstrated that HMOs bind efficiently to a diverse range of pathogens (reviewed by Zopf and Roth, 1996; Sharon and Ofek, 2000; Newburg *et al*., 2005; Kunz and Rudloff, 2006; Hickey, 2012; Bode, 2015; Craft and Townsend, 2018, 2019; Morozov *et al.*, 2018) and their toxins (reviewed by Newburg, 2009; El-Hawiet *et al*., 2015). In addition, the human microbiome evolved concurrently with the evolution of human epithelial surface glycans and milk oligosaccharides (Schluter and Foster, 2012; Moeller *et al*., 2016; Duranti *et al*., 2019; Sakanaka *et al.*, 2019). Milk oligosaccharides are not found as freely occurring oligosaccharides anywhere else in nature but almost exclusively in milk (with exception of tiny quantities being found in urine and blood from lactating mothers) (Newburg, 2000; Bode, 2006; Wise *et al.*, 2018; Jantscher-Krenn *et al.*, 2019).

It is important to note that the genetic polymorphism that forms the basis of the Secretor and Lewis phenotype diversity reflects events of heredity over evolutionary times that caused partial to total loss of the enzyme function in some proportion of the population. Maintenance of the genetic polymorphism of these traits in the population indicate opposing trends in selective pressures either from environmental (*e.g*., regional prevalence of infectious agents) or parent-offspring conflicts (Gagneux and Varki, 1999; Bishop and Gagneux, 2007; Varki *et al.*, 2009; Springer and Gagneux, 2013; Springer and Gagneux, 2016). With simpler words: the fact that 20% of mothers do not provide alpha-1,2-linked fucosylated HMOs to their infant doesn't mean that these HMOs would not be of benefit to their child; rather it reflects that the mother herself can be at a disadvantage of possessing the Secretor phenotype.

1.5 Basis for Quantitative Data of HMOs in Human Milk

The concentrations of HMOs in human milk across lactation phases and across geographies have been investigated in many studies. In 2017, the mean concentrations of 33 oligosaccharides in human milk were systematically and comprehensively reviewed (Thurl *et al.*, 2017). These data provide valuable insight on the patterns of HMO expression in mothers globally and observed wide cross-individual and inter-laboratory variations. The analysis included work up to 2016, identified 48 full-text articles that reported quantitative data of HMOs and included 21 in the systematic review after inclusion/exclusion criteria were applied.

In the years since 2016 many additional studies from all over the world were reported increasing our understanding of global trends and hence an update of the literature base was herein undertaken.

In total **65** publications were identified, and the sections below detail the methods of evaluation of the entire scientific literature identified, followed by a summary of the concentrations of each individual HMO from the total of selected publications.

1.5.1 Scientific Literature Base

A literature search was conducted, identifying peer-reviewed, original scientific papers in the English language published between 1993 and December 2020. An initial collection of 65 articles were identified and entered into a literature base. From this, data relevant to the assessment of selected HMO concentrations in human milk were further identified with the following inclusion and exclusion criteria (see Table 1.5.1-1).

Table 1.5.1-1 Literature Search – Inclusion and Exclusion Criteria

Inclusion	Exclusion
• Peer-reviewed original scientific articles in the English language • Containing data for selected HMOs from human milk • Containing data from at least 10 different donors	• Data from animal milks • Relative quantitative data only • Containing data from less than 10 donors **Additional Exclusion for Secretor-Phenotype HMOs** • Data from unknown milk phenotype • Data only in mothers with non-Secretor milk phenotype • Data only in mixed milk phenotype (pooled samples)

For abbreviations, please see the **List of Abbreviations.**

An overview of all papers included and excluded in the present analysis is presented in Table 1.5.1-2 below.

Table 1.5.1-2 List of Scientific Articles Reporting Individual HMO Concentrations in Human Milk

#	Citation	Global Region	Gestation	Milk Phenotype Group	Analytical Method	Number HMOs	Neutral / Acidic HMOs	Earliest Sampling Day	Latest Sampling Day	Number Donors
1	**Kunz and Rudloff (1993)**	Europe	Term	Mixed (pooled samples)	HPAEC-PAD	8	Neutral & Acidic	15	60	**n.a.**
2	**Thurl *et al.* (1996)**	Europe	Term	1 (Se+/Le+)	HPAEC-PAD	20	Neutral & Acidic	15	60	**1**
3	**Chaturvedi *et al.* (1997)**	LATAM	Term	Mixed (pooled samples)	RP-HPLC-UV	12	Neutral	30	60	**50**
4	**Coppa *et al.* (1999)**	Europe	Term	1 (Se+/Le+)	HPAEC-PAD	21	Neutral & Acidic	4	90	**18**
5	**Kunz *et al.* (1999)**	Europe	Term	Mixed (pooled samples)	HPAEC-PAD	10	Neutral & Acidic	14	28	**10**
6	**Nakhla *et al.* (1999)**	N. Amer.	Term & Preterm	Mixed & Le+	HPAEC-PAD	10	Neutral	0	33	**13**
7	**Erney *et al.* (2000)**	Asia	Term	Mixed & SEC	HPAEC-PAD	9	Neutral	0	452	**381**
8	**Kunz *et al.* (2000)**	Europe	Term	Mixed (pooled samples)	HPAEC-PAD	8	Neutral & Acidic	2	19	**4**
9	**Shen *et al.* (2000)**	N. Amer.	Term	Mixed (pooled samples)	HPCE-UV	5	Acidic	n.a.	n.a.	**n.a.**
10	**Chaturvedi *et al.* (2001)**	N. Amer.	Term	SEC	HPLC-UV	12	Neutral	1	7	**84**
11	**Erney *et al.* (2001)**	AM & EUR	Term	Mixed (pooled samples)	HPAEC-PAD	9	Neutral	1	100	**368**
12	**Martín-Sosa *et al.* (2003)**	Europe	Term	Mixed (pooled samples)	HPLC-UV	7	Acidic	1	32	**12**
13	**Sumiyoshi *et al.* (2003)**	Asia	Term	Mixed (pooled samples)	HPLC-UV	6	Neutral	4	100	**16**
14	**Morrow *et al.* (2004)**	LATAM	Term	Mixed (pooled samples)	RP-HPLC-UV	4	Neutral	1	100	**93**
15	**Musumeci *et al.* (2006)**	Africa & Europe	Term	Mixed (pooled samples)	HPAEC-PAD	2	Neutral & Acidic	1	3	**103**
16	**Asakuma *et al.* (2007)**	Asia	Term	Mixed (pooled samples)	HPLC-UV	9	Acidic	1	3	**20**
17	**Bao *et al.* (2007)**	N. Amer.	Term	Mixed (pooled samples)	CE-UV	12	Acidic	1	4	**14**
18	**Sjögren *et al.* (2007)**	Europe	Term	Mixed (pooled samples)	HPLC-UV	9	Neutral	2	4	**20**
19	**Asakuma *et al.* (2008)**	Asia	Term	SEC	HPLC-UV	10	Neutral	1	3	**12**
20	**Leo *et al.* (2009)**	other	Term	Mixed (pooled samples)	RP-HPLC-FL	11	Neutral	5	60	**8**
21	**Leo *et al.* (2010)**	other	Term	Mixed (pooled samples)	HPLC-UV	17	Neutral & Acidic	5	155	**16**
22	**Thurl *et al.* (2010)**	Europe	Term	1-3	HPAEC-PAD	20	Neutral & Acidic	3	90	**30**
23	**Asakuma *et al.* (2011)**	Asia	Term	Mixed (pooled samples)	HPLC-FL	10	Neutral	30	120	**57**
24	**Coppa *et al.* (2011)**	Europe	Term	1-4	HPAEC-PAD	8	Neutral	25	35	**39**
25	**Gabrielli *et al.* (2011)**	Europe	Preterm	1-4	HPAEC-PAD	23	Neutral & Acidic	4	30	**63**
26	**Galeotti *et al.* (2012)**	Europe	Term	1-4	ESI-MS & HPLC-FL	21	Neutral & Acidic	4	30	**4**
27	**Bao *et al.* (2013)**	N. Amer.	Term	1 (Se+/Le+)	LC-MS	11	Neutral	3	29	**4**
28	**Smilowitz *et al.* (2013)**	N. Amer.	Term	Mixed, SEC & NON-SEC	NMR	10	Neutral	90	90	**52**
29	**Galeotti *et al.* (2014)**	Europe	Term	1-4	CE-UV	16	Neutral & Acidic	4	30	**9**
30	**Hong *et al.* (2014)**	N. Amer.	Term	SEC & NON-SEC	LC-MS	24	Neutral & Acidic	35	35	**30**
31	**Marx *et al.* (2014)**	N. Amer.	term & preterm	Mixed (pooled samples)	HPLC-FL	7	Neutral & Acidic	n.a.	n.a.	**119**
32	**Sakaguchi *et al.* (2014)**	Asia	Term	n.a.	LC-MS/MS-FL	6	Acidic	10	90	**1**
33	**Van Niekerk *et al.* (2014)**	Africa	preterm	SEC & NON-SEC	HPLC-UV	15	Neutral & Acidic	4	28	**82**
34	**Alderete *et al.* (2015)**	N. Amer.	Term	Mixed (pooled samples)	HPLC-FL	16	Neutral & Acidic	30	150	**25**
35	**Monti *et al.* (2015)**	Europe	Term	n.a.	CE-UV	3	Acidic	n.a.	n.a.	**2**

Table 1.5.1-2 List of Scientific Articles Reporting Individual HMO Concentrations in Human Milk

#	Citation	Global Region	Gestation	Milk Phenotype Group	Analytical Method	Number HMOs	Neutral / Acidic HMOs	Earliest Sampling Day	Latest Sampling Day	Number Donors
36	**Olivares *et al.* (2015)**	Europe	Term	SEC & NON-SEC	CE-FL	8	Neutral	30	30	**24**
37	**Spevacek *et al.* (2015)**	N. Amer.	Term & Preterm	Mixed (pooled samples)	NMR	10	Neutral & Acidic	0	28	**25**
38	**Austin *et al.* (2016)**	Asia	Term	Mixed (pooled samples)	UHPLC-FL	10	Neutral & Acidic	5	240	**446**
39	**Aakko *et al.* (2017)**	Europe	Term	SEC	HPLC-FL	16	Neutral & Acidic	1	4	**11**
40	**Kunz *et al.* (2017)**	Europe	Term	SEC & NON-SEC	HPAEC-PAD	16	Neutral & Acidic	1	60	**32**
41	**McGuire *et al.* (2017)**	4 Continents	Term	SEC & NON-SEC	HPLC-FL	19	Neutral & Acidic	14	150	**410**
42	**Sprenger *et al.* (2017)**	Asia	Term	Mixed, SEC & NON-SEC	HPAEC-PAD	5	Neutral & Acidic	30	120	**50**
43	**Williams *et al.* (2017)**	N. Amer.	Term	Mixed (pooled samples)	HPLC-FL	7	Neutral & Acidic	120	180	**16**
44	**Azad *et al.* (2018)**	N. Amer.	Term	Mixed, SEC & NON-SEC	HPLC-FL	19	Neutral & Acidic	90	120	**427**
45	**Elwakiel *et al.* (2018)**	Asia	Term	1 (Se+/Le+)	CE-FL	8	Neutral & Acidic	7	140	**22**
46	**Ma *et al.* (2018)**	Asia	Term	Mixed (pooled samples)	HPLC–MRM–MS	10	Neutral & Acidic	2	365	**46**
47	**Nijman *et al.* (2018)**	N. Amer.	Term	Mixed (pooled samples)	HPAEC-PAD	9	Neutral & Acidic	3	42	**10**
48	**Austin *et al.* (2019)**	Europe	Term & Preterm	Mixed, SEC & NON-SEC	LC-FL	17	Neutral & Acidic	7	112	**500**
49	**Huang *et al.* (2019)**	Asia	Term	Mixed (pooled samples)	UHPLC-FL	12	Neutral & Acidic	1	34	**33**
50	**Larsson *et al.* (2019)**	Europe	Term	SEC	HPLC-FL	16	Neutral & Acidic	150	270	**22**
51	**McJarrow *et al.* (2019)**	Asia	Term	Mixed, SEC & NON-SEC	HPLC-MS	11	Neutral & Acidic	5	180	**41**
52	**Paganini *et al.* (2019)**	Africa	Term	Mixed, SEC & NON-SEC	HPAEC-PAD	13	Neutral & Acidic	n.a.	n.a.	**80**
53	**Samuel *et al.* (2019)**	Europe	Term	Mixed (pooled samples)	LC-FL	18	Neutral & Acidic	2	120	**290**
54	**Tonon *et al.* (2019a)**	LATAM	Term	Mixed (pooled samples)	LC-ESI-MS	14	Neutral & Acidic	n.a.	n.a.	**10**
55	**Tonon *et al.* (2019b)**	LATAM	Term	1-3	LC-MS	16	Neutral & Acidic	25	46	**77**
56	**Ayoub Moubareck *et al.* (2020)**	other	Term	Mixed (pooled samples)	HPAEC-PAD	6	Neutral & Acidic	0	90	**30**
57	**Borewicz *et al.* (2020)**	Europe	Term	Mixed (pooled samples)	HPAEC-PAD	17	Neutral & Acidic	14	84	**24**
58	**Ferreira *et al.* (2020)**	LATAM	Term	Mixed (pooled samples)	HPLC-FL	17	Neutral & Acidic	2	119	**147**
59	**Hassinger *et al.* (2020)**	N. Amer.	Preterm	Mixed (pooled samples)	LC-MS	1	Acidic	5	5	**48**
60	**Lefebvre *et al.* (2020)**	Europe	Term	SEC & NON-SEC	LC-FL	24	Neutral & Acidic	90	360	**156**
61	**Lagström *et al.* (2020)**	Europe	Term	Mixed, SEC & NON-SEC	HPLC-FL	17	Neutral & Acidic	90	90	**802**
62	**Saben *et al.* (2020)**	N. Amer.	Term	Mixed, SEC & NON-SEC	HPLC-FL	19	Neutral & Acidic	60	60	**136**
63	**Torres Roldan *et al.* (2020)**	LATAM	Preterm	Mixed (pooled samples)	HPLC-FL	19	Neutral & Acidic	1	30	**55**
64	**Wang *et al.* (2020)**	Asia	Term	Mixed (pooled samples)	HPAEC-PAD	18	Neutral & Acidic	n.a.	n.a.	**30**
65	**Wu *et al.* (2020)**	Asia	Term	SEC & NON-SEC	HPAEC-PAD	18	Neutral & Acidic	3	168	**59**

For abbreviations, please see the **List of Abbreviations.**

1.5.2 Data Extraction

From the original papers, the study characteristics and quantitative data (as per Table 1.5.2-1) were extracted for further evaluation as described in the sections below.

Table 1.5.2-1 Data Extracted from the Literature Base

Study Characteristics	HMO Quantitative Data
• Authors and year of publication • Global Region • Sample size • Maternal Characteristics (Secretor Status) • Gestational Characteristics (term or pre-term)	• Analytical Method of Quantitation • Lactational period of sampling • Mean or median HMO concentrations, what available • Standard deviation or standard error, if available • 95% confidence levels or interquartile range, if available • Upper range of observed concentrations, if available

For abbreviations, please see the **List of Abbreviations.**

All HMO concentration data were entered into the database in grams per liter. Concentrations expressed in millimoles per liter were converted by multiplying the molecular mass of the HMO into g/L. Data presented in non-numerical form (in graphical format) were estimated manually to the closest 0.1 g/L increment.

A simple proxy for the 95% confidence level (CL) was calculated by adding twice the standard deviation to the reported mean.

1.6 Quantitative Data Pertaining to LNFP-I Concentration in Secretor Milk

1.6.1 LNFP-I: Studies Included and Excluded

The selection criteria focused on the assessment of safety/tolerability from an evaluation of naturally occurring *exposure ranges* in the population (*i.e.*, representative sections of mother/child dyads). In case of the Secretor phenotype HMOs (*e.g.*, 2'-FL, LNFP-I, LNDFH-I) approximately 20% of the female world population do not express Secretor phenotype HMOs into their breast milk at any significant level (so-called "non-Secretor mothers") − due to the genetic polymorphism of the *fut2* alleles. This skews the measured average levels of Secretor phenotype HMOs in mixed (pooled) milk samples. However, the remaining 80% of the female world population (referred to as "Secretor mothers") do excrete Secretor phenotype HMOs into their breast milk, and it was considered that 80% of the population are representative of the entire population regarding assessment of the **safety/tolerance** of Secretor phenotype HMOs for two reasons:

1) infants from "Secretor mothers" can themselves be either Secretor or non-Secretors (through the *fut2* allele of the father) and are therefore neither genetically restricted nor adapted to prefer milk from non-Secretor mothers (genetic laws of inheritance − allele shuffling from both parents − do not establish a consistent link from mother to infant genotype)

2) consistent with 1) there is no data in the literature to suggest that infants from non-Secretor mothers would be at any disadvantage (tolerability or other) to receive LNFP-I or 2'-FL in their diet. The evolutionary history of safe practice of wet-nursing suggests against it. Possibly to the contrary, recent emerging clinical data may suggest benefits for infants from non-Secretor mothers from receiving LNFP-I and/or 2'-FL in their diet (not to be discussed here)

Therefore, and in alignment with Thurl *et al*., 2017, in case of Secretor phenotype HMOs only milk compositional data from "Secretor mothers" was considered. An advantage of this approach is that the observed data in Secretor samples follows principally a typical Gauss distribution (since the non-linear effect of non-Secretor samples that do not contain any Secretor phenotype HMO will not skew the Gauss distribution) and in consequence it is possible to calculate an approximation of the 95% CL from the mean and the standard deviation.

Table 1.6.1-1 provides the overview on included studies and justification for study exclusion for the data analysis of LNFP-I concentrations in breast milk.

Table 1.6.1-1 Included and excluded studies for LNFP-I concentration in Breast Milk

#	Citation	Milk Group	Number Sampled Donors	Included/Excluded LNFP-I
1	**Kunz and Rudloff (1993)**	Mixed (pooled samples)	**n.a.**	Number of samples
2	**Thurl *et al*. (1996)**	1 (Se+/Le+)	**1**	Number of samples
3	**Chaturvedi *et al*. (1997)**	Mixed (pooled samples)	**50**	Pooled samples
4	**Coppa *et al*. (1999)**	1 (Se+/Le+)	**18**	**Included**
5	**Kunz *et al*. (1999)**	Mixed (pooled samples)	**10**	Pooled samples
6	**Nakhla *et al*. (1999)**	Mixed & Lewis+	**13**	Pooled samples
7	**Erney *et al*. (2000)**	Mixed & SEC	**381**	**Included**
8	**Kunz *et al*. (2000)**	Mixed (pooled samples)	**4**	Number of samples
9	**Shen *et al*. (2000)**	Mixed (pooled samples)	**n.a.**	No data LNFP-I, 2'-FL
10	**Chaturvedi *et al*. (2001)**	SEC	**84**	**Included**
11	**Erney *et al*. (2001)**	Mixed (pooled samples)	**368**	Pooled samples
12	**Martín-Sosa *et al*. (2003)**	Mixed (pooled samples)	**12**	No data LNFP-I, 2'-FL
13	**Sumiyoshi *et al*. (2003)**	Mixed (pooled samples)	**16**	Pooled samples
14	**Morrow *et al*. (2004)**	Mixed (pooled samples)	**93**	Pooled samples
15	**Musumeci *et al*. (2006)**	Mixed (pooled samples)	**103**	Pooled samples
16	**Asakuma *et al*. (2007)**	Mixed (pooled samples)	**20**	No data LNFP-I, 2'-FL
17	**Bao *et al*. (2007)**	Mixed (pooled samples)	**14**	Pooled samples
18	**Sjögren *et al*. (2007)**	Mixed (pooled samples)	**20**	Pooled samples
19	**Asakuma *et al*. (2008)**	SEC	**12**	**Included**
20	**Leo *et al*. (2009)**	Mixed (pooled samples)	**8**	Number of samples
21	**Leo *et al*. (2010)**	Mixed (pooled samples)	**16**	Pooled samples
22	**Thurl *et al*. (2010)**	1-3	**30**	**Included**
23	**Asakuma *et al*. (2011)**	Mixed (pooled samples)	**57**	Pooled samples
24	**Coppa *et al*. (2011)**	1-4	**39**	**Included**
25	**Gabrielli *et al*. (2011)**	1-4	**63**	**Included**
26	**Galeotti *et al*. (2012)**	1-4	**4**	Number of samples
27	**Bao *et al*. (2013)**	1 (Se+/Le+)	**4**	Number of samples
28	**Smilowitz *et al*. (2013)**	Mixed, SEC & NON-SEC	**52**	**Included**
29	**Galeotti *et al*. (2014)**	1-4	**9**	Number of samples
30	**Hong *et al*. (2014)**	SEC & NON-SEC	**30**	**Included**
31	**Marx *et al*. (2014)**	Mixed (pooled samples)	**119**	Pooled samples
32	**Sakaguchi *et al*. (2014)**	n.a.	**1**	No data LNFP-I, 2'-FL
33	**Van Niekerk *et al*. (2014)**	SEC & NON-SEC	**82**	**Included**
34	**Alderete *et al*. (2015)**	Mixed (pooled samples)	**25**	Pooled samples
35	**Monti *et al*. (2015)**	n.a.	**2**	Number of samples
36	**Olivares *et al*. (2015)**	SEC & NON-SEC	**24**	Number of samples per data point
37	**Spevacek *et al*. (2015)**	Mixed (pooled samples)	**25**	Pooled samples
38	**Austin *et al*. (2016)**	Mixed (pooled samples)	**446**	Pooled samples
39	**Aakko *et al*. (2017)**	SEC	**11**	**Included**
40	**Kunz *et al*. (2017)**	SEC & NON-SEC	**32**	**Included**

Table 1.6.1-1 Included and excluded studies for LNFP-I concentration in Breast Milk

#	Citation	Milk Group	Number Sampled Donors	Included/Excluded LNFP-I
41	**McGuire *et al*. (2017)**	SEC & NON-SEC	**410**	**Included**
42	**Sprenger *et al*. (2017)**	Mixed, SEC & NON-SEC	**50**	**Included**
43	**Williams *et al*. (2017)**	Mixed (pooled samples)	**16**	Pooled samples
44	**Azad *et al*. (2018)**	Mixed, SEC & NON-SEC	**427**	**Included**
45	**Elwakiel *et al*. (2018)**	1 (Se+/Le+)	**22**	**Included**
46	**Ma *et al*. (2018)**	Mixed (pooled samples)	**46**	Pooled samples
47	**Nijman *et al*. (2018)**	Mixed (pooled samples)	**10**	Pooled samples
48	**Austin *et al*. (2019)**	Mixed, SEC & NON-SEC	**500**	**Included**
49	**Huang *et al*. (2019)**	Mixed (pooled samples)	**33**	Pooled samples
50	**Larsson *et al*. (2019)**	SEC	**22**	**Included**
51	**McJarrow *et al*. (2019)**	Mixed, SEC & NON-SEC	**41**	**Included**
52	**Paganini *et al*. (2019)**	Mixed, SEC & NON-SEC	**80**	Non-sensical data
53	**Samuel *et al*. (2019)**	Mixed (pooled samples)	**290**	Pooled samples
54	**Tonon *et al*. (2019a)**	Mixed (pooled samples)	**10**	Pooled samples
55	**Tonon *et al*. (2019b)**	1-3	**77**	**Included**
56	**Ayoub Moubareck *et al*. (2020)**	Mixed (pooled samples)	**30**	Pooled samples
57	**Borewicz *et al*. (2020)**	Mixed (pooled samples)	**24**	Pooled samples
58	**Ferreira *et al*. (2020)**	Mixed (pooled samples)	**147**	Pooled samples
59	**Hassinger *et al*. (2020)**	Mixed (pooled samples)	**48**	No data LNFP-I, 2'-FL
60	**Lefebvre *et al*. (2020)**	SEC & NON-SEC	**156**	**Included**
61	**Lagström *et al*. (2020)**	Mixed, SEC & NON-SEC	**802**	**Included**
62	**Saben *et al*. (2020)**	Mixed, SEC & NON-SEC	**136**	**Included**
63	**Torres Roldan *et al*. (2020)**	Mixed (pooled samples)	**55**	Pooled samples
64	**Wang *et al*. (2020)**	Mixed (pooled samples)	**30**	Pooled samples
65	**Wu *et al*. (2020)**	SEC & NON-SEC	**59**	**Included**

For abbreviations see **List of Abbreviations**.

1.6.2 LNFP-I: Full Data Overview

Table 1.6.2-1 below shows the extracted quantitative data relating to LNFP-I concentrations in breast milk of Secretor mothers, presented chronologically from year of publication (oldest to newest) and allowing insights into implications of geographical location, gestation type, lactational period and sample size.

Please note: in cases where mean and median data was both provided only the mean is reported in the table. In cases when the data was provided in molar concentration it has been converted to g/L.

Table 1.6.2-1 Concentrations of LNFP-I (g/L) in Breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE/SEM)	95% CL (or IQR)	Upper Range
4	**Coppa *et al.* (1999)**	**Europe**	**Term**	**4**	**90**	**18**	**Mean (g/L) with standard deviation**				
4				4	4			**1.36**	0.18	1.72	n.a.
4				10	10			**1.36**	0.22	1.80	n.a.
4				30	30			**0.99**	0.25	1.49	n.a.
4				60	60			**0.97**	0.61	2.19	n.a.
4				90	90			**1.35**	0.69	2.73	n.a.
6	**Erney *et al.* (2000)**	**4 Continents & WORLD**	**Term**	**0**	**452**	**381**	**Mean (g/L)**				
6		Asia		0	452	**80**		**1.20**	n.a.	n.a.	n.a.
6		Europe		0	452	**68**		**1.05**	n.a.	n.a.	n.a.
6		LATAM		0	452	**197**		**1.25**	n.a.	n.a.	n.a.
6		N. Amer.		0	452	**36**		**0.74**	n.a.	n.a.	n.a.
6		WORLD		0	452	**381**		**1.17**	n.a.	n.a.	n.a.
6		WORLD		0	2	**19**		**1.35**	n.a.	n.a.	n.a.
6		WORLD		3	10	**62**		**1.70**	n.a.	n.a.	n.a.
6		WORLD		11	30	**178**		**1.33**	n.a.	n.a.	n.a.
6		WORLD		31	452	**122**		**0.58**	n.a.	n.a.	n.a.
6		Asia		3	10	**25**		**1.81**	n.a.	n.a.	n.a.
6		Europe		3	10	**14**		**1.56**	n.a.	n.a.	n.a.
6		LATAM		3	10	**19**		**1.73**	n.a.	n.a.	n.a.
6		Asia		11	30	**20**		**1.20**	n.a.	n.a.	n.a.
6		Europe		11	30	**21**		**1.06**	n.a.	n.a.	n.a.
6		LATAM		11	30	**129**		**1.39**	n.a.	n.a.	n.a.
6		Asia		31	452	**24**		**0.49**	n.a.	n.a.	n.a.
6		Europe		31	452	**25**		**0.61**	n.a.	n.a.	n.a.
6		LATAM		31	452	**49**		**0.62**	n.a.	n.a.	n.a.
6		N. Amer.		31	452	**24**		**0.51**	n.a.	n.a.	n.a.
6		Asia		0	2	**11**		**1.42**	n.a.	n.a.	n.a.
6		Asia		3	10	**25**		**1.81**	n.a.	n.a.	n.a.
6		Asia		11	30	**20**		**1.20**	n.a.	n.a.	n.a.
6		Asia		31	452	**24**		**0.49**	n.a.	n.a.	n.a.
6		Europe		3	10	**14**		**1.56**	n.a.	n.a.	n.a.
6		Europe		11	30	**21**		**1.06**	n.a.	n.a.	n.a.
6		Europe		31	452	**25**		**0.61**	n.a.	n.a.	n.a.
6		LATAM		3	10	**19**		**1.73**	n.a.	n.a.	n.a.
6		LATAM		11	30	**129**		**1.39**	n.a.	n.a.	n.a.
6		LATAM		31	452	**49**		**0.62**	n.a.	n.a.	n.a.
6		N. Amer.		31	217	**24**		**0.51**	n.a.	n.a.	n.a.

Table 1.6.2-1 Concentrations of LNFP-I (g/L) in Breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE/SEM)	95% CL (or IQR)	Upper Range
9	**Chaturvedi *et al.* (2001)**	**N. Amer.**	**Term**	**1**	**7**	**84**	**Mean (g/L)**				
9				1	7			**2.10**	n.a.	n.a.	n.a.
9				8	14			**2.20**	n.a.	n.a.	n.a.
9				22	28			**1.30**	n.a.	n.a.	n.a.
9				91	98			**1.60**	n.a.	n.a.	n.a.
9				175	182			**0.70**	n.a.	n.a.	n.a.
9				259	266			**0.40**	n.a.	n.a.	n.a.
9				336	343			**0.30**	n.a.	n.a.	n.a.
17	**Asakuma *et al.* (2008)**	**Asia**	**Term**	**1**	**3**	**12**	**Mean (g/L) with standard deviation**				
17				1	1			**1.47**	1.01	3.49	3.16
17				2	2			**2.08**	1.67	5.42	6.42
17				3	3			**1.67**	1.03	3.73	3.94
17				1	3			**1.74**	1.24	4.21	6.42
20	**Thurl *et al.* (2010)**	**Europe**	**Term**	**3**	**90**	**30**	**Mean (mol/L)**				
20				3	3	**21**		**2.00**	n.a.	n.a.	n.a.
20				8	8	**19**		**2.25**	n.a.	n.a.	n.a.
20				15	15	**17**		**1.64**	n.a.	n.a.	n.a.
20				22	22	**16**		**1.72**	n.a.	n.a.	n.a.
20				30	30	**14**		**1.48**	n.a.	n.a.	n.a.
20				60	60	**12**		**1.06**	n.a.	n.a.	n.a.
20				90	90	**10**		**0.94**	n.a.	n.a.	n.a.
20				3	90	**109**		**1.58**	n.a.	n.a.	n.a.
20				3	90	**17**		**3.18**	n.a.	n.a.	n.a.
22	**Coppa *et al.* (2011)**	**Europe**	**Term**	**25**	**35**	**39**	**Mean (g/L) with standard deviation**				
22						**10**		**1.18**	0.30	1.78	n.a.
22						**6**		**1.25**	0.32	1.89	n.a.
23	**Gabrielli *et al.* (2011)**	**Europe**	**Preterm**	**4**	**30**	**63**	**Mean (g/L) with standard deviation**				
23				4	4	**35**		**1.99**	0.92	3.83	n.a.
23				10	10	**35**		**1.89**	1.30	4.49	n.a.
23				20	20	**35**		**1.68**	1.19	4.06	n.a.
23				30	30	**35**		**1.40**	1.01	3.42	n.a.
26	**Smilowitz *et al.* (2013)**	**N. Amer.**	**Term**	**90**	**90**	**52**	**Mean (mol/L) with standard deviation and range**				
26								**0.18**	0.11	0.40	n.a.
28	**Hong *et al.* (2014)**	**N. Amer.**	**Term**	**35**	**35**	**30**	**Mean (g/L) with standard deviation**				
28						**10**		**0.48**	0.24	0.96	n.a.

Table 1.6.2-1 Concentrations of LNFP-I (g/L) in Breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE/SEM)	95% CL (or IQR)	Upper Range
30	**Van Niekerk *et al.* (2014)**	**Africa**	**Preterm**	**4**	**28**	**82**	**Mean (g/L) with standard deviation**				
30				4	4	**20**		**0.79**	0.31	1.41	n.a.
30				28	28	**20**		**0.63**	0.34	1.31	n.a.
36	**Aakko *et al.* (2017)**	**Europe**	**Term**	**1**	**4**	**11**	**Mean (g/L) with standard deviation**				
36								**1.83**	0.64	3.11	n.a.
37	**Kunz *et al.* (2017)**	**Europe**	**Term**	**1**	**60**	**32**	**Median (g/L) with IQR**				
37				1	7	**21**		**0.98**	n.a.	n.a.	1.57
37				8	15	**21**		**1.30**	n.a.	n.a.	1.64
37				16	60	**21**		**1.09**	n.a.	n.a.	1.73
38	**McGuire *et al.* (2017)**	**4 Continents**	**Term**	**14**	**150**	**410**	**Mean (mol/l or g/L) with standard error**				
38		Africa				**26**		**1.15**	0.18	1.51	n.a.
38		Africa				**31**		**1.39**	0.20	1.78	n.a.
38		Africa				**26**		**1.15**	0.23	1.60	n.a.
38		Africa				**34**		**1.32**	0.18	1.68	n.a.
38		Africa				**27**		**1.52**	0.24	2.00	n.a.
38		Africa				**34**		**0.96**	0.15	1.26	n.a.
38		LATAM				**42**		**0.97**	0.10	1.16	n.a.
38		Europe				**31**		**1.15**	0.17	1.48	n.a.
38		Europe				**19**		**1.46**	0.19	1.85	n.a.
38		N. Amer.				**28**		**1.01**	0.15	1.32	n.a.
38		N. Amer.				**18**		**1.22**	0.12	1.45	n.a.
39	**Sprenger *et al.* (2017)**	**Asia**	**Term**	**30**	**120**	**50**	**Mean & Median (g/L) with standard deviation and range**				
39				30	30	**34**		**n.a.**	n.a.	n.a.	n.a.
39				60	60	**34**		**n.a.**	n.a.	n.a.	n.a.
39				120	120	**33**		**n.a.**	n.a.	n.a.	n.a.
40	**Azad *et al.* (2018)**	**N. Amer.**	**Term**	**90**	**120**	**427**	**Mean & median (mol/L) with SD & IQR and range**				
40						**307**		**1.04**	0.76	2.56	1.37
41	**Elwakiel *et al.* (2018)**	**Asia**	**Term**	**7**	**140**	**22**	**Mean (g/L) with standard deviation**				
41						**12**		**4.47**	0.64	5.75	n.a.
41						**10**		**3.83**	0.64	5.11	n.a.
41						**22**		**4.15**	**0.64**	**5.43**	n.a.

Table 1.6.2-1 Concentrations of LNFP-I (g/L) in Breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE/SEM)	95% CL (or IQR)	Upper Range
44	**Austin *et al.* (2019)**	**Europe**	**Term & preterm**	**7**	**112**	**500**	**Mean & median (g/L) with SD & IQR and range**				
44			preterm	7	7	**19**		**1.62**	0.67	2.96	2.81
44			preterm	14	14	**19**		**1.17**	0.45	2.07	1.95
44			preterm	21	21	**19**		**1.03**	0.41	1.84	1.63
44			preterm	28	28	**18**		**0.90**	0.35	1.59	1.55
44			preterm	35	35	**19**		**0.84**	0.37	1.59	1.44
44			preterm	42	42	**18**		**0.76**	0.40	1.56	1.84
44			preterm	49	49	**18**		**0.70**	0.38	1.47	1.77
44			preterm	56	56	**18**		**0.65**	0.28	1.21	1.12
44			preterm	70	70	**18**		**0.55**	0.30	1.14	1.44
44			preterm	84	84	**15**		**0.44**	0.26	0.97	0.97
44			preterm	98	98	**15**		**0.45**	0.27	0.99	1.01
44			preterm	112	112	**15**		**0.37**	0.26	0.88	0.93
44			term	7	7	**21**		**2.21**	0.75	3.71	3.60
44			term	14	14	**19**		**1.70**	0.67	3.04	3.07
44			term	21	21	**21**		**1.52**	0.65	2.82	3.02
44			term	28	28	**21**		**1.33**	0.69	2.70	3.16
44			term	35	35	**21**		**1.00**	0.53	2.07	2.66
44			term	42	42	**20**		**0.84**	0.40	1.64	2.05
44			term	49	49	**20**		**0.80**	0.54	1.88	2.62
44			term	56	56	**21**		**0.69**	0.46	1.60	1.96
46	**Larsson *et al.* (2019)**	**Europe**	**Term**	**150**	**270**	**22**	**Median (mol/L) with IQR**				
46				150	150	**15**		**0.80**	n.a.	n.a.	1.52
46				270	270	**12**		**0.58**	n.a.	n.a.	1.07
47	**McJarrow *et al.* (2019)**	**Middle East**	**Term**	**5**	**180**	**41**	**Mean (g/L) with standard deviation**				
47				5	15	**41**		**2.09**	0.78	3.65	n.a.
47				180	180	**40**		**0.69**	0.45	1.59	n.a.
50	**Tonon *et al.* (2019b)**	**LATAM**	**Term**	**25**	**46**	**77**	**Mean (g/L) with standard deviation**				
50				25	46			**0.73**	0.52	1.77	n.a.
50				25	46			**2.03**	1.51	5.05	n.a.
55	**Lefebvre *et al.* (2020)**	**Europe**	**Term**	**90**	**360**	**156**	**Mean & median (g/L) with range**				
55				90	90	**125**		**0.53**	n.a.	n.a.	2.03
55				180	180	**125**		**0.34**	n.a.	n.a.	1.52
55				360	360	**125**		**0.25**	n.a.	n.a.	0.67
56	**Lagström *et al.* (2020)**	**Europe**	**Term**	**90**	**90**	**802**	**Median (mol/L) with IQR**				
56				90	90	**699**		**1.06**	n.a.	n.a.	1.56
57	**Saben *et al.* (2020)**	**N. Amer.**	**Term**	**60**	**60**	**136**	**Median (mol/L) with IQR**				
57						**101**		**1.02**	n.a.	n.a.	1.58

Table 1.6.2-1 Concentrations of LNFP-I (g/L) in Breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE/SEM)	95% CL (or IQR)	Upper Range
59	**Wu *et al.* (2020)**	**Asia**	**Term**	**3**	**168**	**59**	**Mean (g/L) with standard error of measurement**				
59				3	3	**25**		**1.44**	0.08	1.60	n.a.
59				7	7	**27**		**1.49**	0.12	1.73	n.a.
59				21	21	**25**		**1.11**	0.09	1.29	n.a.
59				42	42	**15**		**0.77**	0.07	0.91	n.a.
59				77	77	**10**		**0.44**	0.08	0.60	n.a.

For abbreviations see **List of Abbreviations**.

1.6.3 LNFP-I: General Data Analysis

Of the 24 publications reporting the concentration of LNFP-I in Secretor milk, the **lowest reported mean** was 0.18 g LNFP-I/L (Smilowitz *et al*., 2013) [observed in 52 healthy women enrolled in the Foods for Health Institute Lactation Study at the University of California, Davis (UC-Davis) sampled on Day 90 of lactation] and the **highest reported mean** was 4.47 g LNFP-I/L reported by Elwakiel *et al*. (2018) (observed in Chinese women from the Hohhot region sampled between Day 7 and 140 of lactation).

Equivalent ranges of LNFP-I consumption were calculated using values reported in the U.S. Environmental Protection Agency (EPA) *Exposure Factors Handbook* (U.S. EPA, 2011). Briefly, breast milk consumption data from 7 studies were pooled and indexed to body weight and recommended values were derived assuming exclusively breastfed infants. The highest mean and upper percentile of consumption of breast milk on a body weight basis were observed in infants from birth to < 1 month of 150 and 220 mL/kg body weight/day, respectively. Assuming LNFP-I was present at the highest reported **mean concentration** of 4.47 g/L, this would be equivalent to the mean and upper percentile intake of 671[1] and 983[2] mg/kg body weight/day of LNFP-I, respectively.

1.7 Quantitative Data Pertaining to 2'-FL Concentration in Secretor Milk

1.7.1 2'-FL: Studies Included and Excluded

The selection criteria focused on the assessment of safety/tolerability from an evaluation of naturally occurring *exposure ranges* in the population (*i.e*., representative sections of mother/child dyads). In case of the Secretor phenotype HMOs (*e.g*., 2'-FL, LNFP-I, LNDFH-I) approximately 20% of the female world population do not express Secretor phenotype HMOs into their breast milk at any significant level (so-called "non-Secretor mothers") − due to the genetic polymorphism of the *fut2* alleles. This skews the measured average levels of Secretor phenotype HMOs in mixed (pooled) milk samples. However, the remaining 80% of the female world population (referred to as "Secretor mothers") do excrete Secretor phenotype HMOs into their breast milk, and it was considered that 80% of the population are representative of the entire population regarding assessment of the **safety/tolerance** of Secretor phenotype HMOs for two reasons:

1) infants from "Secretor mothers" can themselves be either Secretor or non-Secretors (through the *fut2* allele of the father) and are therefore neither genetically restricted nor adapted to prefer milk from non-Secretor mothers (genetic laws of inheritance − allele shuffling from both parents − do not establish a consistent link from mother to infant genotype)

2) consistent with 1) there is no data in the literature to suggest that infants from non-Secretor mothers would be at any disadvantage (tolerability or other) to receive LNFP-I or 2'-FL in their diet. The evolutionary history of safe practice of wet-nursing suggests against it. Possibly to the contrary, recent emerging clinical data may suggest benefits for infants from non-Secretor mothers from receiving LNFP-I and/or 2'-FL in their diet (not to be discussed here)

[1] Calculated as 4.47 g LNFP-I/L x 0.150 L breast milk/kg body weight/day = 0.6705 g/kg body weight/day = 671 mg/kg body weight/day.
[2] Calculated as 4.47 g LNFP-I/L x 0.220 L breast milk/kg body weight/day = 0.9834 g/kg body weight/day = 983 mg/kg body weight/day.

Therefore, and in alignment with Thurl *et al*. (2017), in case of Secretor phenotype HMOs only milk compositional data from "Secretor mothers" was considered. An advantage of this approach is that the observed data in Secretor samples follows principally a typical Gauss distribution (since the non-linear effect of non-Secretor samples that do not contain any Secretor phenotype HMO will not skew the Gauss distribution) and in consequence it is possible to calculate an approximation of the 95% CL from the mean and the standard deviation.

Table 1.7.1-1 provides the overview on included studies and justification for study exclusion for the data analysis of 2'-FL concentrations in breast milk.

Table 1.7.1-1 Included and Excluded Studies for 2'-FL Concentration in Breast Milk

#	Citation	Milk Group	Number Sampled Donors	Included/Excluded 2'-FL
1	**Kunz and Rudloff (1993)**	Mixed (pooled samples)	**n.a.**	Number of samples
2	**Thurl *et al*. (1996)**	1 (Se+/Le+)	**1**	Number of samples
3	**Chaturvedi *et al*. (1997)**	Mixed (pooled samples)	**50**	Pooled samples
4	**Coppa *et al*. (1999)**	1 (Se+/Le+)	**18**	**Included**
5	**Kunz *et al*. (1999)**	Mixed (pooled samples)	**10**	Pooled samples
6	**Nakhla *et al*. (1999)**	Mixed & Lewis+	**13**	Pooled samples
7	**Erney *et al*. (2000)**	Mixed & SEC	**381**	**Included**
8	**Kunz *et al*. (2000)**	Mixed (pooled samples)	**4**	Number of samples
9	**Shen *et al*. (2000)**	Mixed (pooled samples)	**n.a.**	No data LNFP-I, 2'-FL
10	**Chaturvedi *et al*. (2001)**	SEC	**84**	**Included**
11	**Erney *et al*. (2001)**	Mixed (pooled samples)	**368**	Pooled samples
12	**Martín-Sosa *et al*. (2003)**	Mixed (pooled samples)	**12**	No data LNFP-I, 2'-FL
13	**Sumiyoshi *et al*. (2003)**	Mixed (pooled samples)	**16**	Pooled samples
14	**Morrow *et al*. (2004)**	Mixed (pooled samples)	**93**	Pooled samples
15	**Musumeci *et al*. (2006)**	Mixed (pooled samples)	**103**	Pooled samples
16	**Asakuma *et al*. (2007)**	Mixed (pooled samples)	**20**	No data LNFP-I, 2'-FL
17	**Bao *et al*. (2007)**	Mixed (pooled samples)	**14**	Pooled samples
18	**Sjögren *et al*. (2007)**	Mixed (pooled samples)	**20**	Pooled samples
19	**Asakuma *et al*. (2008)**	SEC	**12**	**Included**
20	**Leo *et al*. (2009)**	Mixed (pooled samples)	**8**	Number of samples
21	**Leo *et al*. (2010)**	Mixed (pooled samples)	**16**	Pooled samples
22	**Thurl *et al*. (2010)**	1-3	**30**	**Included**
23	**Asakuma *et al*. (2011)**	Mixed (pooled samples)	**57**	Pooled samples
24	**Coppa *et al*. (2011)**	1-4	**39**	**Included**
25	**Gabrielli *et al*. (2011)**	1-4	**63**	**Included**
26	**Galeotti *et al*. (2012)**	1-4	**4**	Number of samples
27	**Bao *et al*. (2013)**	1 (Se+/Le+)	**4**	Number of samples
28	**Smilowitz *et al*. (2013)**	Mixed, SEC & NON-SEC	**52**	**Included**
29	**Galeotti *et al*. (2014)**	1-4	**9**	Number of samples
30	**Hong *et al*. (2014)**	SEC & NON-SEC	**30**	**Included**
31	**Marx *et al*. (2014)**	Mixed (pooled samples)	**119**	Pooled samples
32	**Sakaguchi *et al*. (2014)**	n.a.	**1**	No data LNFP-I, 2'-FL
33	**Van Niekerk *et al*. (2014)**	SEC & NON-SEC	**82**	**Included**
34	**Alderete *et al*. (2015)**	Mixed (pooled samples)	**25**	Pooled samples
35	**Monti *et al*. (2015)**	n.a.	**2**	Number of samples
36	**Olivares *et al*. (2015)**	SEC & NON-SEC	**24**	Number of samples per data point
37	**Spevacek *et al*. (2015)**	Mixed (pooled samples)	**25**	Pooled samples
38	**Austin *et al*. (2016)**	Mixed (pooled samples)	**446**	Pooled samples
39	**Aakko *et al*. (2017)**	SEC	**11**	**Included**
40	**Kunz *et al*. (2017)**	SEC & NON-SEC	**32**	**Included**

Table 1.7.1-1 Included and Excluded Studies for 2'-FL Concentration in Breast Milk

#	Citation	Milk Group	Number Sampled Donors	Included/Excluded 2'-FL
41	**McGuire *et al*. (2017)**	SEC & NON-SEC	**410**	**Included**
42	**Sprenger *et al*. (2017)**	Mixed, SEC & NON-SEC	**50**	**Included**
43	**Williams *et al*. (2017)**	Mixed (pooled samples)	**16**	Pooled samples
44	**Azad *et al*. (2018)**	Mixed, SEC & NON-SEC	**427**	**Included**
45	**Elwakiel *et al*. (2018)**	1 (Se+/Le+)	**22**	**Included**
46	**Ma *et al*. (2018)**	Mixed (pooled samples)	**46**	Pooled samples
47	**Nijman *et al*. (2018)**	Mixed (pooled samples)	**10**	Pooled samples
48	**Austin *et al*. (2019)**	Mixed, SEC & NON-SEC	**500**	**Included**
49	**Huang *et al*. (2019)**	Mixed (pooled samples)	**33**	Pooled samples
50	**Larsson *et al*. (2019)**	SEC	**22**	**Included**
51	**McJarrow *et al*. (2019)**	Mixed, SEC & NON-SEC	**41**	**Included**
52	**Paganini *et al*. (2019)**	Mixed, SEC & NON-SEC	**80**	Non-sensical data
53	**Samuel *et al*. (2019)**	Mixed (pooled samples)	**290**	Pooled samples
54	**Tonon *et al*. (2019a)**	Mixed (pooled samples)	**10**	Pooled samples
55	**Tonon *et al*. (2019b)**	1-3	**77**	**Included**
56	**Ayoub Moubareck *et al*. (2020)**	Mixed (pooled samples)	**30**	Pooled samples
57	**Borewicz *et al*. (2020)**	Mixed (pooled samples)	**24**	Pooled samples
58	**Ferreira *et al*. (2020)**	Mixed (pooled samples)	**147**	Pooled samples
59	**Hassinger *et al*. (2020)**	Mixed (pooled samples)	**48**	No data LNFP-I, 2'-FL
60	**Lefebvre *et al*. (2020)**	SEC & NON-SEC	**156**	**Included**
61	**Lagström *et al*. (2020)**	Mixed, SEC & NON-SEC	**802**	**Included**
62	**Saben *et al*. (2020)**	Mixed, SEC & NON-SEC	**136**	**Included**
63	**Torres Roldan *et al*. (2020)**	Mixed (pooled samples)	**55**	Pooled samples
64	**Wang *et al*. (2020)**	Mixed (pooled samples)	**30**	Pooled samples
65	**Wu *et al*. (2020)**	SEC & NON-SEC	**59**	**Included**

For abbreviations see **List of Abbreviations**.

1.7.2 2'-FL: Full Data Overview

Table 1.7.2-1 below shows the extracted quantitative data relating to 2'-FL concentrations in breast milk of Secretor mothers.

Please note: In cases where mean and median data was both provided only the mean is reported in the table. In cases when the data was provided in molar concentration it has been converted to g/L.

Table 1.7.2-1 Concentrations of 2'-FL (g/L) in breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE)	95% CL (or IQR)	Upper Range
4	**Coppa *et al.* (1999)**	**Europe**	**Term**	**4**	**90**	**18**	**Mean (g/L) with standard deviation**				
4				4	4			**3.93**	1.11	6.15	n.a.
4				10	10			**3.02**	0.88	4.78	n.a.
4				30	30			**2.78**	0.94	4.66	n.a.
4				60	60			**1.84**	0.39	2.62	n.a.
4				90	90			**2.46**	0.44	3.34	n.a.
6	**Erney *et al.* (2000)**	**4 Continents & World**	**Term**	**0**	**452**	**381**	**Mean (g/L)**				
6		Asia		0	452	**80**		**2.07**	n.a.	n.a.	n.a.
6		Europe		0	452	**68**		**2.58**	n.a.	n.a.	n.a.
6		LATAM		0	452	**197**		**2.47**	n.a.	n.a.	n.a.
6		N. Amer.		0	452	**36**		**1.99**	n.a.	n.a.	n.a.
6		WORLD		0	452	**381**		**2.38**	n.a.	n.a.	n.a.
6		WORLD		0	2	**19**		**2.78**	n.a.	n.a.	n.a.
6		WORLD		3	10	**62**		**2.60**	n.a.	n.a.	n.a.
6		WORLD		11	30	**178**		**2.56**	n.a.	n.a.	n.a.
6		WORLD		31	452	**122**		**1.90**	n.a.	n.a.	n.a.
6		Asia		3	10	**25**		**2.26**	n.a.	n.a.	n.a.
6		Europe		3	10	**14**		**2.69**	n.a.	n.a.	n.a.
6		LATAM		3	10	**19**		**2.79**	n.a.	n.a.	n.a.
6		Asia		11	30	**20**		**2.36**	n.a.	n.a.	n.a.
6		Europe		11	30	**21**		**2.38**	n.a.	n.a.	n.a.
6		LATAM		11	30	**129**		**2.61**	n.a.	n.a.	n.a.
6		Asia		31	452	**24**		**1.50**	n.a.	n.a.	n.a.
6		Europe		31	452	**25**		**2.36**	n.a.	n.a.	n.a.
6		LATAM		31	452	**49**		**1.91**	n.a.	n.a.	n.a.
6		N. Amer.		31	452	**24**		**1.69**	n.a.	n.a.	n.a.
6		Asia		0	2	**11**		**2.29**	n.a.	n.a.	n.a.
6		Asia		3	10	**25**		**2.26**	n.a.	n.a.	n.a.
6		Asia		11	30	**20**		**2.36**	n.a.	n.a.	n.a.
6		Asia		31	452	**24**		**1.50**	n.a.	n.a.	n.a.
6		Europe		3	10	**14**		**2.69**	n.a.	n.a.	n.a.
6		Europe		11	30	**21**		**2.38**	n.a.	n.a.	n.a.
6		Europe		31	452	**25**		**2.36**	n.a.	n.a.	n.a.
6		LATAM		3	10	**19**		**2.79**	n.a.	n.a.	n.a.
6		LATAM		11	30	**129**		**2.61**	n.a.	n.a.	n.a.
6		LATAM		31	452	**49**		**1.91**	n.a.	n.a.	n.a.
6		N. Amer.		31	217	**24**		**1.69**	n.a.	n.a.	n.a.

Table 1.7.2-1 Concentrations of 2'-FL (g/L) in breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE)	95% CL (or IQR)	Upper Range
9	**Chaturvedi *et al.* (2001)**	**N. Amer.**	**Term**	**1**	**7**	**84**	**Mean (g/L)**				
9				1	7			**2.70**	n.a.	n.a.	n.a.
9				8	14			**2.90**	n.a.	n.a.	n.a.
9				22	28			**3.50**	n.a.	n.a.	n.a.
9				91	98			**3.50**	n.a.	n.a.	n.a.
9				175	182			**2.70**	n.a.	n.a.	n.a.
9				259	266			**1.80**	n.a.	n.a.	n.a.
9				336	343			**1.20**	n.a.	n.a.	n.a.
17	**Asakuma *et al.* (2008)**	**Asia**	**Term**	**1**	**3**	**12**	**Mean (g/L) with standard deviation**				
17				1	1			**2.49**	1.22	4.93	4.51
17				2	2			**2.01**	1.07	4.15	4.06
17				3	3			**1.58**	0.73	3.04	2.76
17				1	3			**2.03**	1.01	4.04	4.51
20	**Thurl *et al.* (2010)**	**Europe**	**Term**	**3**	**90**	**30**	**Mean (mol/L)**				
20				3	3	**21**		**4.13**	n.a.	n.a.	n.a.
20				8	8	**19**		**3.37**	n.a.	n.a.	n.a.
20				15	15	**17**		**3.04**	n.a.	n.a.	n.a.
20				22	22	**16**		**3.02**	n.a.	n.a.	n.a.
20				30	30	**14**		**2.96**	n.a.	n.a.	n.a.
20				60	60	**12**		**2.82**	n.a.	n.a.	n.a.
20				90	90	**10**		**2.59**	n.a.	n.a.	n.a.
20				3	90	**109**		**3.13**	n.a.	n.a.	n.a.
20				3	90	**17**		**4.57**	n.a.	n.a.	n.a.
22	**Coppa *et al.* (2011)**	**Europe**	**Term**	**25**	**35**	**39**	**Mean (g/L) with standard deviation**				
22						**10**		**2.56**	0.90	4.36	n.a.
22						**6**		**2.66**	0.85	4.36	n.a.
23	**Gabrielli *et al.* (2011)**	**Europe**	**Preterm**	**4**	**30**	**63**	**Mean (g/L) with standard deviation**				
23				4	4	**35**		**7.23**	3.39	14.01	n.a.
23				10	10	**35**		**5.36**	2.58	10.52	n.a.
23				20	20	**35**		**4.72**	2.01	8.74	n.a.
23				30	30	**35**		**4.41**	1.70	7.81	n.a.
26	**Smilowitz *et al.* (2013)**	**N. Amer.**	**Term**	**90**	**90**	**52**	**Mean (mol/L) with standard deviation and range**				
26								**1.56**	0.57	2.71	n.a.
28	**Hong *et al.* (2014)**	**N. Amer.**	**Term**	**35**	**35**	**30**	**Mean (g/L) with standard deviation**				
28						**10**		**3.00**	0.80	4.60	n.a.

Table 1.7.2-1 Concentrations of 2'-FL (g/L) in breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE)	95% CL (or IQR)	Upper Range
30	**Van Niekerk *et al.* (2014)**	**Africa**	**Preterm**	**4**	**28**	**82**	**Mean (g/L) with standard deviation**				
30				4	4	**20**		**1.23**	0.65	2.53	n.a.
30				28	28	**20**		**0.68**	0.28	1.24	n.a.
36	**Aakko *et al.* (2017)**	**Europe**	**Term**	**1**	**4**	**11**	**Mean (g/L) with standard deviation**				
36								**2.73**	0.51	3.76	n.a.
37	**Kunz *et al.* (2017)**	**Europe**	**Term**	**1**	**60**	**32**	**Median (g/L) with IQR**				
37				1	7	**21**		**3.99**	n.a.	n.a.	5.16
37				8	15	**21**		**3.60**	n.a.	n.a.	4.75
37				16	60	**21**		**2.76**	n.a.	n.a.	3.82
38	**McGuire *et al.* (2017)**	**4 Continents**	**Term**	**14**	**150**	**410**	**Mean (mol/l or g/L) with standard error**				
38		Africa				**26**		**1.70**	0.20	2.09	n.a.
38		Africa				**31**		**1.80**	0.17	2.15	n.a.
38		Africa				**26**		**2.22**	0.22	2.66	n.a.
38		Africa				**34**		**2.43**	0.26	2.94	n.a.
38		Africa				**27**		**1.04**	0.10	1.23	n.a.
38		Africa				**34**		**2.04**	0.20	2.45	n.a.
38		LATAM				**42**		**3.26**	0.20	3.67	n.a.
38		Europe				**31**		**2.52**	0.20	2.92	n.a.
38		Europe				**19**		**3.49**	0.25	3.99	n.a.
38		N. Amer.				**28**		**2.97**	0.21	3.39	n.a.
38		N. Amer.				**18**		**3.63**	0.40	4.42	n.a.
39	**Sprenger *et al.* (2017)**	**Asia**	**Term**	**30**	**120**	**50**	**Mean & Median (g/L) with standard deviation and range**				
39				30	30	**34**		**2.17**	0.83	3.83	4.99
39				60	60	**34**		**1.76**	0.64	3.03	3.86
39				120	120	**33**		**1.38**	0.59	2.56	3.51
40	**Azad *et al.* (2018)**	**N. Amer.**	**Term**	**90**	**120**	**427**	**Mean & median (mol/L) with SD & IQR and range**				
40						**307**		**3.13**	1.42	5.96	4.09
41	**Elwakiel *et al.* (2018)**	**Asia**	**Term**	**7**	**140**	**22**	**Mean (g/L) with standard deviation**				
41						**12**		**5.10**	1.49	8.08	n.a.
41						**10**		**3.83**	0.43	4.69	n.a.
41						**22**		**4.47**	**0.96**	**6.39**	n.a.

Table 1.7.2-1 Concentrations of 2'-FL (g/L) in breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE)	95% CL (or IQR)	Upper Range
44	**Austin *et al.* (2019)**	**Europe**	**Term & Preterm**	**7**	**112**	**500**	**Mean & median (g/L) with SD & IQR and range**				
44			Preterm	7	7	**19**		**3.11**	1.92	6.95	5.48
44			Preterm	14	14	**19**		**2.02**	0.78	3.58	3.10
44			Preterm	21	21	**19**		**1.93**	0.76	3.46	3.00
44			Preterm	28	28	**18**		**2.05**	0.88	3.81	3.59
44			Preterm	35	35	**19**		**1.94**	0.72	3.38	3.06
44			Preterm	42	42	**18**		**1.93**	0.87	3.66	3.34
44			Preterm	49	49	**18**		**1.90**	0.75	3.40	2.97
44			Preterm	56	56	**18**		**1.94**	0.83	3.61	3.35
44			Preterm	70	70	**18**		**1.79**	0.81	3.41	3.37
44			Preterm	84	84	**15**		**1.69**	0.85	3.39	3.57
44			Preterm	98	98	**15**		**1.66**	0.83	3.32	2.95
44			Preterm	112	112	**15**		**1.62**	0.87	3.36	3.46
44			Term	7	7	**21**		**3.92**	0.96	5.83	5.57
44			Term	14	14	**19**		**2.72**	0.56	3.85	3.66
44			Term	21	21	**21**		**2.64**	0.71	4.05	4.23
44			Term	28	28	**21**		**2.46**	0.61	3.68	3.71
44			Term	35	35	**21**		**2.39**	0.73	3.85	3.96
44			Term	42	42	**20**		**2.25**	0.72	3.69	3.32
44			Term	49	49	**20**		**2.07**	0.73	3.54	3.07
44			Term	56	56	**21**		**1.96**	0.81	3.58	3.20
46	**Larsson *et al.* (2019)**	**Europe**	**Term**	**150**	**270**	**22**	**Median (mol/L) with IQR**				
46				150	150	**15**		**2.99**	n.a.	n.a.	3.42
46				270	270	**12**		**2.54**	n.a.	n.a.	3.71
47	**McJarrow *et al.* (2019)**	**Middle East**	**Term**	**5**	**180**	**41**	**Mean (g/L) with standard deviation**				
47				5	15	**41**		**2.76**	1.50	5.76	n.a.
47				180	180	**40**		**1.37**	0.75	2.87	n.a.
50	**Tonon *et al.* (2019b)**	**LATAM**	**Term**	**25**	**46**	**77**	**Mean (g/L) with standard deviation**				
50				25	46			**2.20**	0.98	4.16	n.a.
50				25	46			**3.43**	1.75	6.93	n.a.
55	**Lefebvre *et al.* (2020)**	**Europe**	**Term**	**90**	**360**	**156**	**Mean & median (g/L) with range**				
55				90	90	**125**		**2.12**	n.a.	n.a.	4.55
55				180	180	**125**		**1.80**	n.a.	n.a.	3.88
55				360	360	**125**		**1.56**	n.a.	n.a.	2.95
56	**Lagström *et al.* (2020)**	**Europe**	**Term**	**90**	**90**	**802**	**Median (mol/L) with IQR**				
56				90	90	**699**		**3.15**	n.a.	n.a.	4.02
57	**Saben *et al.* (2020)**	**N. Amer.**	**Term**	**60**	**60**	**136**	**Median (mol/L) with IQR**				
57						**101**		**0.77**	n.a.	n.a.	1.05

Table 1.7.2-1 Concentrations of 2'-FL (g/L) in breast Milk Samples of Secretor Phenotype

#	Citation	Global Region	Gestation	Earliest Sampling Day	Latest Sampling Day	Number Donors	Data Provided as	Mean (or Median)	SD (or SE)	95% CL (or IQR)	Upper Range
59	**Wu *et al.* (2020)**	**Asia**	**Term**	**3**	**168**	**59**	**Mean (g/L) with standard error of measurement**				
59				3	3	**25**		**3.02**	0.14	3.30	n.a.
59				7	7	**27**		**2.54**	0.18	2.90	n.a.
59				21	21	**25**		**2.35**	0.15	2.65	n.a.
59				42	42	**15**		**1.96**	0.14	2.24	n.a.
59				77	77	**10**		**1.56**	0.15	1.86	n.a.

For abbreviations see **List of Abbreviations**.

1.7.3 2'-FL: General Data Analysis

Of the 24 publications reporting the concentration of 2'-FL in Secretor milk, the **lowest reported mean** was 0.68 g 2'-FL/L (Van Niekerk *et al*., 2014) (observed in mothers participating at a larger clinical trial in Tygerberg Children's Hospital, Cape Town, South Africa, and who gave birth preterm and were sampled on Day 28 of lactation; all mother/infant dyads included in this study had been pre-selected for infants that had developed necrotizing enterocolitis) and the **highest reported mean** was 7.23 g 2'-FL/L reported by Gabrielli *et al*. (2011) [observed in samples of women that were investigated by the Polytechnic University of Marche, Ancona, Italy, and who delivered preterm newborns at 25 to 30 weeks of gestation (mean gestational age: 27.9 weeks) and were sampled on the morning of Day 4 of lactation].

Equivalent ranges of 2'-FL consumption were calculated using values reported in the U.S. EPA *Exposure Factors Handbook* (U.S. EPA, 2011). Briefly, breast milk consumption data from 7 studies were pooled and indexed to body weight and recommended values were derived assuming exclusively breastfed infants. The highest mean and upper percentile of consumption of breast milk on a body weight basis were observed in infants from birth to < 1 month of 150 and 220 mL/kg body weight/day, respectively. Assuming 2'-FL was present at the highest reported **mean concentration** of 7.23 g/L, this would be equivalent to the mean and upper percentile intake of 1.08[3] and 1.59[4] g/kg body weight/day of 2'-FL, respectively.

[3] Calculated as 7.23 g 2'-FL/L x 0.150 L breast milk/kg body weight/day = 1.0845 g/kg body weight/day.
[4] Calculated as 7.23 g 2'-FL /L x 0.220 L breast milk/kg body weight/day = 1.59 g/kg body weight/day.

1.8 List of Abbreviations

2'-FL	2'-Fucosyllactose
3-FL	3-Fucosyllactose
3'-SL	3'-Sialyllactose
6'-SL	6'-Sialyllactose
CE-FL	Capillary electrophoresis with fluorescence detection
CE-UV	Capillary electrophoresis with ultraviolet detection
CL	Confidence level
DF	Difucosyl
DFL	Difucosyllactose
DS	Disialyl
ESI-MS	electrospray ionization with mass spectrometry
F	Fucosyl
FSL	3'-Sialyl-3-fucosyllactose
Fuc	L-Fucose
FUT	Fucosyltransferase enzyme
Fut	Gene encoding fucosyltransferase enzyme
Gal	D-Galactose
GlcNAc	*N*-Acetyl-D-glucosamine
HMO	Human milk oligosaccharide
HPCE-UV	High-performance capillary electrophoresis with ultraviolet detection
HPAEC-PAD	High-performance anion-exchange chromatography coupled with pulsed amperometric detection
HPLC-FL	High-performance liquid chromatography coupled with fluorescence detection
HPLC–MRM–MS	High-performance liquid chromatography coupled with multiple-reaction monitoring mass spectrometry
HPLC-MS	High-performance liquid chromatography coupled with mass spectrometry detection
HPLC-UV	High-performance liquid chromatography coupled with UV-light detection
IQR	Interquartile range
LATAM	Latin America
LC-ESI-MS	Liquid chromatography electrospray ionization with mass spectrometry
LC-FL	Liquid chromatography coupled with fluorescence detection
LC-MS	Liquid chromatography with mass spectrometry
LC-MS/MS-FL	Liquid chromatography with tandem mass spectrometry coupled with fluorescence detection
Le	Lewis
LNH	Lacto-*N*-hexaose
LNnH	Lacto-*N*-neohexaose
LNT	Lacto-*N*-tetraose
LNDFH	Lacto-*N*-difucohexaose
LNFP	Lacto-*N*-fucopentaose
LNnT	Lacto-*N*-neotetraose
LNT	Lacto-*N*-tetraose
LST	Sialyl-lacto-*N*-tetraose
n.a.	Not available

N. Amer.	North America
NICU	Neonatal intensive care unit
NMR	Nuclear magnetic resonance
NON-SEC	Non-secretor phenotype
RP-HPLC-FL	Reversed phase high-performance liquid chromatography coupled with fluorescence detection
RP-HPLC-UV	Reversed-phase high-performance liquid chromatography coupled with UV-light detection
S	Sialyl
SD	Standard deviation
SEM	Standard error of the mean
Se	Secretor
SEC	Secretor phenotype
SL	Sialyllactose
TF	Trifucosyl
UV	Ultraviolet
U.S. EPA	United States Environmental Protection Agency

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GRAS Panel Evaluation of Lacto-*N*-Fucopentaose I (LNFP-I) and 2'-Fucosyllactose (2'-FL) (LNFP-I/2'-FL) Mixture for Use in Infant Formula and Conventional Food Products

03 May 2021

INTRODUCTION

Glycom A/S (Glycom) convened a panel of independent scientists (the "GRAS Panel"), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of data and information pertinent to the safety of the company's lacto-*N*-fucopentaose I (LNFP-I) and 2'-fucosyllactose (2'-FL) mixture (LNFP-I/2'-FL), produced by fermentation using a modified strain of *Escherichia coli* K-12 DH1, and to determine whether the intended uses of LNFP-I/2'-FL in non-exempt term infant formula and various conventional food and beverage products, as described in Table A-1, would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Professor Emeritus, Virginia Commonwealth University School of Medicine), Dr. George C. Fahey (Professor Emeritus, University of Illinois), and Dr. Ronald Kleinman (Professor, Harvard Medical School).

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of all publicly available scientific data and information compiled from a comprehensive search of the scientific literature performed by Glycom and presented to the GRAS Panel in a dossier titled "*Generally Recognized as Safe Status of Lacto-N-fucopentaose I (LNFP-I) and 2'-Fucosyllactose (2'-FL) (LNFP-I/2'-FL)*", which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of LNFP-I/2'-FL and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, the history of consumption from human milk, and the safety of LNFP-I/2'-FL.

Following its independent and collective critical evaluation, and on the basis of scientific procedures, the GRAS Panel unanimously concluded that LNFP-I/2'-FL, produced by fermentation using a modified strain of *E. coli* K-12 DH1, meeting food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS for use in non-exempt term infant formula and conventional food and beverage products as described in Table A-1. A summary of the information critically evaluated by the GRAS Panel is presented below.

SUMMARY AND BASIS FOR GRAS

LNFP-I/2'-FL is obtained from a single microbial fermentation; thus, it is not a blend of separately produced compounds. It also contains small quantities of D-lactose and L-fucose, and minor amounts of other related and fully characterized carbohydrates, which are produced during the fermentation process. Glycom intends to market LNFP-I/2'-FL in the United States (U.S.) marketplace as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products (see Table A-1). The LNFP-I/2'-FL ingredient is produced by fermentation using a modified strain of *E. coli* K-12 and contains no less than 90% of specified saccharides, which is characterized by the sum of LNFP-I, 2'-FL, lacto-*N*-tetraose (LNT), difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose in the final product.

LNFP-I is a pentasaccharide consisting of L-fucose, two D-galactose, D-glucosamine, and D-glucose. 2'-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. The reported mean concentration of LNFP-I in human milk ranged from 0.18 to 4.47 g/L. The reported mean concentration of 2'-FL in human milk ranged from 0.68 to 7.23 g/L. LNFP-I and 2'-FL, obtained from microbial fermentation, are chemically and structurally identical to LNFP-I and 2'-FL that are naturally present in human milk, as confirmed by 1H- and 2D-nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Therefore, LNFP-I/2'-FL has an established long history of safe consumption as a component of human milk in infants on the basis that LNFP-I and 2'-FL manufactured by Glycom is chemically identical to LNFP-I and 2'-FL naturally present in human milk.

The GRAS Panel critically reviewed details of the manufacturing process for LNFP-I/2'-FL. The ingredient is manufactured in compliance with cGMP and incorporates a Hazard Analysis Critical Control Point (HACCP) management system. The manufacturing process can be broadly divided into two stages.

In Stage 1 (upstream processing), D-lactose and D-glucose[1] are converted to LNFP-I/2'-FL by the adapted cellular metabolism of the production microorganism, which uses D-glucose as an energy and carbon source and D-lactose as a substrate for LNFP-I/2'-FL biosynthesis. The production strain is a derivative of *E. coli* K-12 DH1, which is a non-pathogenic laboratory strain with a well characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). This strain was further optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various carbohydrates. The strain was genomically modified by insertion of a fucosyl-transferase from *Helicobacter pylori* that catalyzes the transfer of fucose to the 3 position of lactose and insertion of a major facilitator superfamily (MFS) transporter from *Serratia marcescens* that enhances the efflux of newly formed LNFP-I/2'-FL out of the cell. No antibiotic resistance genes are present, and antibiotics or inducer molecules are not used. The GRAS Panel noted that the identities of the introduced genes and their expression products (*i.e.*, enzymes) are well characterized, and based upon their known functions would not confer toxicogenic/pathogenic properties to the host organism. The recombinant proteins were further characterized using bioinformatic tools and are not homologous to amino acid sequences of known or putative toxins or allergens.

[1] Alternatively, D-sucrose, or glycerol.

In Stage 2 (downstream processing), a series of purification, isolation, and concentration steps are used to generate the final high-purity LNFP-I/2'-FL ingredient. All processing aids and food contact articles used in Stage 1 and 2 are used in accordance with an appropriate federal regulation, have been previously determined to be GRAS, or have been the subject of an effective food contact notification. Quality control measures are in place during the entire purification and isolation process to ensure that the final batches of LNFP-I/2'-FL released conform with the product specifications. LNFP-I/2'-FL produced by fermentation is chemically identical to LNFP-I and 2'-FL in human milk. There have been no modifications to the molecular structure of LNFP-I/2'-FL during the manufacturing process from that of LNFP-I and 2'-FL that is present in human milk.

Glycom has established food-grade specifications for LNFP-I/2'-FL. The specifications for LNFP-I/2'-FL include parameters related to physical properties, specified saccharides, water, and microbiological contaminants. The main components of the ingredient are LNFP-I (min. 50%), 2'-FL (min. 15%), LNT (max. 5%), difucosyl-D-lactose (max. 1%), 3-fucosyllactose (max. 1%), D-lactose (max. 10%), L-fucose (max. 1%), LNFP-I fructose isomer (max. 1.5%), and 2'-fucosyl-D-lactulose (max. 1%). Specifications have been established for carbohydrate-type compounds and residual proteins originating from the fermentation and downstream purification processes. All analytical methods are internationally recognized or have been validated internally. The GRAS Panel reviewed the results from six batches of LNFP-I/2'-FL and concluded that the manufacturing process produces a consistent material in conformance with the established product specifications.

The ingredient also has been evaluated for the presence of fermentation metabolites (*i.e.*, biogenic amines, amino acids, and their metabolites), microbial endotoxins, and residual proteins, the results of which demonstrate that Glycom's LNFP-I/2'-FL is free from these potential contaminants at levels of toxicological concern. The results of batch analyses also confirmed the absence of heavy metals. There was no appreciable carry-over of minerals from fermentation (*i.e.*, anions, trace elements), or quantifiable levels of residual DNA, in the ingredient.

The GRAS Panel reviewed bulk stability data of LNFP-I/2'-FL being tested under real-time conditions of 25°C and 60% relative humidity (RH) for 5 years, and accelerated conditions of 40°C and 75% RH for 2 years. LNFP-I/2'-FL under real-time conditions was stable for composition, sensory and microbiological parameters throughout a 3-year stability study, and similarly through 6-months of a 5-year stability study. No appreciable degradation of LNFP-I/2'-FL and no changes in impurity profile were noted through 6-months of a 2-year accelerated stability study. Stress/forced stability studies of LNFP-I/2'-FL performed at 80°C for 28 days of storage at two different humidity conditions (ambient and high humidity) demonstrated negligible degradation products throughout the storage period. Stress/forced stability studies of aqueous solutions of LNFP-I/2'-FL performed at 60°C under varying pH values for up to 28 days demonstrated that LNFP-I/2'-FL is most stable at a pH range between 3 and 5. The stability of LNFP-I/2'-FL is currently being evaluated in a commercially representative infant formula for 12 months, with interim stability data supporting that LNFP-I/2'-FL is stable in infant formula for 1 month of storage.

LNFP-I/2'-FL is intended to be added to non-exempt term infant formula[2], foods targeted to young children[3], as well as uses in specific conventional food and beverage products used by the general population (see Table A-1; use levels are expressed upon the use levels of LNFP-I). The maximum use levels in term infant formulas are proposed on the basis of providing similar levels of LNFP-I to those consumed by breastfed infants. Use of this ingredient in infant formula (*i.e.*, infants up to 12 months), toddler formulas[4] (*i.e.*, young children older than 12 months) and beverages targeted to young children will provide a use level of LNFP-I of 1.5 g/L in ready-to-drink and reconstituted products, and up to 8.33 g/kg for products other than beverages for infants and young children (*e.g.*, baby foods). LNFP-I/2'-FL is also intended for use in food and beverages targeted towards the general U.S. population at levels up to 1.5 g/L or 20 g/kg, and foods for special dietary use (*e.g.*, meal replacement bars) at levels up to 2.0 g/L or 20 g/kg. The corresponding exposure values for 2'-FL consumption are calculated from LNFP-I intakes based on the average values of 2'-FL and LNFP-I across five batches of LNFP-I/2'-FL.

The GRAS Panel reviewed data related to the estimated dietary exposure to LNFP-I based on an assessment of the anticipated intake of LNFP-I/2'-FL as an ingredient under the intended conditions of use as described in Table A-1. The dietary intakes of the ingredient were estimated using the information from the 2017-2018 cycle of the National Health and Nutrition Examination Survey (NHANES) based on the proposed food uses and use levels of LNFP-I/2'-FL as described in Table A-1. A summary of the dietary intake estimates is provided in Table 1. The corresponding amount of 2'-FL from this ingredient has been determined based on the average values of 2'-FL and LNFP-I across 5 batches of LNFP-I/2'-FL (*i.e.*, 26.5/61.9 = 0.428). Considering the highest estimated mean and 90th percentile body weight intakes (infants aged 7 to <12 months) of 285 and 533 mg LNFP-I/kg body weight/day, this is equivalent to 122[5] and 228[6] mg/kg body weight/day of 2'-FL. Glycom has notified the Agency of the GRAS uses of 2'-FL as an ingredient in non-exempt term infant formula, toddler formulas, and other conventional foods under GRNs 546 and 650. Exposure analyses conducted for all GRAS uses resulted in estimated mean and 90th percentile body weight intakes of 2'-FL in infants aged 7 to <12 months of 188.9 and 295.8 mg/kg body weight/day, respectively (U.S. FDA, 2016).

Table 1 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	174	361	76.2	139	229	388
Infants	7 to <12 m	277	533	97.5	122	285	533
Toddlers	1 to 2 y	70	132	98.9	291	71	132
Children	3 to 11 y	29	60	97.4	967	29	60
Female Teenagers	12 to 19 y	9	21	92.4	407	10	24
Male Teenagers	12 to 19 y	12	23	95.2	415	12	23
Female Adults of Childbearing Age	20 to 40 y	8	17	87.7	609	9	18
Female Adults	20 to 64 y	9	20	87.0	1,402	10	22

[2] Infant formula products to which LNFP-I/2'-FL would be added are most likely to be formula containing partially hydrolyzed cow's milk protein as a protein base.
[3] Nestlé boost kid essentials, Nestlé Nutren junior, or Pediasure by Abbott Nutrition.
[4] Formula products targeting young children older than 12 months of age.
[5] Calculated as 285 mg LNFP-I/kg body weight/day x 0.428 2'-FL/LNFP-I = 122 mg 2'-FL/kg body weight/day.
[6] Calculated as 533 mg LNFP-I/kg body weight/day x 0.428 2 '-FL/LNFP-I = 228 mg 2'-FL/kg body weight/day.

Table 1 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Male Adults	20 to 64 y	9	22	89.0	1,252	10	22
Elderly	65 y and older	8	18	88.0	891	9	18
Total Population	2 y and older	12	27	89.9	5,478	13	29

LNFP-I = lacto-*N*-fucopentaose; bw = body weight; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

The GRAS Panel critically evaluated published data and information characterizing the safety of LNFP-I/2'-FL. This information included a discussion of the safety of the production strain, the metabolic fate of human milk oligosaccharides (HMOs), product-specific toxicity studies on LNFP-I/2'-FL, and an allergenicity assessment of the ingredient. The GRAS Panel noted that all HiMOs manufactured by Glycom are produced from a production organism originating from the same optimized host strain (*E. coli* DH1 MDO) and human-identical milk oligosaccharides (HiMOs) produced by Glycom using modified strains of this lineage have been the subject of extensive toxicological testing without evidence of test article-induced toxicity. The production methods employed by Glycom are based on fermentation processes that utilize food-grade lactose as a substrate and defined carbon and nitrogen sources. The existing toxicological studies conducted with Glycom's portfolio of HiMOs produced by fermentation [*e.g.*, 2'-FL, lacto-*N*-neotetraose (LNnT), 2'-fucosyllactose/difucosyllactose (2'-FL/DFL), LNT, 3'-siallylactose (3'-SL), 6'-siallylactose (6'-SL)] support the safety of the platform strain (MDO) lineage. The introduced genetic modifications for HiMO synthesis produce a predictable pattern of metabolites and intended fermentation products that are identifiable and are not of concern for imparting unexpected pleiotropic effects to fermentation products produced from this host. Based on the established safety of the strain lineage, the compositional characterization of the production process and ingredient composition (*i.e.*, identical to human oligosaccharides), and the fact that Glycom's HiMOs will be used in infant formula at levels that are equivalent to concentrations in human milk, toxicological testing was not necessary to support the safety of the ingredient. However, LNFP I/2'-FL has been tested in a comprehensive series of toxicological studies, including a bacterial reverse mutation assay, an *in vitro* mammalian cell micronucleus test in human lymphocytes and an adapted subchronic (90-day) oral toxicity study in neonatal rats (Phipps *et al*., 2020).

The manufactured LNFP-I and 2'-FL components of the final ingredient are structurally identical to their naturally occurring counterparts in human milk. The absorption, distribution, and metabolism of HMOs have been the subject of extensive investigation (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al.*, 2000; Chaturvedi *et al.*, 2001; Rudolff and Kunz, 2012) and it can be concluded that HMOs, including LNFP-I and 2'-FL, do not undergo any significant digestion in the upper gastrointestinal tract. Very small quantities of ingested HMOs have been reported to be absorbed intact (approximately 1 to 2% of the total amount of HMO ingested) and are excreted unchanged in urine. The data supports limited absorption of LNFP-I/2'-FL and the quantities absorbed would not be different from those occurring in breastfed infants.

The GRAS Panel critically evaluated the results of a 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of LNFP-I/2'-FL when administered orally, by gavage, to neonatal rats from Day 7 of age (Phipps et al., 2020). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of GLP (OECD, 1998) and the most recent version of OECD Test Guideline 408 (OECD, 2018), but was adapted by using neonatal animals (as LNFP-I/2'-FL is primarily intended for use in infant formula). Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (vehicle – water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day LNFP-I/2'-FL, by oral gavage at a dose volume of 10 mL/kg body weight, once daily for 90 days, until the day

before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose LNFP-I/2'-FL group and identify any effects related to the general fiber-like characteristics of the reference material. The rats were examined for the standard toxicological battery. No test item-related adverse effects were reported; therefore, the no-observed-adverse-effect level (NOAEL) for LNFP-I/2'-FL was concluded to be 5,000 mg/kg body weight/day, the highest dose tested. The GRAS Panel agrees with this NOAEL determination.

The GRAS Panel critically evaluated the results of genotoxicity and mutagenicity tests conducted with LNFP-I/2'-FL. LNFP-I/2'-FL was consistently negative in a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test (Phipps *et al.*, 2020).

Studies in humans evaluating the consumption of LNFP-I/2'-FL have not been published to date. However, clinical studies have been conducted on 2'-FL, which are incorporated by reference to GRNs 546, 571, 650, 735, 749, 815, 852, and 897. Clinical data from randomized controlled studies indicate that 2'-FL is well tolerated and does not give rise to any safety concerns when orally administered to infants at up to 1.2 g/L in infant formula (Puccio *et al.*, 2017 - NCT01715246); in young children (ages 1 to 2.5 years) at 3 g/L in young child formula products (Leung *et al.*, 2020 - Netherlands Trial Register NL4627) and adults at up to 20 g/day (Elison *et al.*, 2016 - NCT01927900).

The allergenic potential of the recombinant proteins expressed by the production strain was assessed using bioinformatic analyses. The amino acid sequences of the recombinant proteins were evaluated using the Basic Local Alignment Search Tool (BLAST) search algorithms of the AllergenOnline database (version 21) of the Food Allergen Research and Resource Program (FARRP) of the University of Nebraska (FARRP, 2021). The online tool allows search by three different search algorithms each with its own alert limit for potential allergenicity: (i) full sequence length (FASTA) comparison with an alert limit of minimum 50% sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of minimum 35% sequence similarity to hint for potential allergenic potential; and (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified. In addition, the purification steps involved in the manufacture of LNFP-I/2'-FL are proven to remove protein (*i.e.*, potential allergen) to below the specified level of <0.01% (w/w). Based on the purification process utilized during the manufacturing process and absence of detectable protein in the ingredient, the GRAS Panel considered the risk of allergenicity to be very low.

Following its independent and collective critical evaluation of the available information of LNFP I/2'-FL, the GRAS Panel unanimously concluded that the available information supports the conclusion presented on the next page.

CONCLUSION

We, the GRAS Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that lacto-*N*-fucopentaose I (LNFP-I) and 2'-fucosyllactose (2'-FL) mixture (LNFP-I/2'-FL), produced by fermentation using a modified strain of *Escherichia coli* K-12 DH1, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice, is Generally Recognized as Safe (GRAS) based on scientific procedures, for use in term infant formula and specified conventional food and beverage products as described in Table A-1.

It is our opinion that other qualified experts would concur with these conclusions.

[redacted]

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5/7/2021
Date

[redacted]

George C. Fahey, Ph.D.
Professor Emeritus
University of Illinois

5/10/21
Date

[redacted]

Ronald E. Kleinman, M.D.
Professor of Pediatrics
Harvard Medical School

Date

5/4/2021

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ATTACHMENT A: INTENDED FOOD USES AND USE LEVELS FOR LNFP-I FROM LNFP-I/2'-FL IN THE UNITED STATES

Table A-1 Proposed Food Uses and Use Levels for LNFP-I from LNFP-I/2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Us Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	240	0.48	2.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.12	1.5
	Toddler Formulas[e]	100[d]	0.12	1.5
	Other Baby Foods for Infants and Young Children	7 to 170	0.06 to 1.42	8.33
	Other Drinks for Young Children	120	0.18	1.5
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	0.8	20.0
	Cereal and Nutrition Bars	40	0.4	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	240	0.24	1.0
Milk Products	Buttermilk*	240	0.24	1.5
	Flavored Milk	240	0.24	1.5
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.48	2.0
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.10 to 0.25	1.5
	Yogurt*	170	0.34	10.0
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.24	1.0

LNFP-I = lacto-*N*-fucopentaose I; CFR = *Code of Federal Regulations*; RACC = Reference Amounts Customarily Consumed; RTE = ready-to-eat; U.S. = United States.

* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2020b). When a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC.

[b] Use level expressed on a LNFP-I basis in the final food, as consumed.

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

[d] RACC not available, 100 mL employed as an approximation.

[e] Formula products targeted toward young children (>12 months of age)

[f] Portion sizes are based on representative products on the U.S. market.

From:	Darch, Maryse
To:	Morissette, Rachel
Cc:	Roehrig, Christoph
Subject:	RE: [EXTERNAL] RE: questions for GRN 001035
Date:	Friday, May 13, 2022 3:52:33 PM
Attachments:	image002.png image003.png image004.png image005.png image006.png image007.png GRN 001035 - Response to FDA Questions - 13May"22.pdf Attachment A - Infant Food Codes.pdf

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Dear Dr. Morissette,

Please find attached Glycom's responses to FDA questions for GRN 001035 received March 31st, 2022.

Please do not hesitate to contact us if any further clarification is required.

Kind regards,
Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
Glycom, the leading HMO expert is part of DSM

From: Darch, Maryse
Sent: Friday, May 13, 2022 11:45 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001035

For Internal Use Only

Dear Rachel,

Thank you for following up. We will be sending the responses to the FDA's questions for GRN 1035 shortly.

Kind regards.

Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, May 13, 2022 11:39 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001035

Dear Maryse,

I just wanted to check when we can expect to receive your responses to our questions for GRN 1035?

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Wednesday, April 13, 2022 12:02 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001035

For Internal Use Only

Dear Rachel,

Thank you for confirming the 1-month extension. Noted that the extension may result in a 90-day extension for the notice. The advanced notice on the effect of the requested extension on FDA timelines is very much appreciated.

Kind regards,
Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, April 13, 2022 11:03 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001035

Dear Maryse,

We can do a 1-month extension, but I want to inform you that there's a strong likelihood that we will be issuing a 90-day extension for this notice if that's the case. The current 180-day date is July 27.

Just want you to be aware. So we will expect to see responses on or before May 13, 2022.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Wednesday, April 13, 2022 10:30 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: questions for GRN 001035

Dear Rachel,

We would like to request an extension of 1 month for providing responses to questions on GRAS Notice No. GRN 001035. Due to the scope of the request, and as many of our colleagues are taking vacation during the Easter holiday, this extension would allow us to provide more robust responses to your questions.

I apologize for the delay, and thank you for your understanding in this matter.

Kind regards,

Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Sent: Thursday, March 31, 2022 10:36 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Darch, Maryse <Maryse.Darch@dsm.com>
Subject: RE: questions for GRN 001035

For Internal Use Only

Dear Rachel,

I confirm receipt of the questions and thank you for sending. We will revert back if we have questions at this time.

Kind regards,
Christoph

Christoph Röhrig | Head of HMO Regulatory Affairs | DSM | Kogle Alle 4 | 2970 Hørsholm | Denmark | christoph.roehrig@dsm.com |
Glycom, the leading HMO expert is part of DSM

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, 31 March 2022 15:37
To: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: questions for GRN 001035

Dear Christoph,

Please see attached our questions for GRN 001035. Let me know if you have questions at this time.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

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Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark

13 May 2022

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notice No. GRN 001035

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 31 March 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of lacto-*N*-fucopentaose I with 2'-fucosyllactose (LNFP-I/2'-FL) filed by the Agency under GRN 001035.

FDA.1. On page 5 of the notice, Footnote 2 in Table 1.3-1 states the following: "Infant formula products to which LNFP-I/2'-FL would be added are most likely to be formula containing partially hydrolyzed cow's milk protein as a protein base." Please clarify what is meant by "most likely." Are there other protein bases to which this product will be added? We note that while some non-exempt infant formulas contain partially hydrolyzed protein, most contain intact protein (e.g., milk-based, soy-based).

Glycom does not intend to restrict the use of LNFP-I/2'-FL according to the type or source of infant formula protein base. Therefore, LNFP-I/2'-FL may be added to non-exempt infant formula containing other protein bases, including products containing intact protein from various sources (*e.g.*, milk-based, soy-based).

The infant formula protein base is determined by the infant formula manufacturer. Therefore, it is the responsibility of the infant formula manufacturer to confirm the applicability and the safety of the addition of LNFP-I/2'-FL to an infant formula product.

FDA.2. *On page 12 of the notice, Table 2.2.2-1 provides an overview of the manufacturing method of LNFP-I/2'-FL that includes two purification steps (Steps 6 and 9). Please specify what impurities are removed during these two purification steps and what purification techniques are used.*

The unit operations of these steps are at the same time to concentrate the LNFP-I/2'-FL product as they are also purification steps. Both steps (Steps 6 and 9) employ the use of nanofiltration or nanofiltration/diafiltration membranes to remove water, minerals, and other potentially small molecule metabolites. Due to the small pore size of the membranes, LNFP-I/2'-FL is retained while water and

small molecules are reduced. Specifically, Step 6 of the manufacturing process leads to the pre-concentration of the LNFP-I/2'-FL ingredient, while Step 9 is a second concentration process where evaporation may also be applied to further concentrate the LNFP-I/2'-FL ingredient.

FDA.3. *Please clarify whether cobaltous salts are added to the fermentation medium used in the manufacture of LNFP-I/2'-FL. Food containing added cobaltous salts is deemed adulterated under 21 CFR 189.120.*

Glycom confirms that cobaltous salts are **not** added to the fermentation medium used in the manufacture of LNFP-I/2'-FL.

FDA.4. *On pages 14-15 of the notice, Table 2.3.1-1 lists specifications established for LNFP-I/2'-FL, along with corresponding analytical methods. Please address the following:*

a. *On page 21 of the notice, Glycom states "More restrictive release specifications (including additional limits for Cronobacter spp., L. monocytogenes, and B. cereus) are established for ingredients intended for addition at the dry blending stage of infant formula manufacture where subsequent heat-treatment is not applied." The specifications in Table 2.3.1-1 do not include limits for Cronobacter spp., Listeria monocytogenes, or Bacillus cereus. Part 2.3.2.4 on page 21 states that LNFP-I/2'-FL is intended to be added at the wet blending stage of the infant formula manufacture (i.e., prior to retort). Please confirm if LNFP-I/2'-FL is intended for use in liquid infant formula only. If Glycom intends to use LNFP-I/2'-FL in powdered infant formula, please provide specifications for Cronobacter spp., L. monocytogenes, and B. cereus, as well as the sample size, method of detection, and the results of at least three non-consecutive batch analyses.*

b. *Table 2.3.1-1 provides the specifications for LNFP-I/2'-FL that include the lower limits for LNFP-I (≥50% w/w), 2'-FL (≥15% w/w), and LNFP-I and 2'-FL combined (≥75% w/w), but does not include the upper limits. Please provide these upper limits for both substances individually and combined.*

c. *Please confirm that all analytical methods used to test for the specification parameters are validated for the stated purpose.*

Part a)

LNFP-I/2'-FL is intended to be added to both liquid and powdered infant formulae. Updated specifications for LNFP-I/2'-FL are provided below in Table 1, which include specifications for *Cronobacter spp.*, *Listeria monocytogenes*, and *Bacillus cereus* for LNFP-I/2'-FL intended for addition at the dry blending stage of infant formula manufacture.

Table 1 Updated Specifications for LNFP-I/2'-FL

Description		
LNFP-I/2′-FL (GlyCare™ LNFP-I / 2FL 8001) is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 75% of lacto-*N*-fucopentaose I and 2'-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	White, white to off-white, off-white	ISO 6658
Identification by retention time	RT of main components correspond to RT of standards ± 3%	Glycom method HPLC-13-002
Assay (water-free) – Specified saccharides[a]	≥ 90.0 w/w %	Glycom method HPLC-13-001, HPLC-13-002, HPAEC-HMO-017
Assay (water-free) – LNFP-I and 2'-FL	≥ 75.0 w/w %	Glycom method HPLC-13-002
Assay (water-free) – LNFP-I	≥ 50.0 w/w %	Glycom method HPLC-13-002
Assay (water-free) – 2'-FL	≥ 15.0 w/w %	Glycom method HPLC-13-002
Lacto-*N*-tetraose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-017
3-Fucosyllactose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017
L-Fucose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017
D-Lactose	≤ 10.0 w/w %	Glycom method HPAEC-HMO-017
Difucosyl-D-lactose	≤ 2.0 w/w %	Glycom method HPAEC-HMO-017
LNFP-I fructose isomer	≤ 1.5 w/w %	Glycom method HPLC-13-001
2'-Fucosyl-D-lactulose	≤ 1.0 w/w %	Glycom method HPLC-13-001
Sum of other carbohydrates	≤ 6.0 w/w %	Glycom method HPAEC-HMO-017
pH (20°C, 5% solution)	4.0 to 7.0	Ph. Eur. 2.2.3
Water	≤ 8.0 w/w %	Glycom method KF-001
Ash, sulfated	≤ 0.5 w/w %	Ph. Eur. 2.4.14
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Ph. Eur 2.6.14 (LAL kinetic chromogenic assay)
Lead	≤ 0.1 mg/kg	EN 13805; EPA-6020A
Microbiological Specifications		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO-4833-2
Enterobacteriaceae	≤ 10 CFU/g	ISO 21528-2 or NMKL 144
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter (Enterobacter) sakazakii[b]	Absent in 10 g	ISO 22964
Listeria monocytogenes[b]	Absent in 25 g	ISO 11290-1
Bacillus cereus[b]	≤ 50 CFU/g	ISO 7932
Yeasts	≤ 100 CFU/g	ISO 21527-2
Molds	≤ 100 CFU/g	ISO 21527-2

2'-FL = 2'-fucosyllactose; AFNOR = Association Francaise de Normalisation; CFU = colony forming units; E.U. = endotoxin units; HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl Fischer; LNFP-I = lacto-*N*-fucopentaose I; NMKL = Nordisk Metodikkomite for Levnedsmidler; Ph. Eur. = European Pharmacopoeia; RT = retention time.

[a] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

[b] Applicable to LNFP-I/2′-FL that is added during the dry-blending stage of infant formula manufacturing only.

Results for *Cronobacter spp.*, *Listeria monocytogenes*, and *Bacillus cereus* are provided in Table 2 for the same six independent production batches of LNFP-I/2'-FL ((b) (4) [redacted] as those from the GRAS Notice.

Table 2 Updated Batch Results for Microbiological Analysis of LNFP-I/2'-FL

Parameter	Manufacturing Batch No.					
	(b) (4) [redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Cronobacter (Enterobacter) sakazakii (Absence in 10 g)	Absent	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes (Absence in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus (≤ 50 CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10

2'-FL = 2'-fucosyllactose; CFU = colony forming units; LNFP-I = lacto-N-fucopentaose I.

Part b)

Other than for difucosyllactose (DFL) from 2'-FL/DFL (GRN 815), upper limits have not previously been established for HiMOs that have been notified as GRAS to the U.S. FDA and received from the Agency a "no questions" letter. The purpose of the upper limit for DFL (≤ 20.0 w/w%), as described in GRN 815, is to reflect the naturally occuring ratio between 2'-FL and DFL. However, the main purpose of the LNFP-I/2'-FL ingredient is to facilitate the addition of LNFP-I to foods. For this reason, the proposed use levels of the LNFP-I/2'-FL ingredient are expressed on a LNFP-I basis.

The production of 2'-FL as a result of the fermentation process is considered advantageous as it provides an additional source of fucosylated HiMO. The level of 2'-FL in the LNFP-I/2'-FL ingredient could never exceed the level of LNFP-I as the current specification limit dictates that the ingredient must contain at least 50 w/w% LNFP-I. Several sources of 2'-FL have been notified as GRAS to the U.S. FDA and received from the Agency "no response" letters (*e.g.*, GRN 546, 571, 650, 735, 749, 815, 852, 897, 929, and 932). As such, the use of 2'-FL from different sources is governed by the intended conditions of use that are GRAS for 2'-FL, and it would be the responsibility of the food product manufacturer to ensure that the addition levels of LNFP-I and 2'-FL are GRAS under the intended conditions of use.

Maximum use levels of HiMOs that have been determined to be GRAS in the U.S. are based on naturally occuring levels in human milk. The levels of LNFP-I naturally occuring in human milk are lower than those for 2'-FL. This is reflected by the maximum proposed use levels of LNFP-I/2'-FL (expressed on a LNFP-I basis) being lower those that have been determined as GRAS for 2'-FL for the same food uses. The level of 2'-FL added to food products from the intended use of LNFP-I/2'-FL (assuming 50% 2'-FL as a worst-case) would not exceed maximum use levels of 2'-FL that have been notified as GRAS in the U.S. for the same food uses, as presented Table 3 below.

For the purposes of the dietary exposure assessment, it can be theoretically assumed as the worst-case that the ingredient could contain a maximum of 85 w/w% LNFP-I[1] and a maximum of 50 w/w% 2'-FL[2].

[1] Assuming 15 w/w % 2'-FL, the minimum specification limit for 2'-FL [calculation: 100 % - 15 % 2'-FL = 85 % LNFP-I].
[2] Assuming 50 w/w % LNFP-I, the minimum specification limit for LNFP-I [calculation: 100 % - 50 % LNFP-I = 50 % 2'-FL].

These assumptions were applied in the updated dietary exposure assessment, for which a summary of the results is presented in the response to Question 9. As expected from the lower maximum potential use level of 2'-FL from LNFP-I/2'-FL compared to GRAS levels of 2'-FL, the highest potential estimated daily intakes of 2'-FL from LNFP-I/2'-FL are lower than those that have been notified as GRAS for 2'-FL in the U.S. (see response to Question 9).

As the establishment of upper specification limits for LNFP-I, 2'-FL, and LNFP-I/2'-FL combined is not associated with a safety concern, Glycom's preference is to maintain the specifications of LNFP-I/2'-FL as proposed in the GRAS notice. Nevertheless, Glycom is prepared to discuss the specifications for LNFP-I/2'-FL further as needed.

Table 3 Comparison of Maximum Theoretical Use Levels of 2'-FL from LNFP-I/2'-FL to Maximum Authorized Use Levels of 2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	Maximum Use Level (g/kg or g/L)		
		LNFP-I (from LNFP-I/2'-FL)	2'-FL (from LNFP-I/2'-FL)*	2'-FL (Notified as GRAS)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages	2.0	1.0	5
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.0	0.5	1.2
Infant and Toddler Foods	Term Infant Formulas	1.5	0.75	2.4
	Toddler Formulas	1.5	0.75	2.4
	Other Baby Foods for Infants and Young Children	8.33	4.17	12
	Other Drinks for Young Children	1.5	0.75	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	20.0	10.0	40
	Cereal and Nutrition Bars	20.0	10.0	12
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	1.0	0.5	1.2
Milk Products	Buttermilk	1.5	0.75	1.2
	Flavored Milk	1.5	0.75	1.2
	Milk-Based Meal Replacement and Nutritional Beverages	2.0	X1.0	5
	Yogurt Drinks, Probiotic Drinks	1.5	0.75	5.3
	Yogurt	10.0	5.0	5.3
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.0	0.5	1.2

2'-FL = 2'-fucosyllactose; GRAS = generally recognized as safe; LNFP-I = Lacto-N-fucopentaose I; U.S. = United States.
* Assuming 50% 2'-FL as a worst-case.

Part c)

Validation is ongoing for Glycom methods HPLC-13-001, HPLC-13-002, and HPAEC-HMO-017, and is anticipated to be completed in July.

FDA.5. *On page 14 of the notice, the specifications in Table 2.3.1-1 include an upper limit of 6% for "sum of other carbohydrates". Please provide information regarding the chemical identities of these other carbohydrates and discuss why they are not a safety concern when present at levels up to 6% of LNFP-I/2'-FL.*

Glycom monitors the levels of other carbohydrates carefully using a sensitive analytical technique (HPAEC-HMO-017) at a Level of Reporting (LoR) of 0.03 w/w %. These other carbohydrate compounds are generally structurally related to LNFP-I and 2'-FL (target products), LNT and lactose (substrates), and glucose (the energy source). The top contributors (up to 1.47 w/w %) to the "sum of other carbohydrates" are isomaltose (an end-product of starch digestion) and 2''-fucosyl-lacto-N-triose I (general structural pattern observed in HMOs). Individually, all other carbohydrates are present at low levels. Other contributors include 3'-galactosyl-lactose, 6-β-glucofuranosyl-glucose (6-β-Gluf-Glu), lacto-*N*-triose II, anomerically reduced LNFP-I ("LNFP-I-tol"), and sugars found in nature such as gentiobiose, nigerose, panose, and trehalose.

Analytical results from six independent production batches of LNFP-I/2'-FL (b) (4) [redacted] are presented in Table 2.3.2-1 of the GRAS notice. The 'sum of the other carbohydrates' is well below the proposed upper limit of 6 w/w % for this parameter (average of 1.89 ± 0.61 w/w %; maximum of 2.72 w/w %), as demonstrated in Table 4. Nevertheless, as each individual measurement is subject to a combination of standard deviation of measurement and a degree of variation across fermentations, we are proposing some margin for the upper limit of the "sum of other carbohydrates" parameter. A specification limit for "sum of other carbohydrates" of 6 w/w % has previously been established for other HiMOs (*e.g.*, 2'-FL/DFL – GRN 815).

Table 4 Analytical Results for 'Sum of Other Carbohydrates' from 6 Independent Production Batches of LNFP-I/2'-FL

Parameter	Specification	Manufacturing Batch No.						
		(b) (4) [redacted]						
Sum of other carbohydrates	≤ 6.0 w/w %	2.64	1.43	1.51	1.53	2.72	1.51	1.89 ± 0.61

2'-FL = 2'-fucosyllactose; AVE = average; LNFP-I = lacto-*N*-fucopentaose I; SD = standard deviation.

FDA.6. *On page 16 of the notice, Table 2.3.2-1 contains the results of six batch analyses of LNFP-I/2'-FL. We note that some of the results for 3-FL and L-fucose are reported as <0.03%, for sulfated ash as <0.01%, and for residual protein as <0.0017%. On page 41 of the notice in Part 6.1 Glycom states that 0.0017% (17 mg/kg) is the limit of detection (LOD) for the modified Bradford method used to measure residual protein. Please clarify the LODs for 3-FL, L-fucose, and sulfated ash.*

LODs for 3-FL and L-fucose are not yet available as validation of the Glycom method HPAEC-HMO-017 is ongoing. The limits reported from batch analyses for these parameters in the GRAS notice are LoRs, which can be higher than LODs. The LOD for sulphated ash is ≤ 0.01%.

FDA.7. *On page 20 of the notice, Table 2.3.2.3-1 contains the results for non-carbohydrate residues from six batch analyses of LNFP-I/2'-FL. Please address the following:*

a. *The levels of iron in the six analyzed batches are reported as follows: 1, <0.5, <1, <0.1, <10, and <1%. We assume that the same analytical method was used to analyze all six batches and, as such, the LOD is expected to be the same. Please explain why the results for iron in five out of six batches were reported as ranges and not as exact measured values even though, based on the data provided in Table 2.3.2.3-1, some of these levels seem to be above the LOD of the analytical method. Similarly, please explain why the results provided for phosphate, ammonium, magnesium, manganese, zinc, and molybdenum suggest variable LODs.*

b. *The level of calcium in batch No.* (b) (4) *is reported as <0.000%. Please clarify this value.*

Part a)

The results of analysis for the same parameter have been determined by internal and different accredited laboratories (Wessling Hungary Kft., Eurofins) for the different batches of LNFP-I/2'-FL. Test methods and LODs for iron, phosphate, ammonium, magnesium, manganese, zinc, and molybdenum are provided in Table 5 by laboratory.

Inconsistency of reported levels of these non-carbohydrate residues is due to a combination of different time of measurement and the fact that these parameters are not considered required parameters of the as the levels are below levels of concern. Therefore, the LODs were not pre-defined but rather dependent on the implemented method of measurement in each accredited laboratory.

Table 5 Test Methods and Laboratory LOD/LOQ

Parameter	Specification	Method	Laboratory	LOD/LOQ
Phosphate (w/w %)	Not applicable. Measurement only.	GOST 31867-2012	Internal	0.0005 w/w %
		MSZ EN ISO 6878:2004	Wessling	0.001 w/w %
Ammonium (w/w %)	Not applicable. Measurement only.	GOST 31869-2012	Internal	0.001 w/w %
		MSZ ISO 7150-1:1992	Wessling	0.005 w/w %
Magnesium (w/w %)	Not applicable. Measurement only.	GOST 31869-2012	Internal	0.001 w/w %
		Preparatory: MSZ EN 13805:2015 Measurement: EPA Method 6020A:2007	Wessling	0.001 w/w %
Iron (mg/kg)	Not applicable. Measurement only.	Preparatory: MSZ EN 13805:2015 Measurement: EPA Method 6020A:2007	Wessling	0.5 mg/kg
		DS/EN 13805:2014, DS/EN ISO 11885m:2009	Eurofins	10 mg/kg
Manganese (mg/kg)	Not applicable. Measurement only.	Preparatory: MSZ EN 13805:2015 Measurement: EPA Method 6020A:2007	Wessling	0.1 mg/kg

Table 5 Test Methods and Laboratory LOD/LOQ

Parameter	Specification	Method	Laboratory	LOD/LOQ
		DS/EN 13805m:2014, DS/EN ISO 17294m:2016	Eurofins	1 mg/kg
Zinc (mg/kg)	Not applicable. Measurement only.	Preparatory: MSZ EN 13805:2015 Measurement: EPA Method 6020A:2007	Wessling	0.1 mg/kg
		DS/EN 13805m:2014, DS/EN ISO 17294m:2016	Eurofins	0.5 mg/kg
Molybdenum (mg/kg)	Not applicable. Measurement only.	Preparatory: MSZ EN 13805:2015 Measurement: EPA Method 6020A:2007	Wessling	0.1 mg/kg
		DS/EN 13805m:2014, DS/EN ISO 17294m:2016	Eurofins	0.2 mg/kg

LOD = limit of detection; LOQ = limit of quantification.

Part b)

We thank the FDA for catching this mistake. The level of calcium in batch No. (b) (4) is below the LOD for calcium of <0.001 w/w %.

FDA.8. *On page 5 of the notice, Table 1.3-1 lists the intended use levels of LNFP-I from LNFP-I/2'-FL. Please address the following:*

a. *We note that the provided intended maximum use levels of LNFP-I on a g/RACC basis and on a g/kg (or g/L) basis are not equivalent for several food categories: Cereal and Nutrition Bars, Buttermilk, Flavored Milk, and Yogurt. For example, according to Table 1.3-1, the maximum use level of 0.34 g/RACC of yogurt is equivalent to 10 g/kg of yogurt. Based on our calculations, the maximum use level on a g/kg basis should be 2 g/kg of yogurt. Please re-check the intended use levels for LNFP-I, provide an updated Table 1.3-1 with correct use levels, and update the dietary exposure estimates, if necessary.*

b. *Please clarify whether the intended uses of LNFP-I/2'-FL include any foods for infants and young children that are subject to regulation by the U.S. Department of Agriculture (USDA).*

c. *To clarify the intended uses in foods for infants and young children, please provide a list of NHANES food codes that were considered under the following categories in Table 1.3-1:*
   - *Term Infant Formulas*
   - *Toddler Formulas*
   - *Other Baby Foods for Infants and Young Children*
   - *Other Drinks for Young Children*

Part a)

The proposed use levels of LNFP-I from LNFP-I/2'-FL provided on a g/kg (or g/L) basis in Table 1.3-1 of the GRAS notice, applied in the dietary exposure assessment, are correct. Therefore, there is no change

to the dietary exposure estimates of LNFP-I from the proposed conditions of use of LNFP-I/2'-FL, presented in section 3.2.2 of the notice.

The proposed use levels of LNFP-I from LNFP-I/2'-FL provided on a g/RACC basis were included for reference only. These were back-calculated from the proposed use level of LNFP-I from LNFP-I/2'-FL provided on a g/kg (or g/L) basis and reference amounts customarily consumed (RACCs) per eating occasion established in 21 CFR §101.12. Use levels expressed on a g/RACC have been corrected in the revised Table 1.3-1 below.

Table 1.3-1 Proposed Food Uses and Use Levels for LNFP-I from LNFP-I/2'-FL in the U.S. [REVISED]

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	240	0.48	2.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.15	1.5
	Toddler Formulas[e]	100[d]	0.15	1.5
	Other Baby Foods for Infants and Young Children	7 to 170	0.06 to 1.42	8.33
	Other Drinks for Young Children	120	0.18	1.5
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	0.8	20.0
	Cereal and Nutrition Bars	40	0.8	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	240	0.24	1.0
Milk Products	Buttermilk*	240	0.36	1.5
	Flavored Milk	240	0.36	1.5
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.48	2.0
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.12 to 0.31	1.5
	Yogurt*	170	1.7	10.0
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.24	1.0

CFR = *Code of Federal Regulations*; LNFP-I = lacto-*N*-fucopentaose I; RACC = Reference Amounts Customarily Consumed per Eating Occasion; RTE = ready-to-eat; U.S. = United States.

* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2020a). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.

[b] Use level expressed on a LNFP-I basis in the final food, as consumed.

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

[d] RACC not available, 100 mL employed as an approximation.

[e] Formula products targeted toward young children (> 12 months of age)

[f] Portion sizes are based on representative products on the U.S. market.

Part b)

Glycom does not intend to add LNFP-I/2'-FL to any meat, poultry and/or egg products intended for infants and young children that are subject to regulation by the U.S. Department of Agriculture (USDA).

Part c)

NHANES food codes applied in the dietary exposure assessment for the requested food categories are provided in Attachment A.

FDA.9. *On page 35 of the notice, Part 3.2 discusses dietary exposure to LNFP-I and 2'-FL. Please address the following:*

a. *The dietary exposure to 2'-FL from LNFP-I/2'-FL was estimated using a value of 0.428, which corresponds to the ratio of average levels of 2'-FL and LNFP-I across six batches of LNFP-I/2'-FL provided in Table 2.3.2.2-1 on page 18 of the notice. We note that the ratios for individual batches range from 0.261 to 0.550 and thus the ratio of 0.428 does not represent the worst-case scenario dietary exposure to 2'-FL. Using the upper limit for 2'-FL that we requested to include in the specifications for LNFP-I/2'-FL (see 4b above), please estimate the mean and 90th percentile eaters-only dietary exposure to 2'-FL for infants aged 0 to 6 months and 7 to 12 months, toddlers 1 to 2 years, and for the population 2 years and older.*

b. *Please provide the mean and 90th percentile dietary exposure estimates to LNFP-I/2'-FL (the whole ingredient) from its intended uses in food for the eaters-only subpopulations listed above. The estimates should be based on the lower limit for LNFP-I to account for the worst-case dietary exposure scenario.*

As indicated in the response to Question 4b), it can be theoretically assumed as the worst-case that the ingredient could contain a maximum of 50 w/w% 2'-FL[3]. This means that the maximum theoretical addition level of 2'-FL from LNFP-I/2'-FL is equivalent to the proposed use level of LNFP-I from LNFP-I/2'-FL, and that the maximum theoretical use level of the whole ingredient is double that of LNFP-I.

The resulting maximum theoretical addition levels of 2'-FL and LNFP-I/2'-FL (the whole ingredient), used to calculate the worst-case scenario dietary exposures, are provided by food use in Table 6 below.

Table 6 Maximum Theoretical Use Levels of 2'-FL and LNFP-I/2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	Proposed Use Level of LNFP-I (g/kg or g/L)	Maximum theoretical Use Level of 2'-FL (g/kg or g/L)[a]	Maximum Theoretical Use Level of LNFP-I/2'-FL (g/kg or g/L)[b]
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	2.0	2.0	4.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.0	1.0	2.0
Infant and Toddler Foods	Term Infant Formulas	1.5	1.5	3.0
	Toddler Formulas[d]	1.5	1.5	3.0
	Other Baby Foods for Infants and Young Children	8.33	8.33	16.66
	Other Drinks for Young Children	1.5	1.5	3.0
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	20.0	20.0	40.0

[3] Assuming 50 w/w % LNFP-I, the minimum specification limit for LNFP-I [calculation: 100 % - 50 % LNFP-I = 50 % 2'-FL].

Table 6 Maximum Theoretical Use Levels of 2'-FL and LNFP-I/2'-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	Proposed Use Level of LNFP-I (g/kg or g/L)	Maximum theoretical Use Level of 2'-FL (g/kg or g/L)[a]	Maximum Theoretical Use Level of LNFP-I/2'-FL (g/kg or g/L)[b]
	Cereal and Nutrition Bars	20.0	20.0	40.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	1.0	1.0	2.0
Milk Products	Buttermilk*	1.5	1.5	3.0
	Flavored Milk	1.5	1.5	3.0
	Milk-Based Meal Replacement and Nutritional Beverages[c]	2.0	2.0	4.0
	Yogurt Drinks, Probiotic Drinks	1.5	1.5	3.0
	Yogurt*	10.0	10.0	20.0
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.0	1.0	2.0

LNFP-I/2'-FL = Lacto-*N*-fucopentaose-I/2'- Fucosyllactose; 2'-FL = 2'- Fucosyllactose; LNFP-I = Lacto-N-fucopentaose I; CFR = Code of Federal Regulations; U.S. = United States.

* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[a] Assuming 50% 2'-FL as a worst-case, based on the minimum LNFP-I specification limit (50 w/w %). The resulting maximum theoretical use level of 2'-FL is equivalent to the proposed use level of LNFP-I from LNFP-I/2'-FL.

[b] Assuming the ingredient is composed of 50% LNFP-I and 50% 2'-FL as a worst-case, the resulting maximum theoretical use level of LNFP-I/2'-FL (the whole ingredient) is double the proposed use level of LNFP-I from LNFP-I/2'-FL.

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

[d] Formula products targeted toward young children (> 12 months of age)

Part a)

As described above, LNFP-I/2'-FL could theoretically be comprised of 50% LNFP-I (lower limit specification for LNFP-I) and 50% 2'-FL (theoretical upper limit specification for 2'-FL). Not only does this mean that the maximum theoretical addition level of 2'-FL is equivalent to the proposed use level of LNFP-I from 2'-FL/LNFP-I, but also that the worst-case estimated daily intakes of 2'-FL would be the same as those calculated for LNFP-I (section 3.2.2 of the notice). The consumer-only results are reproduced in Table 7 below for the population groups requested but are expressed on a 2'-FL basis.

Table 7 Summary of the Estimated Daily Intake of 2'-FL from Proposed Food Uses of LNFP-I/2'-FL in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 m	76.2	139	1.50	2.88	229	388
Infants	7 to < 12 m	97.5	122	2.56	4.76	285	533
Toddlers	1 to 2 y	99.0	300	0.86	1.58	71	132

Table 7 Summary of the Estimated Daily Intake of 2'-FL from Proposed Food Uses of LNFP-I/2'-FL in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Total Population	2 y and older	89.8	5,523	0.76	1.66	13	29

2'-FL = 2'-Fucosyllactose; LNFP-I/2'-FL = Lacto-N-fucopentaose-I/2'- Fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

It is highly unlikely that the LNFP-I/2'-FL ingredient would contain 50 w/w % 2'-FL due to the presence of other carbohydrate by-products, water, and ash. Despite this theoretical worst-case approach, intakes resulting from the maximum theoretical use level of 2'-FL from the intended use of LNFP-I/2'-FL (assuming 50% 2'-FL) would not exceed the highest estimated daily intakes of 2'-FL that have been notified as GRAS in the U.S. for similar food uses (see GRNs 546 and 650). Notably, the highest estimated daily intake of 2'-FL from all proposed food uses of up to 987.1 mg/kg body weight/day (8.36 g/day) at the 90th percentile in infants 7 to 12 months was within the established safe background dietary range of 2'-FL intakes from mature human breast milk (GRN 546).

Part b)

If LNFP-I/2'-FL could theoretically be comprised of 50% LNFP-I (lower limit specification for LNFP-I) and 50% 2'-FL (theoretical upper limit specification for 2'-FL), resulting in the same amount of 2'-FL added as LNFP-I, then the the worst-case estimated daily intakes of LNFP-I/2'-FL (the whole ingredient) would be double those calculated for LNFP-I (section 3.2.2 of the notice). Hence, the consumer-only results of LNFP-I (or 2'-FL above) were multiplied by 2 and are expressed on a LNFP-I/2'-FL basis in Table 8 below.

Table 8 Summary of the Estimated Daily Intake of LNFP-I/2'-FL from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 m	76.2	139	3.00	5.76	458	776
Infants	7 to < 12 m	97.5	122	5.12	9.52	570	1,066
Toddlers	1 to 2 y	99.0	300	1.72	3.16	142	264
Total Population	2 y and older	89.8	5,523	1.52	3.32	26	58

LNFP-I/2'-FL = Lacto-N-fucopentaose-I/2'- Fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

FDA.10. *Given the upper and lower limits for 2'-FL, please provide a statement that if LNFP-I/2'-FL meeting these specifications is used in infant formula or formula for young children (>12 months) in combination with other ingredients containing 2'-FL, the use levels of 2'-FL-containing ingredients will be adjusted accordingly to ensure that the total amount of 2'-FL will not exceed the level of 2.4 g/L currently concluded to be GRAS for use in infant formula and formula for young children.*

The purpose of adding LNFP-I/2'-FL – and other human-identical milk oligosaccharides – to infant formula and formula for young children is to produce formula products that are compositionally representative of human breast milk. To this end, 2'-FL from LNFP-I/2'-FL is partially substitutional to other sources of 2'-FL that have GRAS status for use in formula products (e.g., GRN 546, 571, 650, 735, 749, 852, 897, 932), and thus may be added together with other sources of 2'-FL to achieve the maximum GRAS use level of 2.4 g/L. Glycom notes that all infant formulas marketed in the U.S. must meet federal nutrient requirements and infant formula manufacturers must notify the FDA prior to marketing a new formula. Under Section 412(d)(1) of the Federal Food, Drug, and Cosmetic Act (FFDCA), a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula, including a listing of each new or changed ingredient. Accordingly, the manufacturer will need to provide the Agency with assurance that each ingredient meets the requirements of § 106.40(a), including any ingredient that is GRAS for its intended use. It is therefore Glycom's view that existing regulations governing the pre-market clearance requirements for infant formula in the United States are sufficient to ensure that the total amount of 2'-FL will not exceed the level of 2.4 g/L currently concluded to be GRAS for use in infant formula and formula for young children.

FDA.11. *On page 34 of the notice, Glycom states: "Glycom has selected a 1.5 g LNFP-I/L intended use level in infant formula that falls within the 95th percentile of mean values. This approach ensures that the majority of infants will be provided with concentrations that fall within the population means and is well below the upper ranges of concentrations that have been reported for human milk samples, therefore, ensuring that the intended use levels are safe and well tolerated by all infants."*

However, we note the following:

a. *Published peer-reviewed systematic reviews of human milk oligosaccharide (HMO) levels in human milk report mean and median concentrations of LNFP-I that are significantly below the proposed use level of 1.5 g/L. For example, Thurl et al., 2017 compiled data from secretor mothers and reported LNFP-I levels of 0.82 g/L and 0.83 g/L on lactation days 30-60 and 60-100, respectively. Furthermore, Soyyilmaz et al., 2021 examined HMO concentrations in pooled human milk samples of mature milk (days 15-90) and reported median and "mean of means" LNFP-I levels of 0.93 g/L and 0.83 g/L, respectively.*

b. *While Glycom provided data from a number of publications in Appendix A, it was not clear how the dataset was analyzed to make the conclusion that 1.5 g LNFP-I/L falls with the 95th percentile of mean values, especially given that the dataset appeared to have not been stratified by period of lactation.*

c. *Soyyilmaz et al., 2021 also estimated the mean total HMO fraction to be 11.3 g/L in mature milk (days 15-90) using data from 12 publications. Summing the mean of means from their dataset for the individual HMOs (days 15-90) resulted in a total HMO level that approximated (or was slightly lower than) the mean total HMO fraction of 11.3 g/L. This indicates that the majority of the HMO fraction is comprised of a relatively low number of individual HMOs. For these high abundance HMOs, it would seem that using levels much higher than the estimated mean of means would result in a summed total of HMO levels far greater than the average total HMO fraction determined empirically (assuming these HMOs were to be added in combination).*

d. *To our knowledge none of the published studies reported quantified and tracked levels of HMOs from a single mother-infant dyad throughout the period of lactation. Given that the general trend is for HMO levels, including LNFP-I, to decline throughout lactation (i.e. Soyyilmaz et al. 2021; Gu et al., 2021), it is plausible, due to lack of empirical evidence, that a mother who produced milk with high levels of LNFP-I during the sampling period may not necessarily produce the same high levels of LNFP-I throughout the entire period of lactation. Thus, from the available data, it is not clear any particular infant would be continually exposed to high levels of LNFP-I (i.e., almost twice the levels of the reported means) as proposed in this GRAS notice.*

e. *Unlike for 2'-FL, there are no clinical studies indicating that constant levels of LNFP-I (including at the proposed use level of 1.5 g/L) 2 are safe and tolerable for all infants 0-6 months old when added to infant formula.*

When taken together, it is not clear whether a proposed use level of LNFP-I, which is significantly higher than the mean, mean of means, and median from published systematic reviews on HMO levels

in human milk, would be considered generally recognized as safe for all infants consuming infant formula as a sole source of nutrition from 0-6 months. Given that the intended use level is not based on the reported mean, mean of means, or median from the published systematic reviews by Thurl et al., 2017 and/or Soyyilmaz et al., 2021, please provide a rationale discussing why the intended use level is safe for young infants based on evidence considered generally available and generally accepted.

The rationale on the safety of the intended use level of 1.5 g LNFP-I/L in infant formula, taking into consideration the natural variation of LNFP-I, is discussed by point below.

Part a)

The two systematic reviews Thurl *et al*. (2017) and Soyyilmaz *et al*. (2021) focused their analyses on providing representative and reliable mean concentrations of HMOs in either milk samples of the sub-population of Secretor mothers (representing most mothers) or an entire population approach ("pooled Secretor and Non-Secretor milk"), respectively. In Glycom's opinion both approaches provide valuable guidance on setting appropriate use levels of individual HMOs in infant formula products by infant formula manufacturers.

In Glyom's view, however, reliable mean concentrations (mean of means) are without doubt generally recognized as safe – and are thus a highly conservative basis of safety – but are not the limiting upper levels that can be generally recognized as safe.

A less conservative and yet justified basis of safety should, in Glycom's opinion, consider two additional aspects:

- The observed **variability** of HMO levels across populations (information that is lost by a mean of means approach); and
- That the basis of safety can be established for all lactation phases by the **highest exposure on body weight basis**.

Regarding these two aspects:

Variability: it appears unreasonable to assume that milk of different mothers would be incompatible between infants as the long history of wet nursing would also strongly suggest. On the contrary the compatibility of milk from different mothers between infants can probably be considered as generally recognized as safe and may be subject to criticism if presumed otherwise. The scientific basis, however, for this historic observation is that there is no genetic link between the HMO composition of a mother and her infant. Many publications (including the systematic reviews referred to above) have established that the main driver of HMO composition of milk are the polymorphisms observed in the population for the genetic factors underlying the Secretor and Lewis phenotypes, which are genes that are inherited in heterozygous fashion from mother and father and hence eliminate a direct link between one mother's HMO composition and her infant. This suggests that variability must be considered when establishing the upper limits of safety. Glycom considers that the 95 % confidence limit (CL) of mean values that is calculated by extrapolation from standard deviations assuming a Gauss distribution forms a reasonable basis of safety. The variability of individual HMO levels has been generally established and is well recognized, as are 95 % confidence levels recognized as these are often reported in publications

evaluating HMO levels in milk (see sections on LNFP-I and 2'-FL Full Data Overview in Appendix A of the notice).

Highest exposure on body weight basis: it has been generally established (and is generally recognized) that the levels of LNFP-I decline over the course of lactation (e.g. by Soyyilmaz et al., 2021). It has also been generally established that highest intake levels of breastmilk on a bodyweight basis occur during lactation days 14-27(EFSA, 2017a)[4]. As highlighted by part b of this question, we acknowledge that the lactational dependency of LNFP-I levels and worst-case milk intakes have not been sufficiently presented and discussed in Appendix A to our initial notice and we want to address these points now in response to question 11b.

Part b)

The dataset provided in Appendix A of the notice for LNFP-I has now been stratified by lactational period following the division into colostrum (0-5 days), transitional milk (6-14 days), mature milk (15-90 days) and late milk (> 90 days). These are similar to the division of phases in the meta-analysis by Hester *et al.* (2012) on the energy and macronutrient composition of human milk cited in the EFSA Scientific Opinion on the safety and suitability for use by infants of follow-on formulae with a protein content of at least 1.6 g/100 kcal(EFSA, 2017b).[5] In addition to these, the highest-intake phase of milk on a body weight basis (14-27 days) has also been included. The lactation phases evaluated are summarized in Table 9, and the levels of LNFP-I in Secretor milk by lactation phase (as well as a summary across all phases) are presented in the Tables that follow.

Table 9 Lactation Phases Evaluated

Lactation Phase Letter	Lactation Phase Descriptor	Lactation Days
A	Colostrum	0-5
B	Transitional milk	6-14
C	Mature milk	15-90
D	Late milk	> 90
E	Highest intake	14-27

[4] See page 3 of the Opinion: *"The EFSA SC concluded that high infant formula consumption per body weight is derived from 95th percentile consumption with the first weeks of life being the time of the **highest relative consumption on a body weight basis**."*

[5] To highlight the decline of LNFP-I levels over lactation the initial mature phase was sub-divided into 15-90 days and late milk (> 90 days).

Lactation Phase A (Colostrum)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	4	4	1,4	0,2	1,7
19	Thurl	Br. J. Nutr.	2010	21	Europe	3	3	2,0	n.a.	n.a.
23	Gabrielli	Pediatr.	2011	42	Europe	4	4	2,0	0,9	3,8
40	Aakko	Benef. Microbes	2017	11	Europe	1	4	1,8	0,6	3,1
57	Wu	Curr. Dev. Nutr.	2020	25	Asia	3	3	1,4	0,1	1,6
							MEAN	**1,7**	**0,4**	**2,5**
							MEDIAN	**1,8**	**0,4**	**2,4**
							MAX	**2,0**	**0,9**	**3,8**
							COUNT	**5**	**4**	**4**

Lactation Phase B (Transitional Milk)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	10	10	1,4	0,2	1,8
19	Thurl	Br. J. Nutr.	2010	19	Europe	8	8	2,3	n.a.	n.a.
23	Gabrielli	Pediatr.	2011	42	Europe	10	10	2,1	1,3	4,6
37	Kunz	JPGN	2017	32	Europe	8	15	1,3	n.a.	1,6
47	Austin	Nutrients	2019	78	Europe	7	14	1,7	0,6	2,9
48	McJarrow	Nutrients	2019	41	Asia	5	15	2,1	0,8	3,6
57	Wu	Curr. Dev. Nutr.	2020	27	Asia	7	7	1,5	0,1	1,7
							MEAN	**1,8**	**0,6**	**2,7**
							MEDIAN	**1,7**	**0,6**	**2,4**
							MAX	**2,3**	**1,3**	**4,6**
							COUNT	**7**	**5**	**6**

Lactation Phase C (Mature Milk)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	30	90	1,1	0,5	2,1
19	Thurl	Br. J. Nutr.	2010	69	Europe	15	90	1,4	n.a.	n.a.
22	Coppa	JPGN	2011	16	Europe	25	35	1,2	0,3	1,8
23	Gabrielli	Pediatr.	2011	42	Europe	20	30	1,7	1,1	3,8
30	Hong	Anal. Chem.	2014	10	N. Amer.	35	35	0,5	0,2	1,0
37	Kunz	JPGN	2017	32	Europe	16	60	1,1	n.a.	1,7
47	Austin	Nutrients	2019	267	Europe	21	84	0,9	0,4	1,7
59	Tonon	Nutrients	2019	77	LATAM	25	46	0,9	0,6	2,2
50	Lagström	Am. J. Clin. Nutr.	2020	699	Europe	90	90	1,1	n.a.	1,6
53	Ferreira	Nutrients	2020	75	LATAM	28	50	1,3	n.a.	2,0
57	Wu	Curr. Dev. Nutr.	2020	50	Asia	21	77	0,8	0,1	0,9
58	Saben	Nutrients	2020	35	N. Amer.	60	60	1,0	n.a.	1,6
							MEAN	**1,1**	**0,5**	**1,9**
							MEDIAN	**1,1**	**0,4**	**1,7**
							MAX	**1,7**	**1,1**	**3,8**
							COUNT	**12**	**7**	**11**

Lactation Phase D (Late Milk)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
44	Azad	J. Nutr.	2018	307	N. Amer.	90	120	1,0	0,8	2,6
47	Austin	Nutrients	2019	30	Europe	98	112	0,4	0,3	0,9
48	McJarrow	Nutrients	2019	40	Asia	180	180	0,7	0,5	1,6
56	Larsson	Front Pediatr.	2019	40	Europe	150	270	0,6	n.a.	1,0
57	Wu	Curr. Dev. Nutr.	2020	7	Asia	168	168	0,4	0,1	0,6
							MEAN	**0,6**	**0,4**	**1,3**
							MEDIAN	**0,6**	**0,4**	**1,0**
							MAX	**1,0**	**0,8**	**2,6**
							COUNT	**5**	**4**	**5**

Lactation phases A to D (all data)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	4	4	1,4	0,2	1,7
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	10	10	1,4	0,2	1,8
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	30	90	1,1	0,5	2,1
19	Thurl	Br. J. Nutr.	2010	21	Europe	3	3	2,0	n.a.	n.a.
19	Thurl	Br. J. Nutr.	2010	19	Europe	8	8	2,3	n.a.	n.a.
19	Thurl	Br. J. Nutr.	2010	69	Europe	15	90	1,4	n.a.	n.a.
22	Coppa	JPGN	2011	16	Europe	25	35	1,2	0,3	1,8
23	Gabrielli	Pediatr.	2011	42	Europe	4	4	2,0	0,9	3,8
23	Gabrielli	Pediatr.	2011	42	Europe	10	10	2,1	1,3	4,6
23	Gabrielli	Pediatr.	2011	42	Europe	20	30	1,7	1,1	3,8
30	Hong	Anal. Chem.	2014	10	N. Amer.	35	35	0,5	0,2	1,0
37	Kunz	JPGN	2017	32	Europe	8	15	1,3	n.a.	1,6
37	Kunz	JPGN	2017	32	Europe	16	60	1,1	n.a.	1,7
40	Aakko	Benef. Microbes	2017	11	Europe	1	4	1,8	0,6	3,1
44	Azad	J. Nutr.	2018	307	N. Amer.	90	120	1,0	0,8	2,6
47	Austin	Nutrients	2019	78	Europe	7	14	1,7	0,6	2,9
47	Austin	Nutrients	2019	267	Europe	21	84	0,9	0,4	1,7
47	Austin	Nutrients	2019	30	Europe	98	112	0,4	0,3	0,9
48	McJarrow	Nutrients	2019	41	Asia	5	15	2,1	0,8	3,6
48	McJarrow	Nutrients	2019	40	Asia	180	180	0,7	0,5	1,6
59	Tonon	Nutrients	2019	77	LATAM	25	46	0,9	0,6	2,2
56	Larsson	Front Pediatr.	2019	40	Europe	150	270	0,6	n.a.	1,0
50	Lagström	Am. J. Clin. Nutr.	2020	699	Europe	90	90	1,1	n.a.	1,6
53	Ferreira	Nutrients	2020	75	LATAM	28	50	1,3	n.a.	2,0
57	Wu	Curr. Dev. Nutr.	2020	25	Asia	3	3	1,4	0,1	1,6
57	Wu	Curr. Dev. Nutr.	2020	27	Asia	7	7	1,5	0,1	1,7
57	Wu	Curr. Dev. Nutr.	2020	50	Asia	21	77	0,8	0,1	0,9
57	Wu	Curr. Dev. Nutr.	2020	7	Asia	168	168	0,4	0,1	0,6
58	Saben	Nutrients	2020	35	N. Amer.	60	60	1,0	n.a.	1,6
							MEAN	**1,3**	**0,5**	**2,1**
							MEDIAN	**1,3**	**0,4**	**1,7**
							MAX	**2,3**	**1,3**	**4,6**
							COUNT	**29**	**20**	**26**

Lactation Phase E (Highest Milk Intakes)

#	First author	Journal	Year	No Samples	Regions	Lower day sampl.	Upper day sampl.	MEAN (g/L)	SD (g/L)	MEAN +2SD (g/L)
19	Thurl	Br. J. Nutr.	2010	47	Europe	15	30	1,6	n.a.	n.a.
22	Coppa	JPGN	2011	16	Europe	25	35	1,2	0,3	1,8
23	Gabrielli	Pediatr.	2011	42	Europe	20	20	1,8	1,2	4,1
47	Austin	Nutrients	2019	117	Europe	28	28	1,2	0,5	2,3
57	Wu	Curr. Dev. Nutr.	2020	25	Asia	21	21	1,1	0,1	1,3
							MEAN	**1,4**	**0,5**	**2,4**
							MEDIAN	**1,2**	**0,4**	**2,0**
							MAX	**1,8**	**1,2**	**4,1**
							COUNT	**5**	**4**	**4**

Summary Across All Phases

Lactation phase	PARAMETER	MEAN [g/L]	SD [g/L]	MEAN +2SD [g/L]
A	MEAN	**1,7**	**0,4**	**2,5**
	MEDIAN	**1,8**	**0,4**	**2,4**
	MAX	**2,0**	**0,9**	**3,8**
	COUNT	**5**	**4**	**4**
B	MEAN	**1,8**	**0,6**	**2,7**
	MEDIAN	**1,7**	**0,6**	**2,4**
	MAX	**2,3**	**1,3**	**4,6**
	COUNT	**7**	**5**	**6**
C	MEAN	**1,1**	**0,5**	**1,9**
	MEDIAN	**1,1**	**0,4**	**1,7**
	MAX	**1,7**	**1,1**	**3,8**
	COUNT	**12**	**7**	**11**
D	MEAN	**0,6**	**0,4**	**1,3**
	MEDIAN	**0,6**	**0,4**	**1,0**
	MAX	**1,0**	**0,8**	**2,6**
	COUNT	**5**	**4**	**5**
A-D	MEAN	**1,3**	**0,5**	**2,1**
	MEDIAN	**1,3**	**0,4**	**1,7**
	MAX	**2,3**	**1,3**	**4,6**
	COUNT	**29**	**20**	**26**
E	MEAN	**1,4**	**0,5**	**2,4**
	MEDIAN	**1,2**	**0,4**	**2,0**
	MAX	**1,8**	**1,2**	**4,1**
	COUNT	**5**	**4**	**4**
All DATA in g/L (apart from count)				

Glycom acknowledges that the analysis presented herein has not been published to date. However, Glycom considers the basis for GRAS of the proposed use level of 1.5 g LNFP-I/L in infant formula as being provided by the milk levels extracted from a large number of individual publications.

The stratification by lactation phase is corroborative but allows several additional observations on the distribution of LNFP-I concentrations in Secretor milk over time:

A) Colostrum (0-5 days):
- mean values range from 1.4 to 2.0 g/L (mean of means at 1.7 g/L)
- 95 % CL range from 1.6 to 3.8 g/L (mean of 95 CL % at 2.5 g/L)
- 5 peer-reviewed publications support this data

B) Transitional milk (6-14 days):
- mean values range from 1.3 to 2.3 g/L (mean of means at 1.8 g/L)
- 95 % CL range from 1.6 to 4.6 g/L (mean of 95 CL % at 2.7 g/L)
- 7 peer-reviewed publications support this data

C) Mature milk (15-90 days):
- mean values range from 0.5 to 1.7 g/L (mean of means at 1.1 g/L)
- 95 % CL range from 0.9 to 3.8 g/L (mean of 95 CL % at 1.9 g/L)
- 12 peer-reviewed publications support this data

D) Late milk (>90 days)
- mean values range from 0.4 to 1.0 g/L (mean of means at 0.6 g/L)
- 95 % CL range from 0.6 to 2.6 g/L (mean of 95 CL % at 1.3 g/L)
- 5 peer-reviewed publications support this data

E) Highest milk intake (14-27 days):
- mean values range from 1.1 to 1.8 g/L (mean of means at 1.4 g/L)
- 95 % CL range from 1.3 to 4.1 g/L (mean of 95 CL % at 2.4 g/L)
- 5 peer-reviewed publications support this data

The proposed use level of LNFP-I in infant formula of 1.5 g/L falls well within (or even below) the **range of 95 % CL** for *each* of the analyzed lactation periods. It even falls below the **mean of the 95 % CL** for all lactational phases analyzed.

Glycom would like to highlight that the proposed use level of LNFP-I in infant formula (at 1.5 g/L) does not intend to reflect the *upper* safe level – it is merely meant to reflect a conservative safe level. In Glycom's view there is a conservative margin between the proposed use level and what may become generally recognized as the upper safe level in the future, such as for example the **mean of 95 % CL** from the highest intake per body weight period (2.4 g/L on the basis of data summarized herein).

<u>Part c)</u>

We acknowledge that an approach that would base the safety of individual HMOs on the 95 % CL would meet limitations when a large number of the most abundant HMOs in human milk are combined at which point the total combined HMO level may approach or exceed that of human milk. Such theoretical

scenarios would warrant careful assessment that is not within the scope of our notice. We would also like to point out that such a theoretical scenario remains far from current commercial reality. Nevertheless, additional interpretation of the analysis by Soyyilmaz *et al.* (2021) on total HMOs is provided below.

A similar approach was used by Soyyilmaz *et al.* (2021) for the statistical analysis of total HMOs as for individual HMOs. The concentration of total HMOs in mature milk (lactation days 15 to 90) of 11.3 g/L reported by Soyyilmaz *et al.* (2021) is representative of the mean of means concentration from 12 articles reporting total HMO concentrations (see Supplementary Table S2). Likewise, minimum and maximum concentrations of total HMOs in mature milk reported by Soyyilmaz *et al.* (2021) in Table 8 of the publication, ranging from 8.6 to 16.8 g/L, are representative of minimum and maximum mean concentrations from the 12 articles reporting total HMO concentrations (see Supplementary Table S2).

Minimum and maximum mean concentrations of individual HMOs from mature milk obtained from Supplementary Table S2 of Soyyilmaz *et al.* (2021) are summed in Table 10 below. The sum of maximum mean concentrations of individual HMOs (23.755 g/L) from 57 publications is greater than the maximum mean concentration of total HMOs (16.8 g/L) from 12 publications. Still, the maximum proposed use level of 1.5 g LNFP-I/L of infant formula is much lower than the maximum mean level of LNFP-I from pooled mature human milk (up to 2.14 g/L) reported by Soyyilmaz *et al.* (2021).

Table 10 Sum of the Minimum Mean and Maximum Mean Concentrations from Mature Milk for Individual HMOs from the Soyyilmaz *et al.* (2021) Dataset[a]

Individual HMO	Minimum Mean	Maximum Mean
2'-FL	0.69	4.28
LNDFH-I (DF-LNT)	0.005	2.53
LNFP-I	0.161	2.14
LNFP-II	0.022	1.814
LNT	0.2	1.6
3-FL	0.16	1.9
6'-SL	0	0.736
DSLNT	0	1.122
LNnT	0.065	1.24
LDFT (DFL)	0.04	0.54
FDS-LNH	0.08	0.666
LNFP-III	0.021	0.89
3'-SL	0	0.7
LST c	0	0.3
TF-LNH	0.035	0.394
DS-LNH	0.055	0.23
F-LNH-III	0.109	0.128
F-LNH-II	0.007	0.34
DF-LNH I (a)	0	0.389
LST b	0	0.256
LNFP-V	0.002	0.28

Table 10 **Sum of the Minimum Mean and Maximum Mean Concentrations from Mature Milk for Individual HMOs from the Soyyilmaz *et al.* (2021) Dataset[a]**

Individual HMO	Minimum Mean	Maximum Mean
LNDFH-II	0.016	0.235
LNH	0.006	0.16
S-LNFP II	0.013	0.079
F-LNH-I (MFLNH I)	0	0.14
DF-LNH II (b)	0.051	0.051
F-para-LNH I	0.049	0.049
DF-LNH III (c)	0.008	0.056
LNnH	0.01	0.17
DF-pLNnH	0.041	0.041
LST a	0	0.072
LNFP-VI	0.017	0.017
FSL (3'S-3'FL)	0	0.119
pLNH	0.009	0.009
6'SLN	0.005	0.006
pLNnH	0	0.02
Sum	**1.877**	**23.699**

[a] Values were obtained from supplementary Table S2, 'Mature' tab.

Part d)

Indeed, both Thurl *et al.* (2017) and Soyyilmaz *et al.* (2021) determined that the levels of LNFP-I in human milk decreased over the course of lactation, the former reporting that the concentration of LNFP-I decreased by approximately 2-fold. In addition to temporal changes in HMO concentrations observed throughout lactation, many other factors affect the composition of human milk. For example, the levels of fucosylated HMOs in human milk are strongly determined by maternal genetics, namely the Secretor genotype (expression of the *FUT2* gene) and Lewis phenotype (expression of the *FUT3* gene). The levels of LNFP-I have previously been reported to be very low in non-secretor mothers (Hong *et al.*, 2014; van Niekerk *et al.* 2014). Importantly, Soyyilmaz *et al.* (2021) evaluated published data from pooled human milk samples, and thus included data for milk samples from secretor and non-secretor mother's producing high and low levels of LNFP-I.

As established above, lactation stage and maternal genetics are two important contributors to the variability of LNFP-I concentrations in human milk. The proposed use level of LNFP-I in infant formula of 1.5 g/L is within the 95 % CL range for mean values of LNFP-I in Secretor milk across all stages on lactation. The composition of human milk, as well as the safety of the levels of an HMO such as LNFP-I in human milk, are not directly linked between mother-infant dyads. Rather, the production of various milk components including HMOsis linked to the evolutionary history of the expression of genes in the mammary gland during lactation, which has been described in different populations (Ferrer-Admetlla *et al.*, 2009; Oftedal, 2012; Williams *et al.*, 2021). Furthermore, wet nursing was historically the safest and most common alternative to own mother's milk prior to the introduction of infant formula as an alternative milk source (Stevens *et al.*, 2009). Even today in premature infants, a vulnerable population group, if own mother's milk is not available or is not available in sufficient quantity, pasteurized donor

milk from a donor human milk bank is still generally recommended (Moro *et al.*, 2015). Therefore, there is no indication of a safety concern in infants exposed to higher levels of LNFP-I (within the mean range naturally occuring in human milk) compared to own mother's milk.

Part e)

Human milk is the reference standard for infant nutrition. As such, the GRAS determination of HiMOs for use in infant formula has been historically based on the range of naturally occuring levels of corresponding HMOs in human milk. In several cases, a GRAS determination was obtained in the absence of clinical studies conducted in infants directly evaluating the HiMO (3-FL: GRNs 925 and 951; 3'-SL: GRNs 766, 880, and 921; 6'-SL: GRNs 881 and 922; LNT: GRNs 833 and 923).

FDA.12. *In section 6.2 on page 42 of the notice, Glycom did not indicate the end date of its literature search. We also note that two recent systematic reviews (Zhou et al., 2021 and Soyyilmaz et al., 2021) that discuss HMO levels in human milk were not cited in this GRAS notice. Given that Glycom did not cite Zhou et al. 2021 and Soyyilmaz et al. 2021, which appear relevant to the safety assessment for this ingredient, please provide the complete dates of Glycom's literature search and the search criteria used to ensure that Glycom's search was complete.*

We thank the U.S. FDA for bringing to our attention the two recent systematic reviews evaluating HMO levels in human milk (Zhou *et al.*, 2021 and Soyyilmaz *et al.*, 2021). These studies were not identified in Glycom's literature searches as they were published after the end date of the literature searches, as detailed below.

The literature search indicated in section 6.2 on page 42 of the notice identified studies reporting on relevant safety outcomes for LNFP-I and 2'-FL. For LNFP-I, the literature search included all studies published until July 22nd, 2020 (with no other date restrictions), whereas for 2'-FL the literature search included studies published from May 01, 2019 (the publication date for the EFSA Scientific Opinion on the safety of 2' FL/DFL as a novel food[6]) to July 22nd, 2020. The literature search end date aligns with the EU novel food application for LNFP-I/2'-FL, but was not updated prior to the submission of the GRAS notice to the U.S. FDA. We apologize for this oversight, and can conduct an updated literature search on safety-related studies upon request.

A separate literature search was conducted to identify studies reporting on HMO concentrations in human milk published between 1993 and December 2020, as indicated in section 1.5.1 of Appendix A of the GRAS notice.

Mean HMO levels in pooled milk from 31 countries reported in the systematic review by Soyyilmaz *et al.* (2021) have been addressed in the response to Question 11. While we acknowledge the relevance of systematic review by Zhou *et al.* (2021) to the safety assessment of this ingredient, it addresses HMO concentrations in the Chinese population only. We consider Glycom's analysis of LNFP-I levels in

[6] https://www.efsa.europa.eu/en/efsajournal/pub/5717

Secretor milk from different populations including North America (Appendix A of the notice) to be more relevant in establishing the maximum safe use level of LNFP-I in food.

FDA.13. *If LNFP-I/2'-FL is to be used in combination with other HMOs, specifically 2'-FL, it is unclear from the notice if the use level/dietary exposure to 2'-FL will be additive or will be adjusted to reflect the current maximum notified use level of 2.4 g/L of 2'-FL in infant formula. Please indicate whether Glycom expects the cumulative dietary exposure to 2'-FL from the intended uses in infant formula to increase above the dietary exposure from the current uses.*

As indicated in the response to Question 10, LNFP-I/2'-FL may be supplemented with other sources of 2'-FL to achieve the total amount of 2'-FL currently concluded to be GRAS for use in infant formula and formula for young children (*i.e.*, 2.4 g/L). As such, Glycom does not expect the cumulative dietary exposure to 2'-FL from the intended uses in infant formula to increase above the dietary exposure from the current uses.

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We hope this information adequately addresses the Agency's questions on GRN 001035, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
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Maryse Darch
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Glycom A/S

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Representative Food Codes for Foods for Infants and Young Children from Proposed Food Uses of LNFP-I/2'FL in the U.S. (2017-2018 NHANES Data)

Infant and Toddler Foods

Term Infant Formulas

11710000	Infant formula, NFS
11710050	Infant formula, NS as to form (Similac Expert Care Alimentum)
11710051	Infant formula, ready-to-feed (Similac Expert Care Alimentum)
11710053	Infant formula, powder, made with water, NFS (Similac Expert Care Alimentum)
11710054	Infant formula, powder, made with tap water (Similac Expert Care Alimentum)
11710055	Infant formula, powder, made with plain bottled water (Similac Expert Care Alimentum)
11710056	Infant formula, powder, made with baby water (Similac Expert Care Alimentum)
11710350	Infant formula, NS as to form (Similac Advance)
11710351	Infant formula, ready-to-feed (Similac Advance)
11710352	Infant formula, liquid concentrate, made with water, NFS (Similac Advance)
11710353	Infant formula, powder, made with water, NFS (Similac Advance)
11710354	Infant formula, liquid concentrate, made with tap water (Similac Advance)
11710355	Infant formula, liquid concentrate, made with plain bottled water (Similac Advance)
11710356	Infant formula, liquid concentrate, made with baby water (Similac Advance)
11710357	Infant formula, powder, made with tap water (Similac Advance)
11710358	Infant formula, powder, made with plain bottled water (Similac Advance)
11710359	Infant formula, powder, made with baby water (Similac Advance)
11710360	Infant formula, NS as to form (Similac Advance Organic)
11710361	Infant formula, ready-to-feed (Similac Advance Organic)
11710363	Infant formula, powder, made with water, NFS (Similac Advance Organic)
11710367	Infant formula, powder, made with tap water (Similac Advance Organic)
11710368	Infant formula, powder, made with plain bottled water (Similac Advance Organic)
11710369	Infant formula, powder, made with baby water (Similac Advance Organic)
11710370	Infant formula, NS as to form (Similac Sensitive)
11710371	Infant formula, ready-to-feed (Similac Sensitive)
11710372	Infant formula, liquid concentrate, made with water, NFS (Similac Sensitive)
11710373	Infant formula, powder, made with water, NFS (Similac Sensitive)
11710374	Infant formula, liquid concentrate, made with tap water (Similac Sensitive)
11710375	Infant formula, liquid concentrate, made with plain bottled water (Similac Sensitive)
11710376	Infant formula, liquid concentrate, made with baby water (Similac Sensitive)
11710377	Infant formula, powder, made with tap water (Similac Sensitive)
11710378	Infant formula, powder, made with plain bottled water (Similac Sensitive)
11710379	Infant formula, powder, made with baby water (Similac Sensitive)
11710380	Infant formula, NS as to form (Similac for Spit-Up)
11710381	Infant formula, ready-to-feed (Similac for Spit-Up)
11710383	Infant formula, powder, made with water, NFS (Similac for Spit-Up)
11710470	Infant formula, NS as to form (Similac Expert Care NeoSure)

11710471 Infant formula, ready-to-feed (Similac Expert Care NeoSure)
11710473 Infant formula, powder, made with water, NFS (Similac Expert Care NeoSure)
11710477 Infant formula, powder, made with tap water (Similac Expert Care NeoSure)
11710478 Infant formula, powder, made with plain bottled water (Similac Expert Care NeoSure)
11710479 Infant formula, powder, made with baby water (Similac Expert Care NeoSure)
11710620 Infant formula, NS as to form (Enfamil Newborn)
11710621 Infant formula, ready-to-feed (Enfamil Newborn)
11710626 Infant formula, powder, made with water, NFS (Enfamil Newborn)
11710627 Infant formula, powder, made with tap water (Enfamil Newborn)
11710628 Infant formula, powder, made with plain bottled water (Enfamil Newborn)
11710629 Infant formula, powder, made with baby water (Enfamil Newborn)
11710630 Infant formula, NS as to form (Enfamil Infant)
11710631 Infant formula, ready-to-feed (Enfamil Infant)
11710632 Infant formula, liquid concentrate, made with water, NFS (Enfamil Infant)
11710633 Infant formula, liquid concentrate, made with tap water (Enfamil Infant)
11710634 Infant formula, liquid concentrate, made with plain bottled water (Enfamil Infant)
11710635 Infant formula, liquid concentrate, made with baby water (Enfamil Infant)
11710636 Infant formula, powder, made with water, NFS (Enfamil Infant)
11710637 Infant formula, powder, made with tap water (Enfamil Infant)
11710638 Infant formula, powder, made with plain bottled water (Enfamil Infant)
11710639 Infant formula, powder, made with baby water (Enfamil Infant)
11710660 Infant formula, NS as to form (Enfamil A.R.)
11710661 Infant formula, ready-to-feed (Enfamil A.R.)
11710663 Infant formula, powder, made with water, NFS (Enfamil A.R.)
11710664 Infant formula, powder, made with tap water (Enfamil A.R.)
11710665 Infant formula, NS as to form (Enfamil EnfaCare)
11710666 Infant formula, ready-to-feed (Enfamil EnfaCare)
11710667 Infant formula, powder, made with water, NFS (Enfamil EnfaCare)
11710668 Infant formula, powder, made with plain bottled water (Enfamil A.R.)
11710669 Infant formula, powder, made with baby water (Enfamil A.R.)
11710670 Infant formula, NS as to form (Enfamil Gentlease)
11710671 Infant formula, ready-to-feed (Enfamil Gentlease)
11710673 Infant formula, powder, made with water, NFS (Enfamil Gentlease)
11710674 Infant formula, powder, made with tap water (Enfamil EnfaCare)
11710675 Infant formula, powder, made with plain bottled water (Enfamil EnfaCare)
11710676 Infant formula, powder, made with baby water (Enfamil EnfaCare)
11710677 Infant formula, powder, made with tap water (Enfamil Gentlease)
11710678 Infant formula, powder, made with plain bottled water (Enfamil Gentlease)
11710679 Infant formula, powder, made with baby water (Enfamil Gentlease)
11710910 Infant formula, NS as to form (Gerber Good Start Gentle)
11710911 Infant formula, ready-to-feed (Gerber Good Start Gentle)
11710912 Infant formula, liquid concentrate, made with water, NFS (Gerber Good Start Gentle)
11710913 Infant formula, powder, made with water, NFS (Gerber Good Start Gentle)
11710914 Infant formula, liquid concentrate, made with tap water (Gerber Good Start Gentle)
11710915 Infant formula, liquid concentrate, made with plain bottled water (Gerber Good Start Gentle)

11710916 Infant formula, liquid concentrate, made with baby water (Gerber Good Start Gentle)
11710917 Infant formula, powder, made with tap water (Gerber Good Start Gentle)
11710918 Infant formula, powder, made with plain bottled water (Gerber Good Start Gentle)
11710919 Infant formula, powder, made with baby water (Gerber Good Start Gentle)
11710920 Infant formula, NS as to form (Gerber Good Start Protect)
11710923 Infant formula, powder, made with water, NFS (Gerber Good Start Protect)
11710927 Infant formula, powder, made with tap water (Gerber Good Start Protect)
11710928 Infant formula, powder, made with plain bottled water (Gerber Good Start Protect)
11710929 Infant formula, powder, made with baby water (Gerber Good Start Protect)
11710960 Infant formula, NS as to form (Store Brand)
11710961 Infant formula, liquid concentrate, made with water, NFS (Store Brand)
11710962 Infant formula, powder, made with water, NFS (Store Brand)
11710963 Infant formula, ready-to-feed (Store Brand)
11710964 Infant formula, liquid concentrate, made with tap water (Store Brand)
11710965 Infant formula, liquid concentrate, made with plain bottled water (Store Brand)
11710966 Infant formula, liquid concentrate, made with baby water (Store Brand)
11710967 Infant formula, powder, made with tap water (Store Brand)
11710968 Infant formula, powder, made with plain bottled water (Store Brand)
11710969 Infant formula, powder, made with baby water (Store Brand)
11720310 Infant formula, NS as to form (Enfamil ProSobee)
11720311 Infant formula, ready-to-feed (Enfamil ProSobee)
11720312 Infant formula, liquid concentrate, made with water, NFS (Enfamil ProSobee)
11720313 Infant formula, powder, made with water, NFS (Enfamil ProSobee)
11720314 Infant formula, liquid concentrate, made with tap water (Enfamil ProSobee)
11720315 Infant formula, liquid concentrate, made with plain bottled water (Enfamil ProSobee)
11720316 Infant formula, liquid concentrate, made with baby water (Enfamil ProSobee)
11720317 Infant formula, powder, made with tap water (Enfamil ProSobee)
11720318 Infant formula, powder, made with plain bottled water (Enfamil ProSobee)
11720319 Infant formula, powder, made with baby water (Enfamil ProSobee)
11720410 Infant formula, NS as to form (Similac Isomil Soy)
11720411 Infant formula, ready-to-feed (Similac Isomil Soy)
11720412 Infant formula, liquid concentrate, made with water, NFS (Similac Isomil Soy)
11720413 Infant formula, powder, made with water, NFS (Similac Isomil Soy)
11720414 Infant formula, liquid concentrate, made with tap water (Similac Isomil Soy)
11720415 Infant formula, liquid concentrate, made with plain bottled water (Similac Isomil Soy)
11720416 Infant formula, liquid concentrate, made with baby water (Similac Isomil Soy)
11720417 Infant formula, powder, made with tap water (Similac Isomil Soy)
11720418 Infant formula, powder, made with plain bottled water (Similac Isomil Soy)
11720419 Infant formula, powder, made with baby water (Similac Isomil Soy)
11720610 Infant formula, NS as to form (Gerber Good Start Soy)
11720611 Infant formula, ready-to-feed (Gerber Good Start Soy)
11720612 Infant formula, liquid concentrate, made with water, NFS (Gerber Good Start Soy)
11720613 Infant formula, powder, made with water, NFS (Gerber Good Start Soy)
11720614 Infant formula, liquid concentrate, made with tap water (Gerber Good Start Soy)
11720615 Infant formula, liquid concentrate, made with plain bottled water (Gerber Good Start Soy)

11720616 Infant formula, liquid concentrate, made with baby water (Gerber Good Start Soy)
11720617 Infant formula, powder, made with tap water (Gerber Good Start Soy)
11720618 Infant formula, powder, made with plain bottled water (Gerber Good Start Soy)
11720619 Infant formula, powder, made with baby water (Gerber Good Start Soy)
11720800 Infant formula, NS as to form (Store Brand Soy)
11720801 Infant formula, ready-to-feed (Store brand Soy)
11720802 Infant formula, liquid concentrate, made with water, NFS (Store Brand Soy)
11720803 Infant formula, powder, made with water, NFS (Store Brand Soy)
11720807 Infant formula, powder, made with tap water (Store Brand Soy)
11720808 Infant formula, powder, made with plain bottled water (Store Brand Soy)
11720809 Infant formula, powder, made with baby water (Store Brand Soy)
11740310 Infant formula, NS as to form (Enfamil Nutramigen)
11740311 Infant formula, ready-to-feed (Enfamil Nutramigen)
11740312 Infant formula, liquid concentrate, made with water, NFS (Enfamil Nutramigen)
11740313 Infant formula, powder, made with water, NFS (Enfamil Nutramigen)
11740320 Infant formula, NS as to form (PurAmino)
11740323 Infant formula, powder, made with water, NFS (PurAmino)
11740400 Infant formula, NS as to form (Enfamil Pregestimil)
11740401 Infant formula, ready-to-feed (Enfamil Pregestimil)
11740403 Infant formula, powder, made with water, NFS (Enfamil Pregestimil)
11740511 Infant formula, ready-to-feed, low iron (Enfamil Premature 20 Cal)
11740521 Infant formula, ready-to-feed, with iron (Enfamil Premature 20 Cal)

Toddler Formulas

11720430 Infant formula, NS as to form (Similac Expert Care for Diarrhea)
11720431 Infant formula, ready-to-feed (Similac Expert Care for Diarrhea)
11710480 Infant formula, NS as to form (Similac Go and Grow)
11710481 Infant formula, powder, made with water, NFS (Similac Go and Grow)
11710680 Infant formula, NS as to form (Enfamil Enfagrow Toddler Transitions)
11710681 Infant formula, ready-to-feed (Enfamil Enfragrow Toddler Transitions)
11710683 Infant formula, powder, made with water, NFS (Enfamil Enfragrow Toddler Transitions)
11710687 Infant formula, powder, made with tap water (Enfamil Enfagrow Toddler Transitions)
11710688 Infant formula, powder, made with plain bottled water (Enfamil Enfagrow Toddler Transitions)
11710689 Infant formula, powder, made with baby water (Enfamil Enfagrow Toddler Transitions)
11710690 Infant formula, NS as to form (Enfamil Enfagrow Toddler Transitions Gentlease)
11710693 Infant formula, powder, made with water, NFS (Enfamil Enfagrow Toddler Transitions Gentlease)
11710697 Infant formula, powder, made with tap water (Enfamil Enfagrow Toddler Transitions Gentlease)
11710698 Infant formula, powder, made with plain bottled water (Enfamil Enfagrow Toddler Transitions Gentlease)
11710699 Infant formula, powder, made with baby water (Enfamil Enfagrow Toddler Transitions Gentlease)
11710800 Infant formula, NS as to form (PediaSure)
11710801 Infant formula, ready-to-feed (PediaSure)
11710805 Infant formula, with fiber, NS as to form (PediaSure Fiber)
11710806 Infant formula, with fiber, ready-to-feed (PediaSure Fiber)
11710930 Infant formula, NS as to form (Gerber Graduates Gentle)

11710940 Infant formula, NS as to form (Gerber Graduates Protect)
11720320 Infant formula, NS as to form (Enfamil Enfagrow Toddler Transitions Soy)
11720323 Infant formula, powder, made with water, NFS (Enfamil Enfagrow Toddler Transitions Soy)
11720620 Infant formula, NS as to form (Gerber Graduates Soy)

Other Baby Foods for Infants and Young Children

11480010 Yogurt, whole milk, baby food
11480020 Yogurt, whole milk, baby food, with fruit and multigrain cereal puree, NFS
11480030 Yogurt, whole milk, baby food, with fruit and multigrain cereal puree, plus iron
11480040 Yogurt, whole milk, baby food, with fruit and multigrain cereal puree, plus DHA
13310000 Custard pudding, flavor other than chocolate, baby food, NS as to strained or junior
13311000 Custard pudding, baby food, flavor other than chocolate, strained
13312000 Custard pudding, baby food, flavor other than chocolate, junior
20000070 Meat, baby food, NS as to type, NS as to strained or junior
20000090 Meat sticks, baby food, NS as to type of meat
21701000 Beef, baby food, NS as to strained or junior
21701010 Beef, baby food, strained
21701020 Beef, baby food, junior
22810010 Ham, baby food, strained
22820000 Meat stick, baby food
23410010 Lamb, baby food, strained
23420010 Veal, baby food, strained
24701000 Chicken, baby food, NS as to strained or junior
24701010 Chicken, baby food, strained
24701020 Chicken, baby food, junior
24703000 Turkey, baby food, NS as to strained or junior
24703010 Turkey, baby food, strained
24703020 Turkey, baby food, junior
24705010 Chicken stick, baby food
24706010 Turkey stick, baby food
27601000 Beef stew, baby food, toddler
27610100 Beef and egg noodles, baby food, NS as to strained or junior
27610110 Beef and egg noodles, baby food, strained
27610120 Beef and egg noodles, baby food, junior
27610710 Beef with vegetables, baby food, strained
27610730 Beef with vegetables, baby food, toddler
27640050 Chicken and rice dinner, baby food, strained
27640100 Chicken noodle dinner, baby food, NS as to strained or junior
27640110 Chicken noodle dinner, baby food, strained
27640120 Chicken noodle dinner, baby food, junior
27640810 Chicken, noodles, and vegetables, baby food, toddler
27641000 Chicken stew, baby food, toddler
27642100 Turkey, rice and vegetables, baby food, NS as to strained or junior
27642110 Turkey, rice and vegetables, baby food, strained
27642120 Turkey, rice and vegetables, baby food, junior

27642130 Turkey, rice, and vegetables, baby food, toddler
27644110 Chicken soup, baby food
53801000 Cereal bar with fruit filling, baby food
53803050 Cookie, fruit, baby food
53803100 Cookie, baby food
53803250 Cookie, teething, baby
53803300 Cookie, rice, baby
54350000 Crackers, baby food
54350010 Gerber Finger Foods, Puffs, baby food
54350020 Finger Foods, Puffs, baby food
54360000 Crunchy snacks, corn based, baby food
54408100 Pretzel, baby food
56210000 Cereal, nestum
57820000 Cereal, baby food, jarred, NFS
57820100 Rice cereal, baby food, jarred, NFS
57822000 Mixed cereal with applesauce and bananas, baby food, jarred
57823000 Oatmeal with applesauce and bananas, baby food, jarred
57824000 Rice cereal with applesauce and bananas, baby food, jarred
57824500 Rice cereal with mixed fruit, baby food, jarred
57830100 Gerber Graduates Finger Snacks Cereal, baby food
58503000 Macaroni, tomatoes, and beef, baby food, NS as to strained or junior
58503010 Macaroni, tomatoes, and beef, baby food, strained
58503020 Macaroni, tomatoes, and beef, baby food, junior
58503050 Macaroni with beef and tomato sauce, baby food, toddler
58508000 Macaroni and cheese, baby food, strained
58508300 Macaroni and cheese, baby food, toddler
58509020 Spaghetti, tomato sauce, and beef, baby food, junior
58509100 Ravioli, cheese-filled, with tomato sauce, baby food, toddler
58509200 Macaroni with vegetables, baby food, strained
67100100 Fruit, baby food, NFS
67100110 Fruit bar, with added vitamin C, baby food, toddler
67100200 Tropical fruit medley, baby food, strained
67100300 Apples, baby food, toddler
67101000 Apple-raspberry, baby food, NS as to strained or junior
67101010 Apple-raspberry, baby food, strained
67101020 Apple-raspberry, baby food, junior
67102000 Applesauce, baby food, NS as to strained or junior
67102010 Applesauce, baby food, strained
67102020 Applesauce, baby food, junior
67104000 Applesauce and apricots, baby food, NS as to strained or junior
67104010 Applesauce and apricots, baby food, strained
67104020 Applesauce and apricots, baby food, junior
67104030 Applesauce with bananas, baby food, NS as to strained or junior
67104040 Applesauce with bananas, baby food, strained
67104060 Applesauce with bananas, baby food, junior

67104070 Applesauce with cherries, baby food, strained
67104080 Applesauce with cherries, baby food, junior
67104090 Applesauce with cherries, baby food, NS as to strained or junior
67105030 Bananas, baby food, strained
67106010 Bananas with apples and pears, baby food, strained
67106030 Bananas with orange, baby food, strained
67106050 Banana with mixed berries, baby food, strained
67108000 Peaches, baby food, NS as to strained or junior
67108010 Peaches, baby food, strained
67108020 Peaches, baby food, junior
67108030 Peaches, baby food, toddler
67109000 Pears, baby food, NS as to strained or junior
67109010 Pears, baby food, strained
67109020 Pears, baby food, junior
67109030 Pears, baby food, toddler
67110000 Prunes, baby food, strained
67113000 Apples and pears, baby food, NS as to strained or junior
67113010 Apples and pears, baby food, strained
67113020 Apples and pears, baby food, junior
67114000 Pears and pineapple, baby food, NS as to strained or junior
67114010 Pears and pineapple, baby food, strained
67114020 Pears and pineapple, baby food, junior
67304000 Plums, baby food, NS as to strained or junior
67304010 Plums, baby food, strained
67304020 Plums, baby food, junior
67304030 Plums, bananas, and rice, baby food strained
67304500 Prunes with oatmeal, baby food, strained
67307000 Apricots, baby food, NS as to strained or junior
67307010 Apricots, baby food, strained
67307020 Apricots, baby food, junior
67308000 Bananas, baby food, NS as to strained or junior
67308020 Bananas, baby food, junior
67309000 Bananas and pineapple, baby food, NS as to strained or junior
67309010 Bananas and pineapple, baby food, strained
67309020 Bananas and pineapple, baby food, junior
67309030 Bananas and strawberry, baby food, junior
67404000 Fruit dessert, baby food, NS as to strained or junior
67404010 Fruit dessert, baby food, strained
67404020 Fruit dessert, baby food, junior
67404050 Fruit Supreme dessert, baby food
67404070 Apple yogurt dessert, baby food, strained
67404110 Banana apple dessert, baby food, strained
67404300 Blueberry yogurt dessert, baby food, strained
67404500 Mixed fruit yogurt dessert, baby food, strained
67404550 Cherry cobbler, baby food, junior

67405000 Peach cobbler, baby food, NS as to strained or junior
67405010 Peach cobbler, baby food, strained
67405020 Peach cobbler, baby food, junior
67408010 Banana pudding, baby food, strained
67408500 Banana yogurt dessert, baby food, strained
67410000 Cherry vanilla pudding, baby food, strained
67412000 Dutch apple dessert, baby food, NS as to strained or junior
67412010 Dutch apple dessert, baby food, strained
67412020 Dutch apple dessert, baby food, junior
67413700 Peach yogurt dessert, baby food, strained
67414010 Pineapple dessert, baby food, strained
67414100 Mango dessert, baby food
67415000 Tutti-fruitti pudding, baby food, NS as to strained or junior
67415010 Tutti-fruitti pudding, baby food, strained
67415020 Tutti-fruitti pudding, baby food, junior
67430000 Fruit flavored snack, baby food
67430500 Yogurt and fruit snack, baby food
67501000 Apples and chicken, baby food, strained
67501100 Apples with ham, baby food, strained
67600100 Apples and sweet potatoes, baby food, strained
76102010 Spinach, creamed, baby food, strained
76102030 Broccoli, carrots and cheese, baby food, junior
76201000 Carrots, baby food, NS as to strained or junior
76201010 Carrots, baby food, strained
76201020 Carrots, baby food, junior
76201030 Carrots, baby food, toddler
76202000 Carrots and peas, baby food, strained
76205000 Squash, baby food, NS as to strained or junior
76205010 Squash, baby food, strained
76205020 Squash, baby food, junior
76205030 Squash and corn, baby food, strained
76205060 Corn and sweet potatoes, baby food, strained
76209000 Sweet potatoes, baby food, NS as to strained or junior
76209010 Sweet potatoes, baby food, strained
76209020 Sweet potatoes, baby food, junior
76401000 Beans, green string, baby food, NS as to strained or junior
76401010 Beans, green string, baby food, strained
76401020 Beans, green string, baby food, junior
76401060 Beans, green string, baby food, toddler
76402000 Green beans and potatoes, baby food, strained
76403010 Beets, baby food, strained
76405000 Corn, creamed, baby food, NS as to strained or junior
76405010 Corn, creamed, baby food, strained
76405020 Corn, creamed, baby food, junior
76407000 Mixed vegetables, garden vegetables, baby food, NS as to strained or junior

76407010 Mixed vegetables, garden vegetables, baby food, strained
76407020 Mixed vegetables, garden vegetables, baby food, junior
76409000 Peas, baby food, NS as to strained or junior
76409010 Peas, baby food, strained
76409020 Peas, baby food, junior
76409030 Peas, baby food, toddler
76420000 Potatoes, baby food, toddler
76501000 Vegetables and rice, baby food, strained
76502000 Peas and brown rice, baby food
76602000 Carrots and beef, baby food, strained
76603000 Vegetable and beef, baby food, NS as to strained or junior
76603010 Vegetable and beef, baby food, strained
76603020 Vegetable and beef, baby food, junior
76604000 Broccoli and chicken, baby food, strained
76604500 Sweet potatoes and chicken, baby food, strained
76605000 Vegetable and chicken, baby food, NS as to strained or junior
76605010 Vegetable and chicken, baby food, strained
76605020 Vegetable and chicken, baby food, junior
76607100 Potatoes with cheese and broccoli, baby food, toddler
76611000 Vegetable and turkey, baby food, NS as to strained or junior
76611010 Vegetable and turkey, baby food, strained
76611020 Vegetable and turkey, baby food, junior

Adjusted for reconstitution factor of 8.33

57801000 Barley cereal, baby food, dry, instant
57803000 Mixed cereal, baby food, dry, instant
57804000 Oatmeal cereal, baby food, dry, instant
57805000 Rice cereal, baby food, dry, instant
57805080 Rice cereal with apples, baby food, dry, instant
57805090 Rice cereal with mixed fruits, baby food, dry, instant
57805100 Rice cereal with bananas, baby food, dry, instant
57805500 Brown rice cereal, baby food, dry, instant
57806000 Mixed cereal with bananas, baby food, dry, instant
57806050 Multigrain, whole grain cereal, baby food, dry, instant
57806100 Oatmeal cereal with bananas, baby food, dry, instant
57806200 Oatmeal cereal with fruit, baby food, dry, instant, toddler
57807010 Whole wheat cereal with apples, baby food, dry, instant

Other Drinks for Young Children

67202000 Apple juice, baby food
67202010 Apple juice, with added calcium, baby food
67203000 Apple-fruit juice blend, baby food
67203200 Apple-banana juice, baby food
67203400 Apple-cherry juice, baby food

67203500 Apple-grape juice, baby food
67203600 Apple-peach juice, baby food
67203700 Apple-prune juice, baby food
67203800 Grape juice, baby food
67204000 Mixed fruit juice, not citrus, baby food
67204100 Mixed fruit juice, not citrus, with added calcium, baby food
67205000 Orange juice, baby food
67211000 Orange-apple-banana juice, baby food
67212000 Pear juice, baby food
67230000 Apple-sweet potato juice, baby food
67230500 Orange-carrot juice, baby food
67250100 Banana juice with lowfat yogurt, baby food
67250150 Mixed fruit juice with lowfat yogurt, baby food
67260000 Fruit juice and water drink, with high vitamin C and added calcium, baby food
94300100 Water, baby, bottled, unsweetened

From:	Darch, Maryse
To:	Morissette, Rachel
Cc:	Roehrig, Christoph
Subject:	RE: [EXTERNAL] RE: additional questions for GRN 001035
Date:	Thursday, September 22, 2022 9:11:12 AM
Attachments:	image001.png image002.png image003.png image004.png image006.png image007.png image008.png image009.png image010.png image011.png image013.png image014.png GRN 001035 - Response to FDA Questions - 22Sept"22.pdf

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Dear Rachel,

Please find attached our responses to the additional questions for GRN 001035. Please do not hesitate to contact us if any further clarification is necessary.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, September 21, 2022 3:35 PM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: additional questions for GRN 001035

Hi Maryse,

Just checking in on the responses to our follow-up questions for GRN 1035.

Thanks,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Friday, August 19, 2022 2:53 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: additional questions for GRN 001035

Dear Rachel,

Confirmed that the follow-up questions sent on Monday were received. We anticipate providing responses to the follow-up questions early September.

Kind regards,
Maryse

Maryse Darch | HMO Regulatory – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, August 19, 2022 1:06 PM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: additional questions for GRN 001035

Hi Maryse,

I just wanted to confirm that you received our follow-up questions on Monday.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Morissette, Rachel
Sent: Monday, August 15, 2022 9:53 AM

To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: additional questions for GRN 001035

Dear Maryse,

Please see our additional questions for GRN 001035 below, which are largely based on our phone conversation from July 21, 2022. Also, in response to your email below from August 8, 2022, the use level issue applies to infant formula, with some considerations for "toddler formula" that you can find in question 1 below. All other conventional food use levels are not in question, though you are welcome to lower any use levels you see fit, keeping in mind the considerations raised in question 2 below. Please let me know if you have any further questions at this time.

1. We have reviewed the data and information submitted in GRN 001035, as well as in the May 13, 2022 amendment. While there appears to be sufficient data to support the safety of LNFP-I in healthy term infants at the mean (or mean of means) levels reported for mature human milk,[1] general recognition of safety for use levels far above those means (or mean of means) does not appear to be supported at this time. Therefore, we suggest that the maximum use level be lowered to mean (or mean of means) levels in mature milk as reported in recently published and peer-reviewed systematic reviews.

We further note that all of the previously submitted HMO GRNs that resulted in a No Questions letter with intended uses in "toddler formula" proposed a maximum use level that matched or was lower than the maximum use level in infant formula (i.e., GRNs 000735, 000833, 000880, 000881, 000919). We note that most individual HMO or overall HMO levels decrease with period of lactation (i.e., Soyyilmaz et al. 2021), suggesting that older breastfed infants and toddlers (i.e., >9 months of age) are likely exposed to lower levels of most HMOs, including LNFP-I, as compared to young infants expected to consume infant formula (i.e., 0-6 months of age). If Glycom proposes to retain the use level in "toddler formula" (defined as intended for young children >12 months of age in the notice) as originally proposed in GRN 001035, please provide an explanation why a higher use level is GRAS for this use.

[1] We note that colostrum is much different in composition or function from mature milk (Donovan, 2019). As such, mature milk is generally thought to be the most appropriate human milk reference for formulation of infant formula (Wells, 1996).

2. In the May 13, 2022 amendment, the response to our question 4b states that it can be assumed that LNFP-I/2'-FL could contain a maximum of 50% 2'-FL assuming the minimum specification limit for LNFP-I. In addition, Table 3 provided as part of the response includes calculated maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL for each intended food category. We note that the maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL provided in Table 3 do not match those provided in Table 6 in the response to question 9. We assume that the maximum theoretical use

levels of 2'-FL in Table 3 are the result of an error. Please explain the discrepancy between the maximum theoretical use levels of 2'-FL in Tables 3 and 6. If our assumption is correct, please provide a corrected Table 3.

Should Glycom choose to lower the maximum use level of LNFP-I in infant formula (or in any other food category), please provide the following tables:

- Revised Tables 3 and 6 of the May 13, 2022 amendment.
- Revised Tables 7 and 8 of the May 13, 2022 amendment with updated estimates of dietary exposure to 2'-FL and LNFP-I/2'-FL, respectively.
- Revised Tables 3.2.2-1 and 3.2.2-2 of GRN 001035 with updated estimates of dietary exposure to LNFP-I from the intended uses of LNFP-I/2'-FL.

3. In the May 13, 2022 amendment, the response to our question 4c states that validation of Glycom's internal analytical methods (HPLC-13-001, HPLC-13-002, and HPAEC-HMO-017) used to test for the specification parameters is ongoing and is anticipated to be completed in July 2022. When the validation is completed, please provide a statement that all analytical methods listed in Table 2.3.1-1 of the notice are validated for the stated purpose and that the analytical results provided in GRN 001035 continue to be relevant for Glycom's safety conclusion.

4. In the May 13, 2022 amendment, the response to our question 8c lists the NHANES food codes representing foods for infants and young children, including foods that may be regulated by USDA (e.g., 22810010 Ham, baby food, strained or 21701010 Beef, baby food, strained). Please note that while we would consider the dietary exposure estimate including these food codes as conservative, the intended uses in food products regulated by USDA remain excluded from the scope of GRN 001035.

References:

Donovan, S.M. (2019). Human Milk Proteins: Composition and Physiological Significance. Nestle Nutr Inst Workshop Ser *90*, 93-101.
Wells, J.C.K. (1996). Nutritional considerations in infant formula design. Seminars in Neonatology *1*, 19-26.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Monday, August 8, 2022 2:17 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: request for phone call re: GRN 001035

Dear Rachel,

Thank you again for your time on the call in July to discuss the use level in infant formula proposed in GRN 001035. We look forward to receiving the FDA's follow-up questions for GRN 001035.

In the meantime, I realized that we did not discuss the influence of lowering the proposed use level in infant formula on use levels proposed for other food categories, for example toddler formulas and other drinks for young children. To confirm, is the use level issue only applicable to infant formula?

Thank you in advance for your time and feedback.

Kind regards,
Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, July 14, 2022 10:07 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: request for phone call re: GRN 001035

Thank you. I just sent a Zoom invite. Please let me know if you don't receive it. Looking forward to chatting next week.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Thursday, July 14, 2022 10:02 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: request for phone call re: GRN 001035

Dear Rachel,

Thank you for organizing a call to discuss the use level proposed in GRN 001035. Both myself and Christoph are free for a call on Thursday July 21st from 9-10 am.

We look forward to receiving the FDA's feedback to find a solution to the issue.

Kind regards,
Maryse

Maryse Darch | HMO Regulatory Affairs – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, July 14, 2022 9:36 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: request for phone call re: GRN 001035

Dear Maryse,

We have reviewed Glycom's responses to our questions from May 13 and are requesting a phone call to discuss some issues surrounding the use level proposed in GRN 001035. Would you be available during the following dates and times?

Tues July 19: 9-10 am EDT or 11-12 pm
Wed July 20: 11-1 pm or 2-3 pm
Thurs July 21: 9-10 am
Fri July 22: 9-10 am, 11-12 pm, or 1:30-2:30 pm

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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[1] We note that colostrum is much different in composition or function from mature milk (Donovan, 2019). As such, mature milk is generally thought to be the most appropriate human milk reference for formulation of infant formula (Wells, 1996).

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Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark

22 September 2022

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: Additional Questions for GRAS Notice No. GRN 001035

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s email dated 15 August 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice (and the May amendment) for the intended use of lacto-*N*-fucopentaose I with 2'-fucosyllactose (LNFP-I/2'-FL) filed by the Agency under GRN 001035.

FDA.1. *We have reviewed the data and information submitted in GRN 001035, as well as in the May 13, 2022 amendment. While there appears to be sufficient data to support the safety of LNFP-I in healthy term infants at the mean (or mean of means) levels reported for mature human milk,[1] general recognition of safety for use levels far above those means (or mean of means) does not appear to be supported at this time. Therefore, we suggest that the maximum use level be lowered to mean (or mean of means) levels in mature milk as reported in recently published and peer-reviewed systematic reviews.*

We further note that all of the previously submitted HMO GRNs that resulted in a No Questions letter with intended uses in "toddler formula" proposed a maximum use level that matched or was lower than the maximum use level in infant formula (i.e., GRNs 000735, 000833, 000880, 000881, 000919). We note that most individual HMO or overall HMO levels decrease with period of lactation (i.e., Soyyilmaz et al. 2021), suggesting that older breastfed infants and toddlers (i.e., >9 months of age) are likely exposed to lower levels of most HMOs, including LNFP-I, as compared to young infants expected to consume infant formula (i.e., 0-6 months of age). If Glycom proposes to retain the use level in "toddler formula" (defined as intended for young children >12 months of age in the notice) as originally proposed in GRN 001035, please provide an explanation why a higher use level is GRAS for this use.

[1] We note that colostrum is much different in composition or function from mature milk (Donovan, 2019). As such, mature milk is generally thought to be the most appropriate human milk reference for formulation of infant formula (Wells, 1996).

Glycom is agreeable to lower the proposed use level of LNFP-I from LNFP-I/2'-FL in infant formula and toddler formula to 0.8 g/L, the mean (or mean of means) concentration of LNFP-I in mature milk reported in recently published reviews of human milk oligosaccharide (HMO) levels in human milk (Thurl

et al., 2017; Soyyilmaz *et al*., 2021). The revised food use table (Table 1.3-1 of the GRAS notice) is presented below.

Based on the response to Question 2, the maximum proposed use level of LNFP-I from LNFP-I/2'-FL in 'other drinks for young children' has also been revised from 1.5 to 1.2 g/L.

Table 1.3-1 Proposed Food Uses and Use Levels for LNFP-I from LNFP-I/2'-FL in the U.S. [REVISED]

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	240	0.48	2.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.08	0.8
	Toddler Formulas[e]	100[d]	0.08	0.8
	Other Baby Foods for Infants and Young Children	7 to 170	0.06 to 1.42	8.33
	Other Drinks for Young Children	120	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	0.8	20.0
	Cereal and Nutrition Bars	40	0.8	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	240	0.24	1.0
Milk Products	Buttermilk*	240	0.36	1.5
	Flavored Milk	240	0.36	1.5
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.48	2.0
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.12 to 0.31	1.5
	Yogurt*	170	1.7	10.0
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.24	1.0

2'-FL = 2'-fucosyllactose; CFR = *Code of Federal Regulations*; LNFP-I = lacto-*N*-fucopentaose I; RACC = Reference Amounts Customarily Consumed per Eating Occasion; RTE = ready-to-eat; U.S. = United States.

* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2020a). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.

[b] Use level expressed on a LNFP-I basis in the final food, as consumed.

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

[d] RACC not available, 100 mL employed as an approximation.

[e] Formula products targeted toward young children (> 12 months of age)

[f] Portion sizes are based on representative products on the U.S. market.

FDA.2. *In the May 13, 2022 amendment, the response to our question 4b states that it can be assumed that LNFP-I/2'-FL could contain a maximum of 50% 2'-FL assuming the minimum specification limit for LNFP-I. In addition, Table 3 provided as part of the response includes calculated maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL for each intended food category. We note that the maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL provided in Table 3 do not match those provided in Table 6 in the response to question 9. We assume that the maximum theoretical use levels of 2'-FL in Table 3 are the result of an error. Please explain the discrepancy between the maximum theoretical use levels of 2'-FL in Tables 3 and 6. If our assumption is correct, please provide a corrected Table 3.*

Should Glycom choose to lower the maximum use level of LNFP-I in infant formula (or in any other food category), please provide the following tables:

- *Revised Tables 3 and 6 of the May 13, 2022 amendment.*
- *Revised Tables 7 and 8 of the May 13, 2022 amendment with updated estimates of dietary exposure to 2'-FL and LNFP-I/2'-FL, respectively.*
- *Revised Tables 3.2.2-1 and 3.2.2-2 of GRN 001035 with updated estimates of dietary exposure to LNFP-I from the intended uses of LNFP-I/2'-FL.*

We thank the U.S. FDA for finding the discrepancy in the maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL between Tables 3 and 6 of the May amendment. Indeed, there is an error in the reporting of the maximum theoretical use levels of 2'-FL from LNFP-I/2'-FL in Table 3 from the previous response. It can be theoretically assumed as the worst-case that the ingredient could contain a maximum of 50 w/w% 2'-FL[1]. This means that the maximum theoretical addition level of 2'-FL from LNFP-I/2'-FL is equivalent to the proposed use level of LNFP-I from LNFP-I/2'-FL. The corrected Table 3 from the May amendment, with the revised maximum use level of LNFP-I from LNFP-I/2'-FL in term infant formulas and toddler formulas at 0.8 g/L (as per the response to Question 1), is provided below.

Based on the correction, the use level of LNFP-I from LNFP-I/2'-FL originally proposed for the 'Other Drinks for Young Children' food category results in a maximum theoretical use level of 2'-FL from LNFP-I/2'-FL that slightly exceeds the maximum use level of 2'-FL notified as GRAS for this food category. Therefore, the maximum proposed use level of LNFP-I from LNFP-I/2'-FL in 'Other Drinks for Young Children' has been slightly reduced to 1.2 g/L (see also response to Question 1).

[1] Assuming 50 w/w % LNFP-I, the minimum specification limit for LNFP-I [calculation: 100 % - 50 % LNFP-I = 50 % 2'-FL].

Table 3 **Comparison of Maximum Theoretical Use Levels of 2'-FL from LNFP-I/2'-FL to Maximum Authorized Use Levels of 2'-FL in the U.S. [CORRECTED AND REVISED]**

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	Maximum Use Level (g/kg or g/L)		
		LNFP-I (from LNFP-I/2'-FL)	2'-FL (from LNFP-I/2'-FL)*	2'-FL (GRAS Notices with a "No Questions" letter)
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages	2.0	2.0	12 (GRN 1014)
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.0	1.0	1.5[a] (GRN 815) to 6[b] (GRN 1014)
Infant and Toddler Foods	Term Infant Formulas	0.8	0.8	2.4 (GRN 650)
	Toddler Formulas	0.8	0.8	2.4 (GRN 650)
	Other Baby Foods for Infants and Young Children	8.33	8.33	12 (GRN 650)
	Other Drinks for Young Children	1.2	1.2	1.2 (GRN 650)
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	20.0	20.0	40 (GRN 650)
	Cereal and Nutrition Bars	20.0	20.0	30 (GRN 897)
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	1.0	1.0	1.5[a] (GRN 815)
Milk Products	Buttermilk	1.5	1.5	1.5[a] (GRN 815)
	Flavored Milk	1.5	1.5	1.5[a] (GRN 815)
	Milk-Based Meal Replacement and Nutritional Beverages	2.0	2.0	12 (GRN 1014)
	Yogurt Drinks, Probiotic Drinks	1.5	1.5	1.5[a,c] (GRN 815)
	Yogurt	10.0	10.0	15[a] (GRN 815)
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.0	1.0	1.5[a] (GRN 815)

2'-FL = 2'-fucosyllactose; GRAS = generally recognized as safe; GRN = GRAS notice; LNFP-I = lacto-*N*-fucopentaose I; U.S. = United States.

* Assuming 50% 2'-FL as a worst-case.

[a] Assuming the 2'-FL/DFL mixture contains a minimum of 75 w/w % 2'-FL content (as per the ingredient specification; see GRN 815).

[b] 2'-FL is GRAS at a maximum use level of 6 g/L in sports, isotonic, and energy drinks (see GRN 1014).

[c] Yogurt drinks have previously been categorized under flavored milk drinks (*e.g.*, GRN 932).

As described above, it can be theoretically assumed as the worst-case that the ingredient could contain a maximum of 50 w/w% 2'-FL. This means that the maximum theoretical addition level of 2'-FL from LNFP-I/2'-FL is equivalent to the proposed use level of LNFP-I from LNFP-I/2'-FL, and that the maximum theoretical use level of the whole ingredient is double that of LNFP-I.

The corrected Table 6 from the May amendment, with the revised maximum theoretical addition levels of 2'-FL and LNFP-I/2'-FL (the whole ingredient), used to calculate the worst-case scenario dietary exposures, is provided below.

Table 6 **Maximum Theoretical Use Levels of 2'-FL from LNFP-I/2'-FL in the U.S. [CORRECTED AND REVISED]**

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	Proposed Use Level of LNFP-I (g/kg or g/L)	Maximum theoretical Use Level of 2'-FL (g/kg or g/L)[a]	Maximum theoretical Use Level of LNFP-I/2'-FL (g/kg or g/L)[b]
Beverages and Beverage Bases	Non-Milk-Based Meal and Nutritional Beverages[c]	2.0	2.0	4.0
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.0	1.0	2.0
Infant and Toddler Foods	Term Infant Formulas	0.8	0.8	1.6
	Toddler Formulas[d]	0.8	0.8	1.6
	Other Baby Foods for Infants and Young Children	8.33	8.33	16.66
	Other Drinks for Young Children	1.2	1.2	2.4
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	20.0	20.0	40.0
	Cereal and Nutrition Bars	20.0	20.0	40.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	1.0	1.0	2.0
Milk Products	Buttermilk*	1.5	1.5	3.0
	Flavored Milk	1.5	1.5	3.0
	Milk-Based Meal Replacement and Nutritional Beverages	2.0	2.0	4.0
	Yogurt Drinks, Probiotic Drinks	1.5	1.5	3.0
	Yogurt*	10.0	10.0	20.0
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.0	1.0	2.0

2'-FL = 2'-fucosyllactose; GRAS = generally recognized as safe; LNFP-I = lacto-*N*-fucopentaose I; U.S. = United States.
* LNFP-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.
[a] Assuming 50% 2'-FL as a worst-case, based on the minimum LNFP-I specification limit (50 w/w %). The resulting maximum theoretical use level of 2'-FL is equivalent to the proposed use level of LNFP-I from LNFP-I/2'-FL.
[b] Assuming the ingredient is composed of 50% LNFP-I and 50% 2'-FL as a worst-case, the resulting maximum theoretical use level of LNFP-I/2'-FL (the whole ingredient) is double the proposed use level of LNFP-I from LNFP-I/2'-FL.
[c] Includes ready-to-drink and powder forms.
[d] Formula products targeted toward young children (> 12 months of age)

The revised Table 7 from the May amendment (based on the corrections to Table 3 and 6), which summarizes the maximum theoretical estimated daily intake of 2'-FL (from proposed food-uses of LNFP-I/2'-FL) for infants aged 0 to 6 months and 7 to 12 months, toddlers 1 to 2 years, and for the population 2 years and older on an absolute basis (g/person/day) and on a body weight basis (mg/kg body weight/day), is provided below.

Table 7 Summary of the Estimated Daily Intake of 2'-FL from Proposed Food Uses of LNFP-I/2'FL in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 m	76.2	139	1.01	2.15	150	308
Infants	7 to <12 m	97.5	122	2.21	4.21	246	503
Toddlers	1 to 2 y	99.0	300	0.84	1.57	70	132
Total Population	2 y and older	89.8	5,523	0.76	1.66	13	29

2'-FL = 2'-fucosyllactose; LNFP-I/2-'FL = lacto-*N*-fucopentaose I/2'-fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

The revised Table 8 from the May amendment (based on the corrections to Table 3 and 6), which summarizes the maximum theoretical estimated daily intake of LNFP-I/2'-FL for infants aged 0 to 6 months and 7 to 12 months, toddlers 1 to 2 years, and for the population 2 years and older on an absolute basis (g/person/day) and on a body weight basis (mg/kg body weight/day), is provided below.

Table 8 Summary of the Estimated Daily Intake of LNFP-I/2'-FL from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 m	76.2	139	2.01	4.29	300	616
Infants	7 to <12 m	97.5	122	4.42	8.41	492	1,006
Toddlers	1 to 2 y	99.0	300	1.68	3.14	140	264
Total Population	2 y and older	89.8	5,523	1.53	3.32	26	57

LNFP-I/2'-FL = lacto-*N*-fucopentaose I/2'- fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

The revised Tables 3.2.2-1 and 3.2.2-2 from the GRAS notice, which summarize the estimated daily intake of LNFP-I from proposed food uses of LNFP-I/2'-FL on an absolute basis (g/person/day) and on a body weight basis (mg/kg body weight/day), are provided below.

Table 3.2.2-1 Summary of the Estimated Daily Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	0.77	1.82	76.2	139	1.01	2.15
Infants	7 to <12 m	2.15	4.21	97.5	122	2.21	4.21
Toddlers	1 to 2 y	0.83	1.57	99.0	300	0.84	1.57
Children	3 to 11 y	0.73	1.47	97.4	970	0.75	1.47
Female Teenagers	12 to 19 y	0.53	1.23	92.3	413	0.57	1.23
Male Teenagers	12 to 19 y	0.74	1.48	94.9	417	0.78	1.48
Female Adults of Childbearing Age	20 to 40 y	0.57	1.24	87.7	610	0.65	1.29
Female Adults	20 to 64 y	0.62	1.43	86.9	1,408	0.72	1.51
Male Adults	20 to 64 y	0.81	1.96	89.0	1,260	0.91	2.02
Elderly	65 y and older	0.58	1.39	87.7	904	0.66	1.46
Total Population	2 y and older	0.69	1.57	89.8	5,523	0.76	1.66

LNFP-I = lacto-*N*-fucopentaose I; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

Table 3.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNFP-I from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	114	250	76.2	139	150	308
Infants	7 to <12 m	240	496	97.5	122	246	503
Toddlers	1 to 2 y	69	132	98.9	291	70	132
Children	3 to 11 y	29	59	97.4	967	29	60
Female Teenagers	12 to 19 y	9	21	92.4	407	10	24
Male Teenagers	12 to 19 y	12	23	95.2	415	12	23
Female Adults of Childbearing Age	20 to 40 y	8	17	87.7	609	9	18
Female Adults	20 to 64 y	9	20	87.0	1,402	10	22
Male Adults	20 to 64 y	9	22	89.0	1,252	10	22
Elderly	65 y and older	8	18	88.0	891	9	18
Total Population	2 y and older	12	27	89.9	5,478	13	29

LNFP-I = lacto-*N*-fucopentaose I; bw = body weight; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

The revised estimated daily intakes of 2'-FL, LNFP-I/2'-FL, and LNFP-I from the intended conditions of use of the ingredient provided herein generally decreased or remained similar to those reported in the May 13, 2022 amendment or GRN 001035.

FDA.3. *In the May 13, 2022 amendment, the response to our question 4c states that validation of Glycom's internal analytical methods (HPLC-13-001, HPLC-13-002, and HPAEC-HMO-017) used to test for the specification parameters is ongoing and is anticipated to be completed in July 2022. When the validation is completed, please provide a statement that all analytical methods listed in Table 2.3.1-1 of the notice are validated for the stated purpose and that the analytical results provided in GRN 001035 continue to be relevant for Glycom's safety conclusion.*

There has been delay in the finalization of the validation of Glycom's internal analytical methods for the evaluation of LNFP-I/2'-FL specification parameters due to unforeseen instrument repairs during the summer. Nevertheless, the evaluation of the performance of the internal analytical methods confirms that they are fit for purpose (*i.e.*, to test the relevant 3-FL specification parameters), and that the analytical results provided in GRN 001035 are robust and continue to be relevant to Glycom's safety conclusion. Glycom's 3-FL will not be introduced to the U.S. market under the intended conditions of use until finalization of the validation of all internal methods for the testing of the 3-FL specification parameters.

FDA.4. *In the May 13, 2022 amendment, the response to our question 8c lists the NHANES food codes representing foods for infants and young children, including foods that may be regulated by USDA (e.g., 22810010 Ham, baby food, strained or 21701010 Beef, baby food, strained). Please note that while we would consider the dietary exposure estimate including these food codes as conservative, the intended uses in food products regulated by USDA remain excluded from the scope of GRN 001035.*

Glycom acknowledges that the United States Department of Agriculture (USDA) regulates certain meat, poultry, and egg products which are outside the scope of GRN 001035. As the U.S. FDA noted, the inclusion of NHANES food codes representing foods that may be regulated by USDA was intentional as a conservative measure in the dietary exposure estimate.

--

We hope this information adequately addresses the Agency's questions on GRN 001035, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
Date: 2022.09.22 15:00:42 +02'00'

Maryse Darch
Regulatory & Scientific Affairs Manager
Glycom A/S

REFERENCES

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. DOI:10.3390/nu13082737.

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.

From:	Darch, Maryse
To:	Morissette, Rachel
Cc:	Roehrig, Christoph
Subject:	RE: [EXTERNAL] RE: update on follow-up questions for GRN 001035?
Date:	Thursday, December 22, 2022 1:31:47 PM
Attachments:	image001.png image002.png image003.png image004.png image006.png image007.png image008.png image009.png image010.png image011.png image013.png image014.png GRN 001035 - Response to FDA Questions - 22Dec"22.pdf

Confidential

Dear Rachel,

I am happy to share the updated batch analysis results for non-consecutive batches of LNFP-I/2'-FL using the modified, validated, analytical methods. These are provided in the attached response to the follow-up questions received from the Agency in October. Please do not hesitate to contact us if any further clarification is necessary.

We wish you a wonderful holiday season and happy new year.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, November 30, 2022 8:29 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: update on follow-up questions for GRN 001035?

Thank you for the update. Your draft letter is ready to be signed as soon as we get this information, provided nothing unexpected comes of it.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Wednesday, November 30, 2022 8:26 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: update on follow-up questions for GRN 001035?

Dear Rachel,

Apologies for the delayed response. The validation has resulted in slight modifications to internal

analytical methods for the quantification of carbohydrate specification parameters. As a result, we are in the process of re-analyzing non-consecutive batches of the LNFP-I/2'-FL product using the modified analytical methods. We expect to be able to provide the updated results and finalized response to the FDA's follow-up questions before the December holidays.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Monday, November 21, 2022 3:20 PM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: update on follow-up questions for GRN 001035?

Sounds good. Thank you!

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Monday, November 21, 2022 2:50 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: update on follow-up questions for GRN 001035?

Confidential

Dear Rachel,

The validation is progressing well and nearing completion. We will be able to confirm the timeline of the responses to the remaining questions for GRN 1035 by the end of this week.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, November 18, 2022 7:49 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: update on follow-up questions for GRN 001035?

Dear Maryse,

I just wanted to check in on any updates or estimated timelines when we might receive your responses to our remaining questions for GRN 1035? Have the instrumentation issues been sorted out as you had hoped?

Thanks,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Thursday, October 20, 2022 10:57 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: follow-up questions GRN 001035

Dear Rachel,

I confirm receipt of the follow-up questions discussed during the October 13 teleconference.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, October 20, 2022 10:36 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: follow-up questions GRN 001035

Dear Maryse,

Below are the follow-up questions we discussed during our October 13 teleconference.

1. In the amendment dated September 22, 2022, Glycom informed us that there has been a delay in the validation procedure of the internal analytical methods due to unforeseen instrument repairs. At that time Glycom was not able to provide the statement that all the methods are validated for the intended purpose. As we discussed during a teleconference on October 13, 2022, we would not be able to complete our evaluation of GRN 001035 without Glycom providing an adequate statement. Therefore, we request that upon completion of the validation, Glycom provide a statement confirming that all analytical methods (Table 2.3.1-1 on p. 14 of the notice) that were used to generate the batch analyses are validated for the stated purpose. If the validation procedure results in any modifications to the analytical methods that were used to generate the batch analyses, we would expect that Glycom briefly describe the modifications and provide the results of analyses from a minimum of three non-consecutive batches performed using the modified methods.

2. In addition, we request that Glycom address the following:
    - In Table 1.3-1 (p. 5 of the notice), Glycom listed “Probiotic Drinks” as a proposed food use. Please provide representative examples of food products (or NHANES food codes) that Glycom considered as “Probiotic Drinks” in the dietary exposure assessment.
    - In the amendment dated May 13, 2022, Glycom stated that 2'-FL from LNFP-I/2'-FL is intended to be partially (or fully) substitutional to other sources of 2'-FL that have GRAS status for use in formula products. Please clarify whether 2'-FL from LNFP-I/2'-FL is intended to be partially substitutional to other sources of 2'-FL that have GRAS status for use in other foods listed in Table 1.3-1 (p. 5 of the notice).

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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If you as intended recipient have received this e-mail incorrectly, please notify the sender (via e-mail) immediately.

Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark

22 December 2022

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: Additional Questions for GRAS Notice No. GRN 001035

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s follow-up questions received by email on 20 October 2022 pertaining to Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of lacto-*N*-fucopentaose I with 2'-fucosyllactose (LNFP-I/2'-FL) filed by the Agency under GRN 001035.

FDA.1. *In the amendment dated September 22, 2022, Glycom informed us that there has been a delay in the validation procedure of the internal analytical methods due to unforeseen instrument repairs. At that time Glycom was not able to provide the statement that all the methods are validated for the intended purpose. As we discussed during a teleconference on October 13, 2022, we would not be able to complete our evaluation of GRN 001035 without Glycom providing an adequate statement. Therefore, we request that upon completion of the validation, Glycom provide a statement confirming that all analytical methods (Table 2.3.1-1 on p. 14 of the notice) that were used to generate the batch analyses are validated for the stated purpose. If the validation procedure results in any modifications to the analytical methods that were used to generate the batch analyses, we would expect that Glycom briefly describe the modifications and provide the results of analyses from a minimum of three non-consecutive batches performed using the modified methods.*

Glycom confirms that all internal analytical methods in Table 2.3.1-1 on p. 14 of the notice have been validated for the evaluation of the conformance of each batch of LNFP-I/2'-FL with the stated specifications. The validation procedure resulted in some modifications to the internal analytical methods that were used to generate the results of LNFP-I/2'-FL batch analyses provided in the GRAS notice; these modifications are summarized in Table 1 below.

There is also slight modification to the L-fucose specification parameter, to 'sum of L-fucose and 2'-fucosyl-lactitol', given the difficulty in separating these two carbohydrates by high-performance anion-exchange chromatography (HPAEC). Otherwise, all specification parameters remain the same.

Table 1 **Summary of Modifications to Internal Analytical Methods for Batch Analysis of LNFP-I/2'-FL**

Method	Parameters[a]	Modifications
Glycom method HPLC-13-002	• Assay (water-free) – Specified saccharides • Assay (water-free) – LNFP-I and 2'-FL • Assay (water-free) – LNFP-I • Assay (water-free) – 2'-FL	• New column
Glycom method HPLC-13-001	• Assay (water-free) – Specified saccharides • LNFP-I fructose isomer • 2'-Fucosyl-D-lactulose	• New column • Elution profile changed from isocratic to gradient
Glycom method HPAEC-HMO-017	• Assay (water-free) – Specified saccharides • Lacto-*N*-tetraose • 3-Fucosyllactose • Sum of L-fucose and 2'-fucosyl-lactitol • D-Lactose • Difucosyl-D-lactose • Sum of other carbohydrates	• Slightly increased temperature • Slightly decreased flow rate • Adjusted gradient elution profile

2'-FL = 2'-fucosyllactose; HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; LNFP-I = lacto-*N*-fucopentaose I.
[a] Updated specification parameters are indicated in green.

Results of analyses for specification parameters affected by modifications to internal analytical methods (*i.e.*, Glycom method HPLC-13-002, HPLC-13-001, and HPAEC-HMO-017) are provided in Table 2 below for three non-consecutive batches of LNFP-I/2'-FL. Batch analysis results for carbohydrate parameters remain unchanged or similar following modification to the internal analytical methods. The most perceptible difference is the better separation of LNFP-I fructose isomer and 2'-fucosyl-D-lactulose by high-performance liquid chromatography (HPLC) as a result of newer column technology. The sum of L-fucose and 2'-fucosyl-lactitol remains low and within the 1.0 w/w % specification limit originally indicated for L-fucose alone.

Table 2 **Updated Summary of Product Analyses of LNFP-I/2'-FL**

Parameters[a]	Specification	Method	Manufacturing Batch No.		
			(b) (4) [redacted]	[redacted]	[redacted]
Assay (water-free) – Specified saccharides[b]	≥ 90.0 w/w %	Glycom method HPLC-13-001, HPLC-13-002, HPAEC-HMO-017	94.56	93.44	92.87
Assay (water-free) – LNFP-I and 2'-FL	≥ 75.0 w/w %	Glycom method HPLC-13-002	90.66	88.68	89.06
Assay (water-free) – LNFP-I	≥ 50.0 w/w %	Glycom method HPLC-13-002	62.91	69.50	56.68

Table 2 Updated Summary of Product Analyses of LNFP-I/2'-FL

Parameters[a]	Specification	Method	Manufacturing Batch No.		
			(b) (4) [redacted]	[redacted]	[redacted]
Assay (water-free) – 2'-FL	≥ 15.0 w/w %	Glycom method HPLC-13-002	27.75	19.18	32.38
Lacto-*N*-tetraose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-017	1.55	3.27	1.81
3-Fucosyllactose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017	<0.03	<0.03	<0.03
Sum of L-fucose and 2'-fucosyl-lactitol	≤ 1.0 w/w %	Glycom method HPAEC-HMO-017	0.06	0.05	0.06
D-Lactose	≤ 10.0 w/w %	Glycom method HPAEC-HMO-017	1.33	0.66	0.77
Difucosyl-D-lactose	≤ 2.0 w/w %	Glycom method HPAEC-HMO-017	0.37	0.24	0.65
LNFP-I fructose isomer	≤ 1.5 w/w %	Glycom method HPLC-13-001	0.43	0.40	0.23
2'-Fucosyl-D-lactulose	≤ 1.0 w/w %	Glycom method HPLC-13-001	0.13	0.08	0.15
Sum of other carbohydrates	≤ 6.0 w/w %	Glycom method HPAEC-HMO-017	1.76	1.94	3.23

2'-FL = 2'-fucosyllactose; CFU = colony forming units; E.U. = endotoxin units; LNFP-I = lacto-*N*-fucopentaose I.
[a] Updated specification parameters are indicated in green.
[b] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

FDA.2. *In addition, we request that Glycom address the following:*

- *In Table 1.3-1 (p. 5 of the notice), Glycom listed "Probiotic Drinks" as a proposed food use. Please provide representative examples of food products (or NHANES food codes) that Glycom considered as "Probiotic Drinks" in the dietary exposure assessment.*
- *In the amendment dated May 13, 2022, Glycom stated that 2'-FL from LNFP-I/2'-FL is intended to be partially (or fully) substitutional to other sources of 2'-FL that have GRAS status for use in formula products. Please clarify whether 2'-FL from LNFP-I/2'-FL is intended to be partially substitutional to other sources of 2'-FL that have GRAS status for use in other foods listed in Table 1.3-1 (p. 5 of the notice).*

A single food code in the 2017-2018 cycle of the NHANES was identified as representative of the proposed food use in 'Yogurt Drinks, Probiotic Drinks': 11436000 Yogurt, liquid. Other representative examples of probiotic drink food products not captured by food codes in the 2017-2018 NHANES include Yakult, Activia, and DanActive probiotic drink products.

2'-FL from LNFP-I/2'-FL may also be partially substitutional to other sources of 2'-FL that have GRAS status for use in foods other than formula products listed in Table 1.3-1 (p. 5 of the notice).

From: Darch, Maryse
To: Morissette, Rachel
Cc: Roehrig, Christoph
Subject: [EXTERNAL] RE: follow-up questions for GRN 1035
Date: Thursday, January 12, 2023 2:42:17 PM
Attachments: image001.png
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image010.png
image011.png
GRN 001035 - Response to FDA Questions - 12Jan"23.pdf

Dear Rachel,

Please find attached our responses to the additional questions for GRN 001035. Please do not hesitate to contact us if any further clarification is necessary.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Darch, Maryse
Sent: Monday, January 9, 2023 11:26 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: follow-up questions for GRN 1035

Dear Rachel,

I confirm receipt of the additional questions for GRN 1035. We aim to provide responses before end-of-week.

Kind regards,

Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Monday, January 9, 2023 7:16 AM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: follow-up questions for GRN 1035

Dear Maryse,

Thank you for your responses. Our chemist has a couple follow-up questions based on the new results.

1. In response to Question 1 from the December 22, 2022 amendment, Glycom informed us that the L-fucose specification parameter was replaced with the sum of L-fucose and 2'-fucosyl-lactitol. Please confirm that the analytical results for "specified saccharides" also account for the 2'-fucosyl-lactitol content and that 2'-fucosyl-lactitol should be listed as one of the saccharides in footnote b to Table 2 of the amendment.

2. In response to Question 2 from the December 22, 2022 amendment, Glycom stated that a single food code in the 2017-2018 cycle of the NHANES was identified as representative of the proposed food use in 'Yogurt Drinks, Probiotic Drinks': 11436000 Yogurt, liquid. We note that the following food codes can also be used as representative for dairy-based "probiotic drinks": 11115400 "Kefir, NS as to fat content" and 11112120 "Milk, acidophilus, low fat (1%)." Please clarify if the proposed use of LNFP-I/2'-FL in "probiotic drinks" includes foods such as kefir and acidophilus milk and if the use in this food category is limited to dairy-based "probiotic drinks" only.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark

12 January 2023

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: Additional Questions for GRAS Notice No. GRN 001035

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s follow-up questions received by email on 9 January 2023 pertaining to Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of lacto-*N*-fucopentaose I with 2'-fucosyllactose (LNFP-I/2'-FL) filed by the Agency under GRN 001035.

FDA.1. *In response to Question 1 from the December 22, 2022 amendment, Glycom informed us that the L-fucose specification parameter was replaced with the sum of L-fucose and 2'-fucosyl-lactitol. Please confirm that the analytical results for "specified saccharides" also account for the 2'-fucosyl-lactitol content and that 2'-fucosyl-lactitol should be listed as one of the saccharides in footnote b to Table 2 of the amendment.*

Glycom confirms that the analytical results for "specified saccharides" also account for the 2'-fucosyl-lactitol content following the replacement of the 'L-fucose' specification parameter with 'sum of L-fucose and 2'-fucosyl-lactitol'. The corrected analytical results for the 'Assay (water-free) – Specified saccharides' and 'Sum of other carbohydrates' specification parameters are provided in Table 1 below.

Table 1 Updated Summary of Product Analyses of LNFP-I/2'-FL[a]

Parameters	Specification	Method	Manufacturing Batch No.		
			(b) (4) [redacted]	[redacted]	[redacted]
Assay (water-free) – Specified saccharides[b]	≥ 90.0 w/w %	Glycom method HPLC-13-001, HPLC-13-002, HPAEC-HMO-017	94.63	93.49	92.93
Sum of other carbohydrates	≤ 6.0 w/w %	Glycom method HPAEC-HMO-017	1.70	1.89	3.17

2'-FL = 2'-fucosyllactose; LNFP-I = lacto-*N*-fucopentaose I.
[a] Corrections are indicated in green.
[b] Specified saccharides include LNFP-I, 2'-FL, lacto-*N*-tetraose, difucosyl-D-lactose, 3-fucosyllactose, D-lactose, L-fucose, 2'-fucosyl-lactitol, LNFP-I fructose isomer, and 2'-fucosyl-D-lactulose.

FDA.2. *In response to Question 2 from the December 22, 2022 amendment, Glycom stated that a single food code in the 2017-2018 cycle of the NHANES was identified as representative of the proposed food use in 'Yogurt Drinks, Probiotic Drinks': 11436000 Yogurt, liquid. We note that the following food codes can also be used as representative for dairy-based "probiotic drinks": 11115400 "Kefir, NS as to fat content" and 11112120 "Milk, acidophilus, low fat (1%)." Please clarify if the proposed use of LNFP-I/2'-FL in "probiotic drinks" includes foods such as kefir and acidophilus milk and if the use in this food category is limited to dairy-based "probiotic drinks" only.*

Glycom confirms that the scope of the proposed food uses of LNFP-I/2'-FL includes foods such as kefir and acidophilus milk. The dietary intake assessment included the below food codes for acidophilus milk, but as part of the 'Unflavored Pasteurized and Sterilized Milk' food use.

- 11112120 Milk, acidophilus, low fat (1%)
- 11112130 Milk, acidophilus, reduced fat (2%)

Although the food codes were either unintentionally excluded from the dietary exposure assessment (kefir) or categorized in an alternative food category (acidophilus milks), it should be noted that food codes for kefir and acidophilus milks are not anticipated to be significant contributors to the dietary intakes due to the low number of consumers of these food products in the 2017-2018 cycle of the NHANES (9 consumers of kefir, 1 consumer of low fat acidophilus milk, and 0 consumers of reduced fat acidophilus milk – all consumers, except one, are ≥ 2 years of age). As such, these food codes are expected to have a negligible impact on the estimated daily intakes of LNFP-I, 2'-FL, and the LNFP-I/2'-FL ingredient from the proposed food uses by population group.

Glycom also confirms that the proposed use in 'Yogurt Drinks, Probiotic Drinks' is limited to dairy-based probiotic drinks only.

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We hope this information adequately addresses the Agency's questions on GRN 001035, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
Date: 2023.01.12 14:40:11 -05'00'

Maryse Darch
Regulatory & Scientific Affairs Manager
Glycom A/S