Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

17 January 2022

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 non-crystallized 2'-fucosyllactose (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 non-crystallized 2'-Fucosyllactose (2'-FL) produced by *Escherichia coli* K12 DH1 MDO MAP1001h, 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 non-crystallized 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,

Marta H. Mikš, Ph.D., D.Sc.
Senior Regulatory & Scientific Affairs Manager
Glycom A/S
marta.miks@dsm.com

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 NON-CRYSTALLIZED 2'-FUCOSYLLACTOSE (2'-FL)

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

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

DATE:
17 January 2022

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GRAS Notice for Non-Crystallized 2'-Fucosyllactose (2'-FL)

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

GRAS Notice for Non-Crystallized 2'-Fucosyllactose (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 2'-fucosyllactose (2'-FL), as manufactured by Glycom, 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 1.4 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 2'-FL as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

17 January 2022

Marta H. Mikš, Ph.D., D.Sc.
Senior Regulatory & Scientific Affairs Manager
Glycom A/S
marta.miks@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 the Notified Substance

2'-Fucosyllactose; 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 Background

2'-FL manufactured by Glycom using a microbial fermentation process has previously been determined to be GRAS for use in non-exempt infant formula at a maximum use level of 2,400 mg/L of the ready-to-drink or reconstituted formula, as well as in select conventional food and beverage products. The GRAS conclusion was notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). The 2'-FL described in GRN 650 has become commercialized under the tradename GlyCare™ 2FL 9000 (Glycom A/S, 2016; U.S. FDA, 2016).

The 2'-FL that is the subject of this Notice (tradename GlyCare™ 2FL 8001) is manufactured using downstream manufacturing conditions modified from those described in GRN 650 to produce 2'-FL as a dried amorphous powder without a crystallization step (Glycom A/S, 2016; U.S. FDA, 2016). Throughout the Notice the term "non-crystallized 2'-FL" is used to differentiate it from the crystallized 2'-FL that was the subject of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). Additionally, non-crystallized 2'-FL is manufactured from an optimized variant of the production strain described in GRN 650, although from a highly similar strain belonging to the same *Escherichia coli* K-12 DH1 MDO platform strain family (Glycom A/S, 2016; U.S. FDA, 2016).

1.4 Conditions of Use

Food uses of non-crystallized 2'-FL will be fully substitutional on a wt/wt basis to all GRAS uses of 2'-FL described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). Non-crystallized 2'-FL as described herein is therefore intended for use in term non-exempt infant formulas at a maximum use level of 2,400 mg/L of the ready-to-drink or reconstituted formula. The maximum use level is based on providing a similar level of 2'-FL as that which occurs in mature human breast milk. Non-crystallized 2'-FL also may be used in combination with other human-identical milk oligosaccharides (HiMOs) that have been notified to the Agency as GRAS, *e.g.*, lacto-*N*-neotetraose (LNnT), difucosyllactose (DFL), lacto-*N*-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL), such that levels of each HiMO in a finished infant formula preparation are representative of concentrations that have been measured in human milk, taking into account natural variability. The specific combination of HiMOs will be determined by the infant formula manufacturer based on available clinical data and product development goals.

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 manufacturer of a new infant formula must notify the U.S. FDA at least 90 days before marketing its 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. The manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination [*e.g.*, use of 2'-FL with other HiMOs or with other indigestible oligosaccharides such as galacto-oligosaccharides (GOS)] would be well tolerated as part of the Agency's 90-day notification procedure. Section 412 of the *Federal Food, Drug and Cosmetic Act* therefore ensures that any combination of HiMO, whether used singularly or on an additive basis, will be the subject of corroborative safety and tolerance testing in infants.

Non-crystallized 2'-FL is also intended for use in various conventional food and beverage products across multiple categories, presented in Table 1.4-1. As discussed above, food uses of non-crystallized 2'-FL will be completely substitutional to GRAS uses of 2'-FL that has previously been concluded to be GRAS.

Table 1.4-1 Summary of the Individual GRAS Food Uses and Use Levels for Non-Crystallized 2'-FL in Conventional Food and Beverage Products and Infant Formula*

Food Category	Proposed Food Uses	RACC	GRAS Use Level (g/RACC)	Maximum GRAS Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	240 mL	0.28	1.2
Dairy Product Analogs	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	170 g	0.90	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[a]	0.24	2.4
	Toddler Formulas	100 mL[a]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	1.6	40
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk[b]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavored Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.28	5
	Yogurt	170 g	0.90	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-fucosyllactose; GRAS = Generally Recognized as Safe; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

* Food uses and use levels have been adapted from GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016); with due updates in the RACC to reflect current reference amounts (U.S. FDA, 2021a).

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

[b] Milk is a standardized food in the United States. When the milk is fortified with 2'-FL it will then be classified as a milk product.

1.5 Basis for GRAS

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

1.6 Availability of Information

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

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

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

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

It is Glycom's view that manufacturing batch numbers disclosed in Table 2.3-1 of Part 2 of the GRAS Notice are confidential commercial information and should be exempt from public disclosure under the Freedom of Information Act (5 U.S.C. 552). All other data and information in Parts 2 through 7 of the GRAS Notification do not contain trade secrets and commercial or financial information that are considered privileged or confidential, and may be disclosed to the public.

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

2.1 Identity

2'-FL is a naturally occurring trisaccharide detected in some mammalian milks with the highest concentrations present in human milk and is therefore typically referred to as a human milk oligosaccharide (HMO). 2'-FL is a chemically defined linear trisaccharide consisting of L-fucose, D-galactose, and D-glucose, that occurs as one specific constitutional isomer.

The molecular structure of 2'-FL was first elucidated by Richard Kuhn in 1955 (using classical chemical techniques) (Kuhn *et al.*, 1955) and shortly thereafter by Jean Montreuil (Montreuil, 1956). Since then the structure of 2'-FL has been confirmed independently by others using a range of modern structure characterization techniques, including spectroscopic techniques [*e.g.*, ^{1}H-, ^{13}C, and 2D-nuclear magnetic resonance (NMR)] (Jenkins *et al.*, 1984; Ishizuka *et al.*, 1999; Rundlöf and Widmalm, 2001; Urashima *et al.*, 2002, 2004, 2005; Almond *et al.*, 2004; Wada *et al.*, 2008), mass spectrometric (MS) techniques (Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999), and X-ray crystallography (Kuhn *et al.*, 1956; Svensson *et al.*, 2002).

Based on ^{1}H- and ^{13}C-NMR, MS, and high-performance liquid chromatography with corona charged aerosol detector data (HPLC-cCAD), it is confirmed that 2'-FL produced by microbial fermentation is chemically and structurally identical to 2'-FL present in human breast milk. Further description of the structural and chemical identity of 2'-FL is presented below in Table 2.1-1.

Table 2.1-1 Chemical Identity of 2'-Fucosyllactose

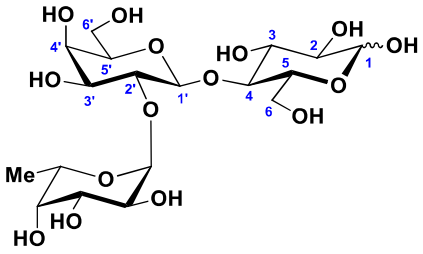
Common Name	2'-Fucosyllactose
Common Abbreviation	2'-FL
Trade Name	GlyCare™ 2FL 8001
Molecular Formula	$C_{18}H_{32}O_{15}$
Molecular Weight	488.44
CAS Registry Number	41263-94-9
CAS Name	*O*-6-Deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose
IUPAC Name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucose
IUPAC Abbreviations	α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-D-Glc (extended) Fuc-(α1-2)-Gal-(β1-4)-Glc (condensed)
Molecular Structure	

Table 2.1-1 Chemical Identity of 2'-Fucosyllactose

Glycan Symbols	
Synonyms	2'-*O*-Fucosyllactose, 2'-*O*-L-Fucosyl-D-lactose, 2'-Fucosidolactose, H-2g (glucose analog of histo-blood group H antigen)
Other Abbreviations	2'-FL (2'FL, 2FL, 2-FL)

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.
Glycan symbols follow Varki *et al.* (2015).

2.2 Manufacturing

2'-FL described in GRN 650 is a high purity crystallized ingredient with a purity of ≥ 94%. Non-crystallized 2'-FL that is the subject of this Notice is produced using the same microbial fermentation process with a highly similar recombinant production strain as described in the supplement to GRN 650 (a direct derivative of the strain of the supplement) (Glycom A/S, 2016; U.S. FDA, 2016). Furthermore, Glycom has implemented a number of unit operation optimizations to the downstream processing (DSP) of 2'-FL that collectively allow for the omission of the crystallization step, and now produces 2'-FL as a dried amorphous powder. A comparison of the original downstream manufacturing conditions and the revised manufacturing conditions for the production of Glycom's non-crystallized 2'-FL preparation are presented below. Where relevant, details of the production organism and other elements of the manufacturing processes that have not changed are incorporated by reference to relevant sections in GRN 650 and its supplement (Glycom A/S, 2016; U.S. FDA, 2016).

2'-FL is manufactured in compliance with current Good Manufacturing Practices (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The raw materials from which 2'-FL is derived include D-lactose as a substrate, with D-glucose[2] and ammonium salts used as energy, carbon, and nitrogen sources for fermentation. The manufacturing process can be broadly divided into two stages: in Stage 1 [upstream processing (USP)], D-lactose is converted to 2'-FL by the cellular enzymes of the 2'-FL production organism. In Stage 2, the DSP, a series of purification and isolation steps generate the final high-purity 2'-FL product.

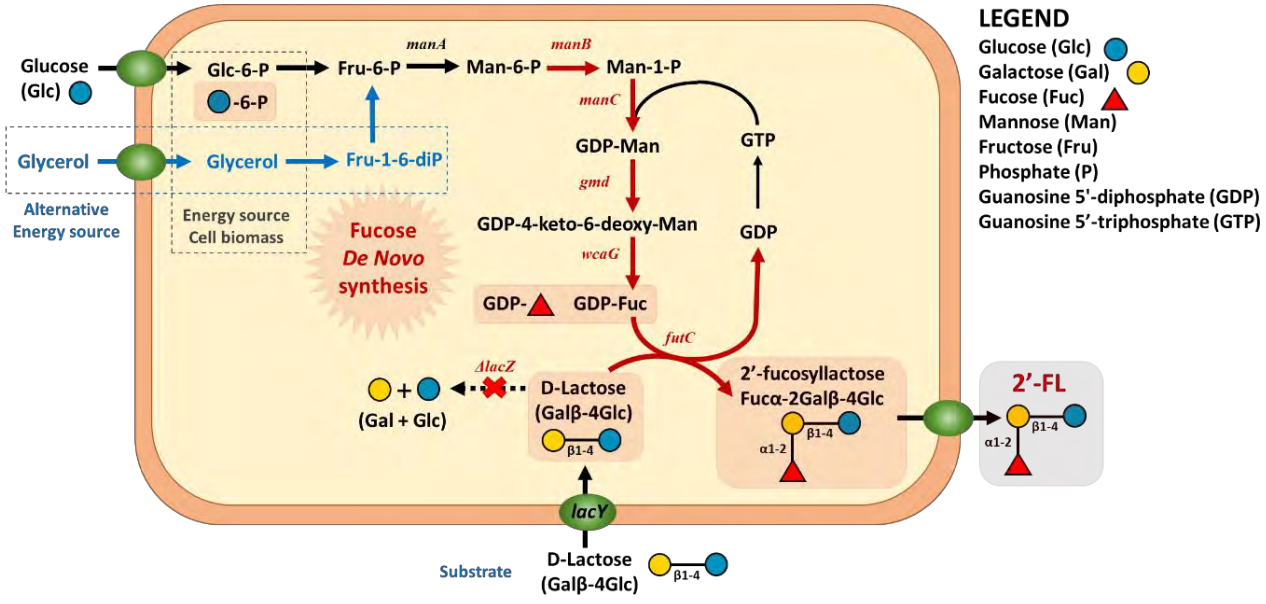
[2] Alternatively, D-sucrose or glycerol.

2.2.1 Description of the Production Microorganism

2'-FL is produced by microbial fermentation using biosynthetic processes introduced into a recombinant strain of *Escherichia coli* (*E. coli*) K-12 DH1. To this end, *E. coli* K-12 DH1 was first optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various sugars, optimizing the strain to become a general HiMO production platform strain (*E. coli* K-12 DH1 MDO). All of Glycom's HiMO manufacturing strains are direct derivatives of this platform strain. The platform strain has then been engineered to furnish the *E. coli* K-12 DH1 MDO MAP1001-series, in particular MAP1001d, allowing for the manufacture of 2'-FL/DFL (detailed information is incorporated here by reference to GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019) or 2'-FL (further information is incorporated here by reference to the supplement to GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016).

The additionally introduced genetic modifications leading from strain *E. coli* K-12 DH1 MDO MAP1001d to the ***E. coli* K-12 DH1 MDO MAP1001h** strain, which is used for the manufacture of non-crystallized 2'-FL, are described here. One copy of a heterologous gene encoding a Major Facilitator Superfamily (MPS) was inserted into the bacterial chromosome of strain *E. coli* K-12 DH1 MDO MAP1001d. The synthetic DNA of the gene was codon optimized at the DNA sequence level to increase the expression of the protein in the production strain. The expressed protein is identical to an MFS protein found in *Rosenbergiella nectarea.* Production of 2'-FL is generated in a biosynthetic reaction involving D-lactose (substrate) and D-glucose[3] (carbon source) as shown Figure 2.2.1-1 below.

Figure 2.2.1-1 Simplified Biosynthesis Pathway of 2'-FL in Recombinant *Escherichia coli* K-12 DH1 MDO MAP1001h



2'-FL = 2'-fucosyllactose.

[3] Alternatively, D-sucrose or glycerol.

Allergen Online[4] was used to compare the FASTA sequence of the introduced protein against known peer-reviewed sequences in the Food Allergy Research and Resource Program (FARRP) allergen database. Searches were conducted using default algorithm settings for (i) matches to the full-length sequences, (ii) matches to 80 amino acid sequence segments (sliding window), and (iii) 8-mer sequence alignments. Full-length FASTA matches with E-values of (<1e-7) and/or sequence identity greater than 50% were considered potentially cross-reactive with the aligned sequence. In accordance with Codex guidelines[5], FASTA also was used to search for 80 amino acid sliding window segments aligning with a match > 35% identity to a protein in the allergen database. In addition, the identification of 8 contiguous amino acids common to the expressed enzyme and a known allergen might be considered a potential risk for immunological cross-reactivity (JECFA, 2016)[6]. No hits above 50% sequence identity with the full protein were found, no hits above 35% sequence identity of 80-mer sliding windows were found, and no hits of sequence identity in 8-mer sliding window peptides were found.

The strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection under deposit number DSM 33313.

2.2.2 Description of the Production Process

2.2.2.1 Manufacturing Stage 1: Fermentation Procedure

The manufacturing process for 2'-FL can be broadly divided into two stages and include upstream processes (Stage 1), which relate to the fermentation and biosynthesis of 2'-FL, as well as downstream processes (Stage 2), which are largely characterized as processes for purification of the ingredient.

In Stage 1 of the manufacturing process, D-lactose and D-glucose[7] are converted by the engineered metabolic pathway of the production microorganism into 2'-FL by cellular fermentation. The fermentation is maintained for several days until in-process controls indicate a favorable ratio of 2'-FL to other carbohydrates and high consumption of D-lactose. 2'-FL is excreted into the fermentation broth, and the microbial biomass containing the production organism is then removed from the culture supernatant containing 2'-FL by ultrafiltration/diafiltration and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of in-process controls and then further purified by the second stage of the production process, the DSP. Detailed information on the fermentation procedures including descriptions of the raw materials is incorporated by reference to Sections II.B.2 through II.B.4 of GRN 650 and Sections 2.2.2 and 2.2.3 of GRN 815 (Glycom A/S, 2016, 2018; U.S. FDA, 2016, 2019).

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

[5] Codex Alimentarius (2003). Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms. (CAC/GL 46-2003; Adopted in 2003, Annexes II and III adopted in 2008). Rome Italy: Codex Alimentarius. Available at: http://www.fao.org/fao-who-codexalimentarius/standards/list-of-standards/en/.

[6] JECFA (2016) Potential allergenicity of enzymes: change to the number of amino acids in segments used in allergen dat abase searches. Eightieth meeting. WHO technical report series; no. 995.

[7] Alternative options of raw materials for energy and carbon source are D-sucrose and glycerol.

2.2.2.2 Manufacturing Stage 2: Purification and Isolation

As discussed, changes to the manufacture of 2'-FL as described in GRN 650 *vs*. non-crystallized 2'-FL as described in this Notice relate to implementation of manufacturing changes to produce a dried amorphous product that does not require crystallization (Glycom A/S, 2016; U.S. FDA, 2016). Omission of the crystallization step has a significant impact on certain aspects of the production process since a crystallization operation comes at a significant premium on cost, waste management (solvents), and through-put of the manufacturing line.

Glycom notes that omission of the crystallization step is feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improves the performance of each purification step) accompanied by a drying procedure (*e.g.*, freeze- or spray-drying, but not limited to these), which will be used instead of crystallization to isolate 2'-FL during DSP. The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH), and these modifications do not change the overall process.

The change eliminates the use of the acetic acid solvent in the production of 2'-FL and has not only an impact on the overall costs of the process, but also has a tremendous impact from an environmental perspective, as it eliminates the use of significant amounts of acetic acid per kg of produced 2'-FL (*ca*. 3.0 kg acetic acid per kg of final 2'-FL) and therefore significantly reduces waste associated with HiMO manufacturing and produces a finished product that is absent of solvent residues.

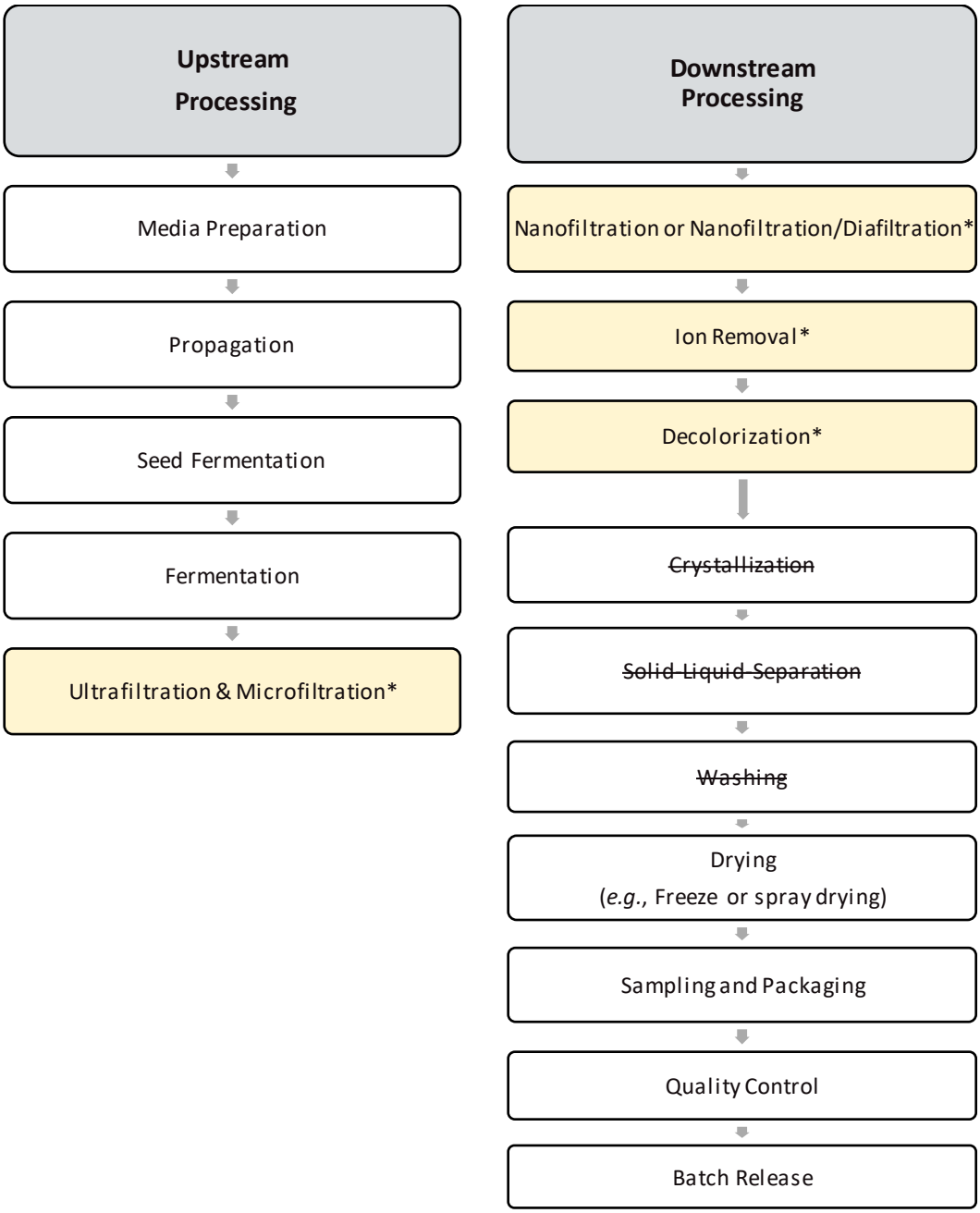
The primary impact of refining the manufacturing process to enable the omission of the crystallization step is the production of a non-crystallized 2'-FL product with slightly higher levels of 2'-FL biosynthesis-related carbohydrates, namely D-lactose (≤ 10.0%) and difucosyl-D-lactose (DFL; ≤ 5.0%); this is in comparison to 2'-FL that is crystallized, where lower levels of D-lactose (≤ 3.0%) and DFL (≤ 1.0%) are obtained. D-Lactose and DFL are naturally present in human milk, and therefore are not considered as "impurities" in the non-crystallized 2'-FL ingredient. As component of the 2'-FL/DFL mixture (notified to the Agency as GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019), the DFL specification level is set at ≤ 20%.[8]

Glycom has analytical data demonstrating that the use of its improved purification procedures in lieu of crystallization largely limits the changes to D-lactose and DFL and does not alter the levels of other potential production strain metabolites or process-related residues (*e.g.*, residual protein, DNA, endotoxins), a conclusion that is attributed to the fact that several purification steps for removal of these compounds (*e.g.*, ion adsorption and de-colorization) are maintained.

As discussed in more detail in Part 6 of this Notice, there are no safety concerns related to the aforementioned purity variation since the differences in purity are predominantly limited to safe and structurally related carbohydrate components. An overview of the revised downstream manufacturing processes for production of 2'-FL is presented below in Figure 2.2.2.2-1.

[8] Please note that primary differences between the non-crystallized 2'-FL and the 2'-FL/DFL mixture (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019) are the stricter DFL content in the case of the non-crystallized 2'-FL (≤ 5% *vs*. ≤ 20%) and the use of another variant of the MAP1001 strain family. A few additional subtle differences are described below in the specifications.

Figure 2.2.2.2-1 Overview of the Downstream Manufacturing Process for Non-Crystallized 2'-Fucosyllactose



* The mandatory purification steps of improved performance have been highlighted in yellow. Steps with strikethrough represent process operations applied for production of the crystallized ingredient that are omitted in the production of the non-crystallized ingredient.

2.2.3 Quality Control

The manufacture of non-crystallized 2'-FL by microbial fermentation is consistent with cGMP and HACCP principles.

Given that the principal raw materials and the final product are well characterized and pure compounds, 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.

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

The production process (including all used processing aids, raw materials, unit operations and filter aids), and the food safety management system comply with Food Safety Systems Certification (FSSC) 22000 and International Organization for Standardization (ISO) 9001.

2.3 Product Specifications and Batch Analysis

The compositional changes resulting from modifications in the production process are largely limited to increased levels of D-lactose and DFL, which consequently lead to a proportional decrease of 2'-FL. Accordingly, the specification for non-crystallized 2'-FL has been revised to reflect these compositional changes (see Table 2.3-1). All methods of analysis are either internationally recognized or developed internally by Glycom. The ingredient is specified as a white to off-white powder or agglomerates with a 2'-FL content of at least 85% based on HPLC-cCAD. Upper limits have been established for the raw materials used in the manufacturing (*e.g.,* D-lactose), the carbohydrates formed during the fermentation (*e.g.*, DFL, L-fucose, 2'-fucosyl-D-lactulose), heavy metals, and microbiological parameters, to ensure the purity of the final product.

Microbiological specifications have been revised from those described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016) to bring the ingredient in alignment with updated specifications that consider the use of Glycom's HiMOs in wet blending of infant formula ingredients during the manufacture of finished powdered products. Glycom has established separate specification parameters for 2'-FL depending on whether the ingredient is utilized in wet-blending or dry-blending of the infant formula production process. The wet-blending stage of manufacturing involves a heat-treatment (retort) step in which sterilization of these microorganisms would occur. Heat-treatments at temperatures above 75°C for 30 seconds will provide a reduction in excess of 10 log units of vegetative microorganisms such as *Salmonella* spp. or *Enterobacteriaceae*, including *Cronobacter sakazakii*; heat-treatments above 100°C will lead to reductions in excess of several hundred log units (WHO, 2006). Microbial specifications used for wet-blending applications will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55 (U.S. FDA, 2021c).

Table 2.3-1 Specifications and Batch Analyses for Non-Crystallized 2'-FL in Comparison to Specifications for Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and Non-Crystallized 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019)

Parameter	Unit	GRAS Specification			Batch Analyses		
		2'-FL GRN 650 (crystallized)	2'-FL/DFL GRN 815 (non-crystallized)	2'-FL (non-crystallized)	[redacted]	[redacted]	[redacted]
Appearance		**Powder, agglomerates, powder with agglomerates**			Complies	Complies	Complies
Color		**White, white to off-white, off-white**			Complies	Complies	Complies
Assay (water-free) specified saccharides[a]	w/w %	≥ 96.0	≥ 92.0	**≥ 90.0**	92.3	92.8	94.4
Assay (water-free) 2'-fucosyllactose	w/w %	≥ 94.0	≥ 75.0	**≥ 85.0**	87.7	88.9	90.3
Difucosyl-D-lactose	w/w %	≤ 1.0	≤ 20.0	**≤ 5.0**	1.90	1.89	1.78
D-Lactose	w/w %	≤ 3.0	**≤ 10.0**		2.03	1.16	2.00
L-Fucose	w/w %	≤ 1.0	**≤ 1.0**		0.06	0.06	< 0.03
2'-Fucosyl-D-lactulose	w/w %	≤ 1.0	≤ 2.0	**≤ 1.5**	0.30	0.55	0.24
pH in 5% solution (20°C)		3.2–5.0	3.5–5.4	**4.0–6.0**	4.7	4.8	5.3
Water	w/w %	≤ 5.0	≤ 6.0	**≤ 9.0**	5.38	5.24	2.3
Ash, sulphated	w/w %	≤ 1.5	≤ 0.8	**≤ 0.8**	< 0.05	0.1	< 0.01
Acetic acid	w/w %	≤ 1.0	NA[b]		NA[b]	NA[b]	NA[b]
Residual proteins by Bradford assay	w/w %	**≤ 0.01**			Complies	Complies	Complies
Residual endotoxins	E.U./mg	**≤ 10**			Complies	Complies	Complies
Lead	mg/kg	≤ 0.1		**≤ 0.05**	0.011	< 0.05	< 0.01
Microbiological Criteria[c]							
Aerobic mesophilic bacteria total count	CFU/g	≤ 500	**≤ 1,000**		Complies	Complies	Complies
Enterobacteriaceae	10 g/CFU/g	Absent	**≤ 10**		Complies	Complies	Complies
Salmonella	25 g	**Absent**			Complies	Complies	Complies
Cronobacter (Enterobacter) sakazakii	10 g	Absent	NA[d]		NA[d]	NA[d]	NA[d]
Listeria monocytogenes	25 g	Absent	NA[d]		NA[d]	NA[d]	NA[d]
Bacillus cereus	CFU/g	≤ 50	NA[d]		NA[d]	NA[d]	NA[d]
Yeasts	CFU/g	≤ 10	**≤ 100**		Complies	Complies	Complies
Molds	CFU/g	≤ 10	**≤ 100**		Complies	Complies	Complies

Table 2.3-1 Specifications and Batch Analyses for Non-Crystallized 2'-FL in Comparison to Specifications for Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and Non-Crystallized 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019)

2'-FL = 2'-fucosyllactose; CFU = colony forming units; DFL = difucosyllactose; E.U. = endotoxin units; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; NA = not applicable.

[a] Assay (water-free) specified saccharides includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose and 2'-fucosyl-D-lactulose. In GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016) the parameter HiMS (human-identical milk oligosaccharides) is used and includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose.

[b] Acetic acid is used as a processing aid during the crystallization step of the production process described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

[c] The microbial specifications listed represent minimum requirements for 2'-FL that is added to infant formula and toddler formula products during the wet-mix stage of the infant formula manufacturing process, and also is suitable for conventional food products used by the general population (*i.e.,* non-infant formula and toddler formula food products). The minimum microbial requirements for 2'-FL that is added during the dry-blending stage of infant formula manufacturing include the following additional parameters: *Cronobacter (Enterobacter) sakazakii* (Absent in 10 g), *Listeria monocytogenes* (Absent in 25 g), and *Bacillus cereus* (not more than 50 CFU/g).

[d] Not applicable when 2'-FL is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior to drying.

2.3.1 Additional Quantitative and Qualitative Analyses

The main changes applied to the production process relative to those described for 2'-FL in GRN 650 involve optimization of the purification steps during DSP, accompanied with another isolation and drying procedure (*e.g.*, freeze- or spray-drying) instead of the crystallization step (Glycom A/S, 2016; U.S. FDA, 2016). Accordingly, previous optional unit operations (*e.g.*, ion adsorption filtration) have become mandatory. Removal of the intact production microorganism occurs early in the process during USP, and all other purification steps of the DSP (*e.g.*, filtration, decolorization) remain in place. As such, the residual levels of the production microorganism or its metabolites remaining in non-crystallized 2'-FL will not be affected by this change in the production process. Data and information characterizing the quantitative and qualitative purity of 2'-FL are therefore incorporated by reference to Sections II.C.4 of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

2.4 Stability

Aside from the minor changes in the levels of some of the existing saccharides in Glycom's non-crystallized 2'-FL (*e.g.*, an increase in D-lactose and DFL), the modification to the processing method (as described above) does not affect the structural/chemical identity of 2'-FL itself. As such, no changes are expected with regard to the stability profile of non-crystallized 2'-FL obtained from a genetically modified strain of *E. coli* K-12, neither during bulk storage nor when incorporated into food matrices. Bulk stability data on non-crystallized 2'-FL confirm the stability over its intended storage period (data provided upon request).

Part 3. § 170.235 Dietary Exposure

Glycom has not changed the intended uses of non-crystallized 2'-FL relative to those previously described for crystallized 2'-FL described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). Dietary exposures to 2'-FL from uses in infant formula and conventional food products are therefore incorporated by reference to Section IV.A of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

The use of non-crystallized 2'-FL as described herein will be completely substitutional to the GRAS uses of 2'-FL described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016); therefore, introduction of the non-crystallized form of 2'-FL to the U.S. market will not change dietary exposures to 2'-FL. While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, such as 2'-FL, LNnT, DFL, LNT, 3'-SL, and 6'-SL, will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. 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 lactational stages considering natural variation. Consequently, 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 human breast milk (Viverge *et al.*, 1985, 1990; Coppa *et al.*, 1999; Gabrielli *et al.*, 2011; Alderete *et al.*, 2015; Xu *et al.*, 2017).

Part 4. § 170.240 Self-Limiting Levels of Use

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

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

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

6.1 Introduction

Glycom has conducted a scientific procedures GRAS evaluation of non-crystallized 2'-FL for use as an ingredient in infant formula and specified conventional food applications marketed to the general population. As described previously in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016), the addition of 2'-FL to infant formula and conventional food products at specified use levels has been concluded to be GRAS on the basis of scientific procedures. The GRAS conclusion was notified to the offices of the U.S. FDA and filed without objection by the Agency under GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). In addition, 2'-FL/DFL [specified to contain 2'-FL at no less than 75% (w/w)] was notified and filed without objection by the Agency under GRN 815 (Glycom A/S, 2018; U.S. FDA, 2019). More recently, 2'-FL produced by *E. coli* K-12 strain MG1655 INB000846 has also been notified to the Agency as GRAS under GRN 897 (notified by DuPont Nutrition and Health) (DuPont Nutrition and Health, 2019; U.S. FDA, 2020). Data and information supporting the safety of 2'-FL are therefore incorporated by reference to Section IV of GRN 650 and Part 6 of GRNs 815 and 897 (Glycom A/S, 2016, 2018; DuPont Nutrition and Health, 2019; U.S. FDA, 2016, 2019, 2020).

Glycom has implemented several revisions to the manufacturing process of non-crystallized 2'-FL, resulting in slight changes to the specification of 2'-FL described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). The predominant change in Glycom's production process of non-crystallized 2'-FL compared to that described previously in GRN 650 is the omission of crystallization, which has become feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improve the performance of each purification step) accompanied with drying steps that will be used instead of crystallization to isolate 2'-FL during DSP (Glycom A/S, 2016; U.S. FDA, 2016). The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH) that do not change the overall process.

Changes to the specifications for 2'-FL are summarized in Table 2.3-1 above. Namely, the proposed changes are justified as follows:

- The increase in the specification limit for D-lactose from ≤ 3.0 to ≤ 10% will not have any impact on the safety of 2'-FL, given that D-lactose is already a major component of milk/dairy, breast milk, and infant formula, and any intake of D-lactose from its presence in 2'-FL would be negligible in comparison to the formulation of an infant formula.

- The increase in the specification limit for DFL from ≤ 1.0 to ≤ 5.0% will not have an impact on the safety of 2'-FL as it has already been concluded to be GRAS in the case of GRN 815 of 2'-FL/DFL with levels of DFL ≤ 20% (Glycom A/S, 2018; U.S. FDA, 2019).

- Due to the increased content of D-lactose and DFL, the level of 2'-FL (on a w/w % basis) is reduced accordingly and Glycom specifies it at ≥ 85.0%.

- As an additional measure of purity, and reflecting that Glycom's non-crystallized 2'-FL still represents a highly purified ingredient, a new specification limit of ≥ 90% for the sum of specified saccharides (*i.e.*, 2'-FL, D-lactose, DFL, L-fucose, and 2'-fucosyl-D-lactulose) is applied.

- Additionally, to harmonize the microbiological criteria across all of Glycom's HiMOs, and to consider the use of this ingredient during the wet blending stage of infant formula manufacturing, the following microbiological criteria were established: aerobic mesophilic bacteria total count ≤ 1,000 CFU/g, *Enterobacteriaceae* ≤ 10 CFU/g, *Salmonella* absent in 25 g, yeasts count ≤ 100 CFU/g, and molds count ≤ 100 CFU/g.

Glycom has presented analytical data, which demonstrate that non-crystallized 2'-FL has no significant difference in nutritional value, metabolism, or level of undesirable substances when compared to the 2'-FL that has GRAS status under GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

The production microorganism used for the synthesis of Glycom's non-crystallized 2'-FL ingredient is directly derived from the production strain that is used to manufacture crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019). Any changes to the production strain have been subject to a comprehensive risk assessment through a combination of bioinformatic evaluation and phenotypic characterization. Since 2'-FL is secreted into the culture medium, and the production strain itself is removed intact during USP (Stage 1), the modifications made to the final DSP steps (improved purification process instead of crystallization) will not have any impact on transfer of components of the production organism (*e.g.*, protein) to the finished product. This is supported by analytical data demonstrating the absence of the production strain and residual protein (*i.e.*, below the limit of quantitation of 0.0017%) in non-crystallized 2'-FL.

For the purposes of identifying any new data relevant to the safety of 2'-FL published since the most recent 2'-FL GRAS determination notified to the U.S. FDA with a no questions response (*i.e.*, GRN 897; DuPont Nutrition and Health, 2019; U.S. FDA, 2020), a comprehensive search of the published scientific literature was conducted on 13 December 2021 spanning the period of September 2019 to December 2021. The search was conducted using the electronic search tool, ProQuest, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS reviews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. A discussion of all newly available published and unpublished studies, both favorable and unfavorable, is presented below.

6.2 Absorption, Distribution, Metabolism, and Excretion (ADME)

The powder properties (amorphous *vs*. crystalline) of the 2'-FL ingredient are of no relevance to its use in infant nutrition products since the production of most infant beverage formulations includes dissolution, heat-treatment, and spray-drying during manufacturing of the food/beverage, converting any crystalline component into an amorphous powder. Moreover, since any potential difference in dissolution properties of amorphous and crystalline 2'-FL is equalized through food formulation processes, there will be no difference in the absorption, distribution, metabolism, and excretion (ADME) profile of the ingredients. Apart from powder properties, the proposed specification varies only slightly in the ratio between certain compositional constituents (*e.g.*, D-lactose), and the amount of D-lactose that would result from the intake of non-crystallized 2'-FL would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula).

For a comprehensive discussion on the ADME profile of 2'-FL the reader is directed to Section IV.D of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

6.3 Toxicological Studies

The general recognition of safety of 2'-FL under the specified conditions of use in term infant formula and conventional food and beverage products is largely based on published studies characterizing the concentrations of 2'-FL in human milk (see Section IV.B of GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016), the corresponding history of safe consumption of 2'-FL by breastfed infants, and data demonstrating that 2'-FL is of high purity and is structurally identical to naturally occurring 2'-FL in breast milk.

The results of published and unpublished toxicological studies in neonatal and mature rats further corroborate the safety of the ingredient. Comprehensive discussions of the published toxicity studies as they apply to the safety of 2'-FL for use in infant formula and foods are incorporated by reference to Section IV.B.5 of GRN 546 (Glycom A/S, 2014; U.S. FDA, 2015), Section IV.E of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016), Part 6 of GRN 815 (Glycom A/S, 2018; U.S. FDA, 2019), and Part 6 of GRN 897 (DuPont Nutrition and Health, 2019; U.S. FDA, 2020). These studies included a 90-day oral toxicity study in neonatal rats, and *in vivo* and *in vitro* genotoxicity assays undertaken on high-purity 2'-FL preparations (or 2'-FL-containing mixtures). Findings from these studies demonstrated that 2'-FL is not genotoxic and is of low toxicity potential following gavage dosing in neonatal pups.

Glycom identified recent published toxicological assessments of mixtures containing 2'-FL (including 2'-FL in combination with 3-fucosyllactose, LNT, 3'-SL, and 6'-SL[9], and 2'-FL in combination with lacto-*N*-fucopentaose I[10]) (Parschat *et al.*, 2020; Phipps *et al.*, 2021). These results confirm that these mixtures were not genotoxic and the no-observed-adverse-effect-levels (NOAELs) were established as the highest dose tested. The absence of adverse or toxicity effects reported in the literature on 2'-FL (and mixtures thereof) in animal models is consistent with its natural presence in human milk.

Glycom has also undertaken corroborative product-specific studies that re-affirm the safety of 2'-FL (as a non-crystallized ingredient produced by the methods described in this Notice). Non-crystallized 2'-FL was subject to a bacterial reverse mutation assay conducted in accordance with the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (*Bacterial reverse mutation test*) (OECD, 2020); an *in vitro* mammalian cell micronucleus assay in accordance with OECD Test Guideline 487 (*In vitro mammalian cell micronucleus test*) (OECD, 2016); and a modified 90-day repeat dose toxicity study in neonatal rats in accordance with OECD Test Guideline 408 (*Repeated dose 90-day oral toxicity study in rodents*) (OECD, 2018).

The results of the genotoxicity studies, summarized in Table 6.3-1, confirmed that non-crystallized 2'-FL was non-mutagenic and not clastogenic or aneugenic under the conditions of the studies (Dunton, 2020a,b [unpublished]).

[9] The test article described in Parschat *et al.* (2020) contained 47% 2'-FL, 16% 3-fucosyllactose, 24% LNT, 4% 3'-SL, and 4% 6'-SL.
[10] The test article described in Phipps *et al.* (2021) contained 59% lacto-*N*-fucopentaose I and 32% 2'-FL.

Table 6.3-1 Summary of Genotoxicity Studies Conducted on Non-Crystallized 2'-FL as Described in this Notice

Test	Concentration	Metabolic Activation	Result
Bacterial reverse mutation (*Salmonella* Typhimurium and *Escherichia coli* tester strains	50, 150, 500, 1,500, and 5,000 µg/plate	± S9	Negative
Micronucleus assay (cultured peripheral human lymphocytes)	65, 200, 650, or 2,000 µg/mL	± S9	Negative

2'-FL = 2'-fucosyllactose; S9 = activation with Aroclor 1254-induced rat liver S9.

Results of the 90-day repeat dose toxicity study in neonatal rats are summarized in the sub-section below.

6.3.1 90-Day Repeat Dose Toxicity Study on Non-Crystallized 2'-FL

A corroborative 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of non-crystallized 2'-FL (produced by the methods described in this Notice), when administered by gavage to neonatal rats from Day 7 of age (MacGregor, 2021 [unpublished]). 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 non-crystallized 2'-FL is primarily intended for use in infant formula) to consider the requirements of *EFSA Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age* (EFSA, 2017), *Guidance for industry: nonclinical safety evaluation of pediatric drug products* (U.S. FDA, 2006), *Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications* (EMEA, 2008), and the *Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs* (MHLW, 2018).

Twenty neonatal Crl:CD(SD) rats (7 days of age; 10 male and 10 female) were orally provided non-crystallized 2'-FL by gavage once daily at concentrations of 0, 1,000, 3,000, or 5,000 mg/kg body weight/day for 90 days. Doses of 2'-FL were corrected to account for "other carbohydrates" within the test article batches (therefore, the highest dose corresponded to a total carbohydrate amount of 5,935 mg/kg body weight/day). Another 10 rats (5/sex) in the vehicle control and high dose groups were dosed daily for 90 days then kept undosed for 4 weeks to assess the reversibility of any observed effects seen in the dosing period. There were 9 deaths during the course of the study, but none of the deaths were considered to be test item-related. Gavage administration of non-crystallized 2'-FL to neonatal rats for at least 90 days at doses up to 5,000 mg/kg body weight/day (5,935 mg/kg body weight/day total carbohydrates), was well tolerated and not associated with any test item-related adverse effects. Therefore, the NOAEL was concluded to be 5,000 mg of 2'-FL/kg body weight/day, the highest dose tested (see Table 6.3.1-1).

Table 6.3.1-1 Summary of Repeat-Dose Oral Toxicology Study Conducted on Non-Crystallized 2'-FL

Species	Dose (mg/kg/day bw)	Duration (days)	NOAEL (mg/kg bw)	Reference
Rat [Crl:CD(SD)]	1,000, 3,000, or 5,000 (2'-FL produced by microbial fermentation – Glycom)	90	5,000*	MacGregor (2021) [unpublished]

2'-FL = 2'-fucosyllactose; bw = body weight; NOAEL = no-observed-adverse-effect level.
* Highest dose tested.

6.4 Other Studies

Glycom's updated literature search identified limited new information from various exploratory studies evaluating putative biological roles/effects of 2'-FL in human nutrition. Overall, there was no new information from investigational studies of 2'-FL *in vitro* or in animal models to suggest that use of 2'-FL as an ingredient in infant formula or conventional foods would be unsafe.

6.5 Human Studies

6.5.1 Controlled Intervention Studies

Several clinical studies in infants receiving infant formula containing 2'-FL alone or in combination with other oligosaccharides [LNnT, GOS, or short-chain fructo-oligosaccharides (scFOS)] and one study in adults administered 2'-FL and/or LNnT have been reviewed previously in GRN 650 (Elison *et al.*, 2016; Puccio *et al.*, 2017), GRN 815 (Marriage *et al.*, 2015; Alliet *et al.*, 2016; Goehring *et al.*, 2016; Steenhout *et al.*, 2016) and GRN 897 (Kajzer *et al.*, 2016; Nowak-Wegrzyn *et al.*, 2019). Comprehensive discussions of these studies can be found in Section IV.F of GRN 650, Section 6.4 of GRN 815 (Glycom A/S, 2016, 2018; U.S. FDA, 2016, 2019), and Section 6.3 of GRN 897 (DuPont Nutrition and Health, 2019; U.S. FDA, 2020).

The results of the updated literature search identified 10 new interventional clinical trials in which endpoints related to the safety of 2'-FL were identified including a study in infants conducted with a HiMO mixture containing 2'-FL. In addition, the results of 2 unpublished studies in infants provided formula containing 2'-FL and *Lactobacillus reuteri* were also identified (Corsello *et al.*, 2020 [abstract]; Hascoët *et al.*, 2021 [abstract]). These studies are summarized in the subsections below and are tabulated in Section 6.5.1.13. Studies exclusively examining benefits of 2'-FL supplementation were not included herein.

6.5.1.1 2'-FL in a 100% Whey, Partially Hydrolyzed Infant Formula (Storm et al., 2019)

A randomized, controlled, double-blind, multi-center study was conducted to evaluate the feeding tolerance of 2'-FL in healthy infants enrolled at 2 weeks of age (± 5 days) [Storm *et al.,* 2019; National Clinical Trial number (NCT) 03307122]. The infants were randomly assigned to receive one of two infant formulas for 6 weeks. Both formulas contained a 100% whey, partially hydrolyzed protein base, and both were supplemented with the probiotic *Bifidobacterium lactis* strain Bb12. Both formulas provided 0.67 kcal/mL and 2.2 g protein/L. The only difference between the two formulas was the addition of 0.25 g/L 2'-FL to the test formula. At the enrollment visit (V0), the Infant Gastrointestinal Symptom Questionnaire (IGSQ) was administered and anthropometric measurements were taken by trained study staff. Caregivers began to feed the infants with either the test or control formula *ad libitum* after V0. Following 42 days of feeding, subjects returned for a second visit (V1). For two days prior to V1, caregivers completed a diary recording the amount of formula intake, stool parameters (frequency, consistency, and whether the infant had difficulties passing bowel movements), the frequency of spit-up and vomit, and duration of crying and fussing. At V1, the IGSQ and anthropometric measurements were repeated.

Seventy-nine infants were enrolled and 63 completed the study per protocol (n=30 in the Test group, n =33 in the Control group). Body weights and lengths of the infants were similar between the Test and Control groups at both baseline (V0) and at the end of the 6-week intervention period (V1). No serious adverse events were reported in the study, and the incidence of adverse events was comparable between the Test and Control groups. No significant differences were observed in the IGSQ scores between the Test and Control groups. There were also no significant differences in the stool frequency or consistency , and the duration of crying and fussing and vomiting frequency were similar between groups. The study authors reported that no safety concerns were noted with either of the study formulas. Partially hydrolyzed infant formula containing 2'-FL and *B. lactis* is well tolerated, though it was also recognized that the level of 2'-FL tested in this study is in the lower ranges of what has been reported in human milk.

6.5.1.2 Use of a Partially Hydrolyzed 100% Whey-Based Infant Formula with Lactobacillus reuteri in Infants with Caregiver-Perceived Intolerance (Czerkies et al., 2019)

The effect of switching exclusively formula-fed infants with perceived formula intolerance to a reduced lactose, partially hydrolyzed 100% whey-based formula (PHF-W) with *Lactobacillus reuteri* and 2'-FL was evaluated in a single-arm and single-blind study (Czerkies *et al.*, 2019; NCT03679234). A total of 50 healthy, full-term infants (27 males, 23 females; 14 to 60 days of age) with caregiver-perceived fussiness (*i.e.*, identified as "very" or "extremely" fussy) were eligible to enroll in the study. The infants switched from their current formula to receive the test formula for 3 weeks. The infants gastrointestinal-related signs and symptoms were measured on the IGSQ, a validated 13-item questionnaire. The IGSQ was performed at baseline and repeated after 3 weeks of test formula consumption. The test formula was a commercially available 100% whey, partially hydrolyzed infant formula (2.2 g protein/100 kcal) with reduced lactose (30% of carbohydrate source), 2'-FL (0.25 g/L reconstituted formula) and L. *reuteri* (1×10^6 CFU/g powder). Caregivers documented the volume of formula consumed and the infant's fussiness after each of the first three feedings with the test formula. The test subjects returned to the study site after 3 weeks and the caregivers completed the IGSQ and formula satisfaction questionnaire. Serious or non-serious adverse events were recorded throughout the study period.

Following 3 weeks of test formula use, the IGSQ significantly decreased from baseline in both the intention-to-treat and per-protocol populations. When compared to baseline, the fussiness score was also significantly decreased after the first, second, and third feedings of the test formula, and by 24 hours after the first site visit. No subjects worsened in their fussiness score at any time point. Ninety-three percent of caregivers stated their infants were comfortable on the test formula, 95% were satisfied with the test formula and all reported their infants liked the test formula. Two infants withdrew from the study due to adverse event (one with hard stools, rash, gastroesophageal reflux disease; one with bronchiolitis). Twelve subjects reported 17 adverse events during the study, with only one adverse event reported as having a probable relationship to the test formula (*i.e.*, hard stools, which led to withdrawal of that subject from the study). Overall, the study authors concluded that the PHF-W formula was associated with a reduction in infant fussiness and improved gastrointestinal comfort after 3 weeks of feeding.

6.5.1.3 Real-World Study in Infants Fed 2'-FL and LNnT (Román Riechmann et al., 2020)

A non-randomized, open-label, prospective study was conducted in healthy, term infants (Román Riechmann *et al.*, 2020; NCT04055363). In this real-world study, infants were enrolled at age 7 days to 2 months and fell into one of three groups: an exclusively formula-fed group, a mixture of formula and human milk fed, or exclusively breastfed infants (serving as a reference population). Formula-fed infants received a partially hydrolyzed, 100% whey, term infant formula (67 kcal/100 mL, 1.9 g protein/199 kcal, 11.5 g carbohydrates/100 kcal, 5.1 g lipids/100 kcal, 1.0 g 2'-FL/L, and 0.5 g LNnT/L) that contained *L. reuteri*[11] (dose not reported), vitamins, and minerals, *ad libitum* for 8 weeks.

Anthropometry measures (weight, length, head circumference) were measured at baseline and at Week 8. z-scores for weight-for-age, length-for-age, head circumference-for-age, and body mass index-for-age were calculated. Gastrointestinal symptoms were evaluated *via* the IGSQ. Adverse events were recorded from the time of enrollment through the end of study.

A total of 66 exclusively formula-fed, 48 mixed-fed, and 45 exclusively breastfed infants were included in the analyses. When comparing baseline characteristics of the enrolled infants, the exclusively formula-fed group was slightly younger at enrollment ($p < 0.01$) and had a higher proportion of male infants ($p > 0.05$) compared to the mixed-fed and breastfed group. Consistent with the slightly younger age group, baseline weight and length were slightly lower in the exclusively formula-fed group. Other baseline anthropometric characteristics were comparable across groups.

Through the study, age-appropriate growth was reported in all groups. Differences in baseline weight and length did not persist by Week 8; there were no significant differences between any groups for any of the anthropometric measures. The composite IGSQ scores showed low gastrointestinal distress in all groups at all time points. No significant differences were reported in four of the subdomains of gassiness, fussiness, crying, and spitting-up/vomiting. In the last subdomain of stooling, the formula-fed and mixed feeding group exhibited a statistically significant different score at baseline compared to exclusively breastfed infants. This was significantly improved at Week 8 in exclusively formula-fed infants, with scores moving closer to the stooling profile of the exclusively breastfed group. Stooling scores in mixed-fed infants remained significantly different at Week 8.

Three patients experienced potentially product-related adverse events, including two instances of cow's milk intolerance (one in the exclusively formula-fed group, one in the mixed-feeding group and one instance of irritability in the exclusively formula-fed group). No serious adverse events were attributed to the study feeding. The authors noted that the incidence of adverse events was low overall and was not significantly different between the groups.

[11] Published as *Lactobacillus reuteri* but in current day is referred to as *Limosilactobacillus reuteri*.

6.5.1.4 Randomized, Double-Blind, Controlled Study in Infants Provided Formula with 2'-FL and Lactobacillus reuteri (Corsello et al., 2020 [abstract])

A randomized, double-blind, controlled trial was conducted in healthy infants who received cow's milk-based infant formula (Corsello *et al.*, 2020 [abstract]; NCT03090360). In the study, 289 infants aged less than 14 days old residing in Italy and Belgium were provided a standard bovine milk-based whey-predominant term infant formula containing *L. reuteri*[12] at 1 x 10^7 CFU/g (control) or the same formula supplemented with 1.0 g/L of 2'-FL (test formula) until Day 180. Subjects were allowed progressive introduction of complementary foods or liquids after Study Day 120 (approximately 4 months of age). A non-randomized group comprising of infants exclusively breastfed to 4 months served as a reference (n=60). The primary outcome was weight gain (in g/day) through 4 months of age. Secondary safety endpoints included additional anthropometric measures, stool pattern, gastrointestinal tolerance (including spitting-up, flatulence), associated behaviors (including crying, fussiness, sleep) *via* a 3-day parent diary, and adverse events. Secondary efficacy endpoints included an analysis of the fecal bacterial species at 1, 2, and 3 months of age, and enterotoxin targets *via* quantitative polymerase chain reaction (qPCR).

Among the 352 infants screened, 289 were randomized to receive either the control or test formula. Fifty-six infants receiving control formula, 44 infants receiving test formula, and 25 breastfed infants did not complete the study. The main reason for non-completion was withdrawal without explanation (n=62), followed by withdrawal by adverse event (n=29), withdrawal with explanation (n=24), lost to follow-up (n=4), and other (n=6). The mean duration of formula intake and the volume of formula consumed per day were comparable between the two formula-fed groups.

The results of the safety measures indicated that weight gain was similar between formula-fed groups and was above the non-inferiority margin (defined as -3 g/day). Anthropometric z-scores for weight-for-age, length-for-age, weight-for-length, and head circumference-for-age were comparable between all groups including the reference group and/or statistical differences were not clinically relevant. Growth measures including weight-for-age, length-for-age, head circumference-for-age, and weight-for-length for all infants were plotted individually against World Health Organization (WHO) reference standards. Both formulas were well tolerated and no differences in stool characteristics were reported. Parent-reported gastrointestinal symptoms and behavioral patterns, and physician-confirmed gastrointestinal adverse events were low and similar between formula groups.

The study authors concluded that *L. reuteri*-containing infant formula supplemented with 2'-FL at 1.0 g/L supported age-appropriate growth and was well tolerated.

[12] Published as *Lactobacillus reuteri* but in current day is referred to as *Limosilactobacillus reuteri*.

6.5.1.5 2'-FL in a Hypoallergenic Extensively Hydrolysed Infant Formula (Ramirez-Farias et al., 2021)

The safety and tolerance of 2'-FL in an extensively hydrolyzed casein-based infant formula was investigated in a prospective, single arm, non-randomized, multi-center study (Ramirez-Farias *et al.*, 2021; NCT03884309). Infants less than 60 days of age with persisting feeding intolerance or suspected food protein allergy sensitivity (or other conditions for which an extensively hydrolyzed formula was deemed appropriate) were enrolled in the study. Infants were excluded if they had an allergy or intolerance to any ingredient in the study product, were receiving oral or inhaled steroids, or were treated with antibiotics or other medications that may affect the growth, gastrointestinal tolerance, and/or development within 2 weeks prior to enrollment. Exclusion criteria also included an adverse maternal, fetal, or participant medical history that were deemed to have potential effects on growth and/or development.

Infants were provided a commercially representative [13] hypoallergenic casein-based, powdered, extensively hydrolyzed formula containing 0.2 g/L of 2'-FL (Abbott Nutrition, Abbott Laboratories, Columbus, OH, USA) for 60 days. At Visit 1, eligible infants already consuming an extensively hydrolyzed formula without 2'-FL were switched to the study formula and were provided the formula *ad libitum* as their sole source of nutrition. Weight, length, and head circumference measurements were collected at study entrance, Visit 3 (30 ± 3 days), and Visit 4 (60 ± 3 days). Formula intake, stool records, and an Infant Feeding and Stooling Pattern Questionnaire were completed at Visit 1, and daily formula intake and stool observations were recorded up to Visit 2 and 3 consecutive days before Visits 3 and 4.

The primary outcome was maintenance of weight-for-age z-score during the study. Secondary variables included mean rank stool consistency, predominant stool color, predominant stool consistency, average number of stools per day, percent of feedings with spit-up/vomit associated with feeding, weight, interval weight gain per day, length, interval length gain per day, head circumference, and interval head circumference gain per day. Clinical symptoms were reviewed at enrollment, Visit 2, and Visit 4. Adverse events and serious adverse events were monitored during the study.

Forty-seven infants were included in the intent-to-treat analysis[14]. Of the 48 infants enrolled, 36 completed the study. Adverse events were reported in 15 (32%) of the infants in the intent-to-treat cohort. Most adverse events were mild in severity and deemed by the study investigators as unrelated to the study formula. Commonly reported adverse events included seborrheic dermatitis (n=5), gastrointestinal reflux disease (n=3), and infantile spit-up (n=2).

The average volume of study formula intake was 754 mL/day at Study Days 1 through 7, 850 mL at Study Days 28 through 30, and 935 mL at Study Days 58 through 60. Weight z-scores showed a statistically significant improvement from Day 1 to 60. Seventy-eight percent of infants maintained their weight-for-age z-scores, exceeding the hypothesized percentage of 70%. Clinical history and symptom recording indicated that eczema resolved or improved in 71% of infants and the remaining 29% remained the same. Vomiting resolved or improved in 100%, constipation resolved or improved in 84%, spit-up/gagging/reflux improved in 59%, fussiness improved or resolved in 40%, diarrhea resolved in 50%, and blood in stool resolved in 100% of infants, as evidenced in the clinical history and symptoms records at Day 7. At Day 60, parents reported eczema resolved or improved in 72%, vomiting resolved in 75%, constipation resolved or improved in 83%, spit-up/gagging/reflux improved in 71%, "fussiness" improved or resolved in 60%, and blood in stool remained resolved in 100% of infants. It was noted by the study authors that tolerance measures (including

[13] The study formula, with the exception of the added 2'-FL, is commercially available in the U.S.
[14] Of the 48 infants that were enrolled, one infant did not receive study product.

formula intake, stool characteristics, and symptoms) were comparable to other extensively hydrolyzed formula feeding studies that did not contain 2'-FL.

Overall, the study investigators concluded that "[extensively hydrolyzed formula with added 2'-FL] *was safe and well tolerated and consumption of the formula over 60 days showed improvement and resolution of persistent symptoms*."

6.5.1.6 2'-FL in Combination with Short-Chain Galacto-Oligosaccharides, Long-Chain Fructo-Oligosaccharides, 3'-Galactosyllactose, and Milk Fat in Healthy Term Infants (Vandenplas et al., 2020)

A double-blind, randomized, controlled, parallel group growth equivalence study of a partly fermented infant formula product containing 2'-FL, 3'-galactosyllactose (3'-GL), short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), and milk fat was conducted in healthy term infants from multiple locations in Belgium, Hungary, Poland, Spain, and Ukraine (Vandenplas *et al.*, 2020; NCT03476889). Groups of infants (≤ 2 weeks of age at study initiation) were exclusively fed either a commercially available complete cow's milk-based infant formula (n=86 at test completion) or the same infant formula supplemented with 1 g/L 2'-FL, 0.15 g/L 3'-GL, and anhydrous milk fat (n=90 at test completion), until 17 weeks of age. Both test formulas also contained 8 g/L scGOS/lcFOS (9:1 ratio). A control group of entirely breastfed infants (n=56 at test completion) also was included for reference.

The primary outcome measurement was weight gain, which was recorded at baseline (≤ 2 weeks of age), 4, 8, 12, and 17 weeks of age. Other growth parameters, including length and head circumference, were also measured at the same time intervals. Parameters of gastrointestinal tolerance (*i.e.*, regurgitation, vomiting, stool characteristics) were reported by the parents throughout the test period, and any additional safety outcomes (*i.e.*, adverse events) were reported by the investigators. Dietary intake of the test product also was recorded throughout the test period.

Formula consumption was consistent across the formula test groups, both with respect to intake/day and intake/kg body weight/day. The mean daily weight gain of infants receiving either formula was consistent throughout the test period; however, weight gain was significantly greater in these test groups compared to the breastfed infants. Regarding gastrointestinal tolerance, occurrence of regurgitation was reported to be slightly greater in breastfed infants; 23.4% of infants receiving the 2'-FL formula, 25.8% in the commercially available formula, and 45.8% of infants in the breastfed group at 8 weeks of age experienced frequent regurgitation, the time point where the highest incidence of regurgitation was reported. No significant difference was reported in symptoms of vomiting in any groups. Stool frequency was significantly reduced in both formula groups compared with breastfed infants.

At least one adverse event was reported in 39.3% of infants receiving the 2'-FL test formula, in 31.7% of infants receiving the control formula, and in 24.6% of breastfed infants. No statistical significance was reported in regurgitation, vomiting, frequent watery stools, or infrequent hard stools between either group receiving infant formula. Eleven total serious adverse events were noted in formula-fed infants; seven in infants who received the 2'-FL test formula and four in infants who received the control formula. However, this difference was not statistically significant and was determined to be unrelated to the study product by the investigators. No serious adverse events were reported in breastfed infants.

The study authors concluded that the infant formula containing 2'-FL, 3'-GL, scGOS/lcFOS, and milk fat was safe and well tolerated in healthy term infants, and supportive of adequate infant growth.

6.5.1.7 Randomized, Controlled, Double-Blind Clinical Trial of Young Child Formula Containing 2'-Fucosyllactose, Galacto-Oligosaccharides, and Intact Bioactive Proteins in Children Aged 1 to 2.5 Years (Leung et al., 2020)

The safety of young child formulas containing 2'-FL, GOS, and intact bioactive proteins was examined in a randomized controlled, double-blind, parallel-group designed clinical trial (Leung *et al.*, 2020; clinical trial registry number not reported). Hong Kong-based, healthy, Chinese-ethnic children aged 1 to 2.5 years were randomized to receive one of four interventions (composition of the investigational young child formula products summarized in Table 6.5.1.7-1).

Table 6.5.1.7-1 Composition of Investigational Young Child Formulas (Adapted from Leung *et al.*, 2020)

Component	Young Child Formula-Reference (per 100 mL)	Young Child Formula-A (per 100 mL)	Young Child Formula-B (per 100 mL)	Young Child Formula-C (per 100 mL)
Protein (N x 6.25)	2.7 g	2.7 g	2.7 g	2.7 g
Immunoglobulins	0 g	0.1 g	0.01 g	0 g
Lactoferrin	< 0.002 g	0.17 g	0.01 g	< 0.002 g
Transforming Growth Factor (TGF)-β	0.99 µg	1.5 µg	1.5 µg	0.99 µg
Fat	2.5 g	2.5 g	2.5 g	2.5 g
Milk Fat	0.05 g	1.7 g	0.05 g	0.05 g
Linoleic acid	337 mg	281 mg	337 mg	337 mg
Available carbohydrates	10 g	9.7 g	10 g	9.7 g
Fiber	0.4 g	0.69 g	0.4 g	0.69 g
Galacto-oligosaccharides (GOS)	0.4 g	0.4 g	0.4 g	0.4 g
2'-Fucosyllactose (2'-FL)	0 g	0.3 g	0 g	0.3 g
Energy	73 kcal	72 kcal	73 kcal	72 kcal

During a 6-month intervention period, milk consumption was restricted to two servings of 200 mL/day of the allocated young child formula. Habitual food intake over the last month was recorded by a food frequency questionnaire. During the study, parents/caretakers were instructed to record left-over milk (or extra young child formula consumed), any signs of illness, and medications used. Daily defecation patterns (including time, frequency, and stool's visual appearance), and stool consistency [using the Bristol Stool Scale (BSFS)] were also recorded. Subjects were examined physically at scheduled clinic visits every two months. Nasal swabs and stool samples were collected at baseline and final visits for microbiota analyses. Subjects presenting with respiratory or gastrointestinal symptoms were instructed to return to the clinic for examination by a pediatrician and an additional nasal swab and/or stool sample was collected closest to the second day from the onset of infection. Adverse events were recorded using the International Classification of Diseases and the severity and likelihood of the relationship to the intervention were recorded.

The primary outcomes included the incidence of upper respiratory tract infections (defined as a runny nose and/or cough for ≥ 2 days) and duration of gastrointestinal tract infections (defined as the number of days from first until last excretion of liquid or semi-liquid stools) in these toddlers. Secondary and tertiary outcomes included the mean duration of upper respiratory tract infection; incidence of gastrointestinal tract infections; the incidence and duration of gastrointestinal tract infection meeting the WHO criteria of ≥ 3 liquid/semi-liquid stools within 24 hours; and anthropometric data.

Of 1,157 children screened, a final intention-to-treat cohort of 456 children was included in the analyses. All groups were comparable with respect to baseline characteristics and nutrient intake. A per-protocol analysis was possible in 85 to 89% of subjects. The results showed that all interventions had similar frequencies of adverse events and serious adverse events; and there were no reported cases of product-related adverse events, as judged by investigators and confirmed by the data safety monitoring board. No notable between-group differences were reported in anthropometric changes during the study.

The average numbers of new upper respiratory tract infections for children receiving the reference young child formula and the three other investigational formulas were not statistically different and changes in the mean weekly upper respiratory tract infection incidence also showed no discernible between-group differences. Groups also showed similar mean duration of gastrointestinal tract infection. Analyses for gastrointestinal tract infection duration remained statistically insignificant when controlled for breastfeeding during infancy.

Although the study authors reported an increased number of gastrointestinal tract infection episodes and a shorter time to the first gastrointestinal tract infection in participants receiving young child formula supplemented with 0.3 g/100 mL of 2'-FL, it was noted that these secondary and tertiary outcome findings must be interpreted with caution. Specifically, the reference young child formula used in this study contained 0.4 g/100 mL of GOS and 0.99 µg/100 mL of transforming growth factor-β, precluding the ability to form robust conclusions on the effect of 2'-FL against a true control group. Additionally, with the inclusion of 0.4 g/100 mL of GOS in all formulas and the low overall incidence of upper respiratory tract infections in the study cohort, any statistically significant improvements on top of the reference formula would be difficult to demonstrate as incidences remained lower than expected during the study[15]. Furthermore, given the very healthy study cohort[16] and reference young child formula designed to contain additional health-promoting biomolecules not listed as essential components in standard follow-on formula products (*i.e.*, the addition of GOS and transforming growth factor-β), the ability to observe *clinically relevant* benefits in a healthy population receiving a supplemented base formulation is unlikely given the study design.

No notable between-group differences in pathogen identification and microbiome analyses in nasal and stool samples were reported.

The study authors concluded that the young child formulas tested had similar safety profiles to the reference formula in healthy Chinese toddlers and that further human studies were needed to verify these findings.

[15] Notably, the study was powered to detect differences in treatment groups assuming an incidence rate of 3.5 episodes of upper respiratory tract infections per subject. The actual overall incidence observed in the study was 1.7 to 2.0 episodes per subject in each group.

[16] With respect to the healthy study cohort, exclusion criteria included children that were sick within 2 weeks of screening and children taking antibiotics, anti-viral drugs, montelukast, laxatives, or anti-diarrheal drug within 1 month.

6.5.1.8 Randomized, Placebo-Controlled, Double-Blind, Study in Irritable Bowel Syndrome Patients (Iribarren et al., 2020)

The influence of 2'-FL administration (in combination with LNnT) was explored in a Phase II randomized, placebo-controlled, double-blind study in adults with irritable bowel syndrome (IBS) (Iribarren *et al.*, 2020; NCT02875847). Patients aged 18 to 75 years diagnosed with at least moderate-severity irritable bowel syndrome according to the Rome IV criteria were randomly allocated to receive either a placebo (glucose), or 5 or 19 g/day of a 4:1 ratio of 2'-FL and LNnT for 4 weeks. Patients were followed for an additional 4 weeks after the cessation of the intervention. All subjects were advised to maintain their usual diet and medication regimen throughout the study. During clinic visits at screening, baseline, end of intervention (Week 4) and study end (Week 8), patients completed validated clinical questionnaires to assess the severity of gastrointestinal and psychological symptoms. Body weight, height, adverse events, changes in medication or diet, and compliance were also recorded, and a physical examination was performed by the study physician. Fecal samples were collected during these visits and subject to microbiota profiling.

The primary endpoint of the study was to determine the daily dose of 2'-FL+LNnT associated with an increase in *Bifidobacterium* spp. abundance without aggravating gastrointestinal symptoms, as assessed by the Gastrointestinal Symptom Rating Scale for IBS (GSRS-IBS). Secondary efficacy endpoints included IBS severity [as measured by the IBS Symptom Severity Scale (IBS-SSS)], bowel habits (stool consistency), and anxiety and depression.

In total, 60 patients (20 patients in each arm) were included in the intention-to-treat analysis. Two patients (one from the placebo group and one from the 10 g 2'-FL+LNnT/day group) discontinued prematurely after 2 weeks of intervention due to increased IBS symptoms. Anthropometric and IBS classifications did not differ between the three groups at baseline; however, patients allocated to receive 5 g 2'-FL+LNnT/day demonstrated a lower GSRS-IBS total score at baseline compared to the other groups ($p = 0.03$). After 4 weeks of intervention, the group receiving 10 g 2'-FL+LNnT/day exhibited a statistically significant higher abundance of fecal bifidobacteria as compared to the groups receiving placebo and 5 g 2'-FL+LNnT/day, as well as when compared to baseline; however, these differences did not persist into Week 8 (*i.e.*, following cessation of the intervention). Patients receiving 5 g 2'-FL+LNnT/day did not show a change at Week 4 but had a decreased bifidobacteria abundance at Week 8 compared to baseline. No changes were reported in the placebo group at any timepoint. The severity of overall or individual gastrointestinal symptoms did not differ between the groups at the Week 4 or Week 8 timepoints.

The study authors concluded that daily intake of 10 g of 2'-FL+LNnT over 4 weeks was well tolerated and did not induce worsening of IBS symptoms, bowel habits, anxiety, or depression, and that most patients were able to complete the 4 weeks of intervention without significant side effects.

6.5.1.9 Prospective, Open-label, Single-arm Study in Irritable Bowel Syndrome Patients (Palsson et al., 2020)

The safety and tolerability of 2'-FL (in combination with LNnT) was investigated in a multicenter, open-label trial in which adult volunteers with IBS (70.7% women; mean age of 44 years) were provided the mixture for 12 weeks (Palsson *et al.*, 2020; clinical trial registry number NCT03550742). Volunteers were instructed to consume one 5-g pack of 2'-FL+LNnT (4:1 ratio of 2'-FL and LNnT) per day, either mixed in food, beverage, or taken on its own. Of the 317 patients included in the analysis, 245 reported full adherence to the study intervention. A baseline assessment was conducted to determine stool consistency (measured by BSFS), IBS symptom severity, IBS gastrointestinal symptom rating scale, and IBS quality of life. These surveys were also conducted 4, 8, and 12 weeks after the beginning of intervention. The occurrence of adverse events was monitored throughout the intervention period.

The overall safety of the 2'-FL+LNnT was reported as well tolerated, with the occurrence of adverse events remaining low (*i.e.*, 46 patients reported a combined total of 87 adverse events). Upon reviewing the reported adverse events, the investigators considered 65 adverse events (reported by 33 patients) to be possibly or probably related to the test article. Most adverse events were related to passing gas, abdominal distension, and abdominal pain, in order of occurrence frequency. Eight participants dropped out of the study due to adverse events. One serious adverse event occurred in the test period; however, it was reviewed by the medical safety officer and concluded to be unrelated to the test article.

The study authors therefore concluded that daily administration of 2'-FL+ LNnT (4:1 ratio of 2'-FL and LNnT) was safe and well tolerated in adults with IBS.

6.5.1.10 Randomised, Double-Blind, Controlled Clinical Study Examining Safety of 2'-FL and LNnT in a Liquid Supplement for Premature Infants (Hascoët et al., 2021 [abstract])

The effect of a supplement containing 2'-FL and LNnT on growth, safety, and feeding tolerance was examined in a multi-center, randomized, double-blind, controlled clinical study conducted in France (Hascoët *et al.*, 2021 [abstract]; NCT03607942). In this study, preterm infants (27 to 33 weeks gestation, birth weight < 1,700 g) were randomly allocated to receive either a supplement containing 2'-FL and LNnT in a 10:1 ratio (administered as a total of 0.374 g/kg body weight/day, dissolved in water buffered with a pH-adjusting agent) or an isocaloric placebo supplement consisting of only glucose (0.140 g/kg body weight/day) from randomization (as early as possible) to discharge from the neonatal unit. The primary outcome was feeding tolerance, measured by non-inferiority in days to reach full enteral feeding from birth in the 2'-FL+LNnT group compared to the placebo group (non-inferiority margin of +4 days). Anthropometric z-scores were calculated using Fenton growth standards. Other secondary outcomes include fecal markers of gut health/maturation and microbiota.

A total of 43 infants were allocated to the 2'-FL+LNnT supplement group and 43 to the placebo control group. The mean chronological age at the initiation of supplementation was 6.3 days in the 2'-FL+LNnT group and 6.2 days in the placebo group. The mean total duration of intervention was 41 (range: 2 to 80) days in the 2'-FL+LNnT group and 34.5 (range: 2 to 125) days in the placebo group. Non-inferiority in time to reach full enteral feeding in the 2'-FL+LNnT group versus the placebo was achieved in the full analysis set (least squares mean difference = 2.16 days; 95% confidence level -5.33, 1.00; upper bound of 95% confidence interval < non-inferiority margin). Similar results were observed in the per protocol set. The adjusted mean time to reach full enteral feeding from birth was 2 days shorter in the 2'-FL+LNnT group compared to placebo (12.2 days versus 14.3 days) but this finding did not reach statistical significance

(p=0.177). There was no difference in weight-for-age z-scores between the groups. Length-for-age z-scores were statistically significantly higher in the 2'-FL+LNnT supplement group versus the control group at days 14 (least squares mean difference of 0.29; p=0.037) and 21 (least squares mean difference of 0.31; p=0.037). Head circumference-for-age z-score was significantly higher in the group receiving 2'-FL+LNnT versus the control at discharge (least squares mean difference of 0.42; p=0.007). Gastrointestinal tolerance measures, incidence of gastrointestinal adverse events, incidence of necrotizing colitis, and incidence of other illnesses and infections were similar between groups. No cases of illnesses and infections were deemed related to the intervention.

The study investigators concluded that HMO supplementation was safe and well tolerated in pre-term infants and that the HMO supplement supported early postnatal growth.

6.5.1.11 Safety and Tolerability of 2'-FL Alone and in Combination with LNnT in Children Aged 5 to 12 Years (Fonvig et al., 2021)

A single-center, randomized, controlled, double-blinded, parallel intervention study was conducted to determine the effect of 8 weeks supplementation of either 2'-FL alone or a mixture of 2'-FL and LNnT in a 4:1 mass ratio on fecal microbiota (primary objective) and safety/tolerability (secondary objectives) in 5- to 12-year-old children (Fonvig *et al.*, 2021; NCT02786160). The study population consisted of children aged 6.4 to 12.7 years at enrollment who were admitted to a childhood obesity treatment program. Except from the excess body weight (body mass index z-score ≥ 2.3), the children were healthy and showed no sign of illness or metabolic disorders. A total of 75 eligible children (43 females) were randomized to receive a daily dose of 4.5 g of either 2'-FL alone, a mix of 2'-FL and LNnT in a 4:1 mass ratio ("Mix"), or placebo of powdered glucose (25 children in each group) for 8 weeks. The participants were asked to dissolve the investigational product in at least 50 mL of liquid and consume it in the morning with breakfast. Data on effect during the intervention period was collected at baseline, at an intermediate visit (after 4 weeks of intervention), and at the end of the 8-week intervention.

The results show that the abundance of *Bifidobacterium* in fecal samples was increased from baseline to the intermediate visit and from baseline to the end-of-intervention in the 2'-FL group and from baseline to the intermediate visit in the Mix group. There were no statistically significant differences in the magnitude of this effect between these two intervention groups. No such changes in the abundance of *Bifidobacterium* were observed in the placebo group. In addition, no statistically significant difference in *Bifidobacterium* abundance was seen from the intermediate visit to the end-of-intervention for the 2'-FL group and for the Mix group, indicating that the full bifidogenic effect is reached after 4 weeks of intervention. The bifidogenic effect was primarily mediated through an increased abundance of *Bifidobacterium adolescentis*.

This study shows that a daily intake of 4.5 g of either 2'-FL or the Mix is safe for use in children. Blood samples were collected at baseline, end-of-intervention (Week 8), and during final study visit at 10 ± 1 month after the end-of-intervention visit. All measurements were generally within the normal range expected for the age group at all timepoints assessed; any measurements that deviated from the normal range were minor and were considered clinically irrelevant by the investigators. A few minor statistically significant changes in safety biomarkers (*i.e.*, hematology and clinical chemistry parameters measured in blood) were observed between the baseline and end-of-intervention visit. However, these changes remained within the normal variation and were considered clinically irrelevant by the investigators, with no consistent changes observed in any of the intervention groups. No clinically relevant changes in anthropometric measures, body composition, or vital signs (resting blood pressure) were reported.

An analysis of adverse events indicates no clinically relevant differences between the groups. A total of 75 adverse events were reported by 37 subjects. The number of children experiencing at least one adverse event throughout the study was similar across the intervention groups (12, 13, and 13 subjects in the placebo, 2'-FL, and Mix group, respectively). The number of adverse events also occurred with similar frequency across the intervention groups (24, 24, and 27 in the placebo, 2'-FL, and Mix group, respectively). Of the 75 adverse events reported, 53 adverse events were considered unlikely to be related to the investigational product, 20 were considered possibly related, and 2 (both occurring in the Mix group) were considered "unknown", whereby it was not possible to rate the severity or relationship to the investigational product by the investigator. Of the 20 possibly-related adverse events, 15 were considered to be mild; these were mostly gastrointestinal in nature, and occurred across all groups including the placebo (6, 2, and 7 in the placebo, 2'-FL, and Mix group, respectively). The remaining 5 adverse events were deemed moderate in severity, and they all occurred as a single incidence in one subject in the Mix group (abdominal cramps, abdominal pain, diarrhea, reflux, and flatulence). No serious adverse events occurred. Overall, the reported adverse events raised no safety concerns.

The investigational products did not provoke digestive intolerance in any of the groups as measured by the GSRS, indicating that both 2'-FL and the Mix are well tolerated in children. Mean scores for the individual symptoms remained at levels between "No discomfort at all" and "Mild discomfort," and except from a statistically significant reduction in urgent need to have a bowel movement from baseline to end of intervention in the 2'-FL group, the fluctuations in total GSRS score, scores for individual symptoms and scores for clusters of symptoms were not statistically significant for any of the groups. The BSFS scores showed no between-group differences in proportion of abnormal bowel movements, indicating that the investigational product did not induce digestive distress. The investigational products did not have an impact on change in anthropometric measures or body composition during the 8 weeks of intervention.

Overall, the results from this study demonstrate that supplementation with HiMOs in children (2'-FL alone or in combination with LNnT in a 4:1 ratio) is safe for use at the tested dose without provoking digestive intolerance. This is similar to what has been shown in other studies with HiMOs in infants and adults.

6.5.1.12 Multicenter, Double-Blind, Randomized, Controlled, Parallel-Designed 4-Month Growth, Safety, and Tolerability Study of Formula for Infants Containing 5HMO-Mix (Parschat et al., 2021)

The growth, safety, and tolerability of an infant formula supplemented with a mixture of 5 HiMOs ("5HMO-Mix") during the first 4 months of life has been evaluated in a double-blind, randomized, controlled, multicenter[17] non-inferiority trial (Parschat *et al.*, 2021; Clinical Trial Registry NCT03513744). The 4-month intervention period was followed by a 2-month voluntary follow-up period during which parents could choose to continue intervention up to 6 months. Healthy term infants 14 days of age or younger were eligible to participate in the study. Infants whose mother independently and voluntarily chose not to breastfeed were randomized to receive infant formula with or without the addition of the 5HMO-Mix. In parallel, a group of exclusively breastfed infants were enrolled as a reference group.

[17] Subjects were recruited from 12 sites across Germany (2 sites), Italy (5 sites), and Spain (5 sites) from December 2018 to November 2020.

The basic infant formula providing the macro- and micro-nutrients required for infant nutrition was manufactured in compliance with regulations of the European Union for infant formulae. The Test formula was identical to the basic infant formula apart from the partial replacement of maltodextrin with the 5HMO-Mix. Specifically, the 5-HMO-Mix was added at a concentration providing 5.75 g/L in the reconstituted infant formula. The concentration of individual HiMOs from the 5HMO-Mix manufactured by Chr. Hansen HMO GmbH (Rheinbreitbach, Germany) added to the test formula is presented in Table 6.5.1.12-1 below. The reconstituted control and test formulas contained similar energy within natural and tolerable ranges (68 and 67 kcal/100 mL, respectively), and identical amounts of protein (1.4 g/100 mL), fat (3.6 g/100 mL), carbohydrates (7.2 g/100 mL), lactose (5.2 g/100 mL), vitamins, and other nutrients.

Table 6.5.1.12-1 Concentrations of Individual HiMOs from the 5HMO-Mix in the Powdered and Reconstituted Test Infant Formula (Parschat *et al.*, 2021)

HiMO	Proportion of 5HMO-Mix (%)	Powdered Test Infant Formula (g/100 g)	Reconstituted Test Infant Formula (g/L)
5HMO-Mix	**100**	**4.35**	**5.75**
2'-FL	52	2.26	2.99
3-FL	13	0.57	0.75
LNT	26	1.13	1.5
3'-SL	4	0.17	0.23
6'-SL	5	0.22	0.28

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactoes; 3'-SL = 3'-sialyllactose; 5HMO-Mix = mixture of 5 HMOs; 6'-SL = 6'-sialyllactose; HiMO = human-identical milk oligosaccharide; LNT = lacto-*N*-tetraose.

All infants were fed *ad libitum* according to their assigned feeding group. The total daily intake was recorded by parents in 3-day diaries. In the formula groups, compliance was determined based on the weight of delivered *versus* returned packages of the study product and was defined as the consumption of at least 80% of the anticipated quantity as calculated from the average intake by infants 0 to 4 months of age.

The primary objective of the trial was to demonstrate that infant formula supplemented with the 5HMO-Mix supports normal non-inferior growth during the first 4 months of age by comparing the mean daily body weight gain after 4 months intervention between the formula-fed groups. Secondary outcomes included other anthropometric measures (absolute data, changes, increments, and WHO growth standard z-scores for weight, length, and head circumference), tolerability (stool frequency and consistency assessed using the Amsterdam Stool Chart), digestive tolerance (regurgitation, vomiting, and flatulence), and behavior (fussiness, crying, and awakening at night). Tolerability, digestive tolerance, and behavioral endpoints were evaluated based on parent ratings for pre-determined scales in 3-day diaries, recorded either after (Day 0) or before (Days 14, 28, 56, 84, or 112) each visit. The primary endpoint was evaluated in the full-analysis dataset (FAS)[18] and the per-protocol dataset (PPS)[19]. Growth parameters were evaluated in the FAS, while all other secondary outcomes were evaluated in the safety dataset (SS)[20].

[18] All subjects enrolled in the study who received at least one feeding, had any tolerability data available up to 4 months, and had at least one body weight value at baseline and after baseline.
[19] All subjects from the FAS without any major deviations.
[20] All subjects enrolled in the study who received at least one feeding and had any tolerability data available up to 4 months.

Overall, 341 infants were enrolled in the study, 225 of which were formula-fed and randomized to the formula groups (113 to the 5HMO-Mix Test Group and 112 to the Control Group); the remaining 116 breastfed infants were allocated to the Reference Group. The study was completed by 265 infants (77.7%), while 76 infants discontinued the study (Control Group: n=21; Test Group: n=27; Reference Group: n=28).

The mean daily intake of infant formula on a volume (mL/day) and energy (kcal/day) basis was similar between the Test and Control Groups. The average daily intake of the 5HMO-Mix steadily increased throughout intervention, ranging from 2.6 ± 0.8 g/day at enrollment to 5.2 ± 1.0 g/day at 4 months[21].

The mean daily body weight gain after 4 months of intervention was within the non-inferiority margin of -3 g/day in the Test Group compared to the Control Group for both the FAS and PPS (non-inferiority $p<0.001$). Furthermore, there were no significant differences in any anthropometric measures evaluated between the formula-fed groups throughout intervention.

Stool frequency was similar between the Test Group and breastfed Reference Group from 2 to 4 months; at 4 months, infants from the Control Group passed fewer stools on a daily basis compared to infants from the Test Group ($p=0.0428$) and breastfed infants ($p=0.0136$). A significantly higher frequency of soft stools was observed in the Test Group compared to the Control Group during the first 2 months of intervention ($p<0.05$), while breastfed infants generally had a higher frequency of soft stools compared to both formula-fed groups at most timepoints. There was no difference in flatulence, vomiting, or fussiness without crying between the formula-fed groups. Regurgitation was higher in the Test Group compared to Control from 1 to 4 months ($p<0.05$) but comparable to breastfed infants. Crying was less frequent in the Test Group compared to breastfed infants at most timepoints ($p<0.05$), though no significant difference between the formula-fed groups was observed. Throughout intervention, infants from the formula-fed groups woke less frequently at night compared to breastfed infants ($p<0.05$).

The number and intensity of reported adverse events were similar between all three groups, and there was no significant difference in adverse events categorized according to the Medical Dictionary for Regulatory Activities (MedDRA) by primary system, organ, and class (SOC) between the formula-fed groups. Among specific adverse events, a higher incidence of genital fungal infection was reported in the Test Group (n=5) compared to Control (n=0; $p=0.0290$), and hematochezia and plagiocephaly were more frequent in the Test Group compared to the breastfed Reference Group. For hematochezia, the study authors noted that the overall frequency was low (Test Group: n=5; Control Group: n=2; Reference Group: n=2) and could be caused by factors unrelated to the intervention. The majority of serious adverse events were reported in the Control Group (Control Group: n=9; Test Group: n=3; Reference Group: n=4). In each of the formula-fed groups, two of the reported serious adverse events were determined to be related to the investigational product. In the Test Group, one subject was hospitalized due to choking and gastroesophageal reflux who later recovered and continued the study, and another subject experienced severe diarrhea who was treated with hydrolyzed milk and removed from the study. Both serious adverse events reported in the Control Group resulted in the diagnosis of bovine milk protein allergy.

Overall, the study authors concluded that infant formula supplemented with a mixture of 5 HiMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) at concentrations similar to those naturally occurring in human milk supported normal infant growth and was safe and well tolerated.

[21] Calculated from mean infant formula consumption volumes ranging from 459.7 ± 137.7 mL/day at enrollment to 902.5 ± 170.0 mL/day at 4 months.

6.5.1.13 Summary of Interventional Clinical Studies Identified

Glycom performed a search of the scientific literature for new interventional studies relevant to the safety of 2'-FL. Overall, the new clinical studies examining the effect of the administration of 2'-FL to infants, children, and adults have not identified any safety concerns (see Table 6.5.1.13-1).

Table 6.5.1.13-1 Summary of the Interventional Clinical Studies Conducted on 2'-FL

Study Population and Study Design	Duration of Intervention	Study Groups and Test Articles	References
Studies Conducted in Infants			
79 healthy full-term singleton infants 39–40 per group 14 ± 5 days of age at enrollment Multi-center, randomized, double-blind, controlled study	42 days	***Control Formula:*** 100% whey partially hydrolyzed formula containing *Bifidobacterium lactis* ***Test Formula:*** Same as control, but with 0.25 g/L 2'-FL	Storm *et al.* (2019) ***Clinical trial number*** NCT03307122
50 healthy full-term infants 14–60 days of age at enrollment Single-arm and single-blind study	3 weeks	***Test Formula***: 100% whey partially hydrolyzed formula containing *Lactobacillus reuteri* and 0.25 g/L 2'-FL (single-arm study)	Czerkies *et al.* (2019) ***Clinical trial number*** NCT03679234
159 healthy full-term infants 45–66 per group 7 days–2 months old at enrollment Non-randomized, open-label, prospective study	8 weeks	***Exclusively Formula-fed Group:*** *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g LNnT/L ***Mixed Formula-fed and Breastfed Group***: *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g LNnT/L ***Exclusively Breastfed Group (Reference Group):*** Breastfed enrolled at the same time as formula-fed infants	Román Riechmann *et al.* (2020) ***Clinical trial number*** NCT04055363
289 healthy formula-fed infants 60 healthy breastfed infants (reference group) Less than 14 days of age at enrollment Double-blind, randomized, controlled study	6 months	***Control Formula:*** Cow's milk-based infant formula containing *L. reuteri* (1×10^7 CFU/g) ***Test Formula:*** Same as control, plus 1.0 g/L 2'-FL	Corsello *et al.* (2020) [abstract] ***Clinical trial number*** NCT03090360

Table 6.5.1.13-1 Summary of the Interventional Clinical Studies Conducted on 2'-FL

Study Population and Study Design	Duration of Intervention	Study Groups and Test Articles	References
47 infants with suspected food protein allergy, persistent feeding intolerance, or presenting conditions where an extensively hydrolyzed formula was deemed appropriate 0–60 days of age at enrollment Single arm, non-randomized, multi-center study	60 days	***Test Formula:*** Extensively hydrolyzed casein-based infant formula containing 2'-FL (0.2 g/L)	Ramirez-Farias *et al.* (2021) ***Clinical trial number*** NCT03884309
176 healthy formula-fed infants 56 healthy breastfed infants (reference group) 14 days of age or less at enrollment Double-blind, randomized, controlled study	15 weeks	***Control Formula:*** Cow's milk-based infant formula containing 8 g/L scGOS/lcFOS (9:1 ratio) ***Test Formula:*** Same as control, plus 1.0 g/L 2'-FL, 0.15 g/L 3'-GL, and anhydrous milk fat	Vandenplas *et al*. (2020) ***Clinical trial number*** NCT03476889
86 preterm infants (27 to 33 weeks gestation, birth weight < 1,700 g) 43 per group Average 6 days of age at intervention initiation Multi-center, randomized, double-blind, controlled study	Enrollment to discharge from neonatal unit	***Control Supplement:*** Glucose (0.140 g/kg bw/day) ***Test Supplement:*** 2'-FL and LNnT in 10:1 ratio (0.374 g/kg bw/day)	Hascoët *et al*. (2021) [abstract] ***Clinical trial number*** NCT03607942

Table 6.5.1.13-1 Summary of the Interventional Clinical Studies Conducted on 2'-FL

Study Population and Study Design	Duration of Intervention	Study Groups and Test Articles	References
225 healthy term infants and 116 healthy breastfed infants (Reference Group) 112–113 per formula group ≤ 14 days of age at enrollment Multicenter, randomized, double-blind, controlled, parallel-designed study	4 months	***Control Formula:*** Basic infant formula ***Test Formula:*** Same as control, plus a target HiMO content of 5.75 g/L (2.99 g/L of 2'-FL, 1.5 g/L of LNT, 0.75 g/L of 3-FL, 0.28 g/L of 6'-SL, and 0.23 g/L of 3'-SL) ***Reference Group:*** Breastfed infants	Parschat *et al*. (2021) ***Clinical trial number*** NCT03513744
Studies Conducted in Children			
456 healthy children aged 1–2.5 years Randomized, controlled, double-blind, parallel-design study	6 months	**YCF-Reference:** YCF containing 0.99 µg/100 mL TGF-β, 0.4 g/100 mL GOS **YCF-A:** YCF containing 0.1 g/L immunoglobulins, 0.17 g/100 mL lactoferrin, 1.5 µg/100 mL TGF-β, 0.4 g/100 mL GOS, and 0.3 g/100 mL 2'-FL **YCF-B:** YCF containing 0.1 g/L immunoglobulins, 0.17 g/100 mL lactoferrin, 1.5 µg/100 mL TGF-β, and 0.4 g/100 mL GOS **YCF-C:** YCF containing 0.99 µg/100 mL TGF-β, 0.4 g/100 mL GOS, and 0.3 g/100 mL 2'-FL	Leung *et al.* (2020) ***Clinical trial number*** Not reported
75 obese children (5–12 years old) 25 per group Randomized, controlled, double-blind, parallel intervention study	8 weeks	**Control:** 4.5 g/day glucose **Test group 1:** 4.5 g/day 2'-FL **Test group 2:** 4.5 g/day mixture of 2'-FL plus LNnT in 4:1 ratio (3.6 g/day of 2'-FL plus 0.9 g/day of LNnT)	Fonvig *et al*. (2021) ***Clinical trial number*** NCT02786160

Table 6.5.1.13-1 Summary of the Interventional Clinical Studies Conducted on 2'-FL

Study Population and Study Design	Duration of Intervention	Study Groups and Test Articles	References
Studies Conducted in Adults			
60 adults with irritable bowel syndrome 20 per group Randomized, placebo-controlled, double-blind study	4 weeks	***Control:*** Glucose ***Test Group 1:*** 5 g/day of a 4:1 mixture of 2'-FL and LNnT ***Test Group 2:*** 10 g/day of a 4:1 mixture of 2'-FL and LNnT	Iribarren *et al.* (2020) ***Clinical trial number*** NCT02875847
245 adults with irritable bowel syndrome Open label, single-arm trial	12 weeks	***Test Formula***: 5 g of a 4:1 mixture of 2'-FL and LNnT	Palsson *et al*. (2020) ***Clinical trial number*** NCT03550742

2'-FL = 2'-fucosyllactose; 3 -FL = 3-fucosyllactose; 3'-GL = 3'-galactosyllactose; 3'-SL = 2'-sialyllactose; 6'-SL = 6'-sialyllactose; CFU = colony forming units; GOS = galacto-oligosaccharides; HiMO = human-identical milk oligosaccharide; lcFOS = long-chain fructo-oligosaccharides; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; scGOS = short-chain galacto-oligosaccharides; TGF = transforming growth factor; YCF = young child formula.

6.6 Allergenicity

The production microorganism used in the manufacturing process of non-crystallized 2'-FL is highly similar to that previously described in GRN 815 and in the Supplement to GRN 650 (Glycom A/S, 2016, 2018; U.S. FDA, 2016, 2019). Since 2'-FL is secreted into the culture medium, and the production strain itself is removed intact during USP (Stage 1), the modifications made to the final DSP steps (improved purification process instead of crystallization) will not have any impact on its allergenicity potential. This is supported by analytical data demonstrating that no production strain or residual proteins (*i.e.*, below the limit of quantitation of 0.0017%) remain in non-crystallized 2'-FL.

Nevertheless, the allergenic potential of introduced proteins as a result of the genetic modification of the *E. coli* K-12 host (which itself is recognized as non-allergenic) has been assessed using the search algorithms provided by the Allergen Online tool (ver. 21) of the University of Nebraska (FARRP, 2021). This database was last updated on 14 February 2021 and contains sequences of 2,233 known and putative allergens. 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 ≥ 50% sequence similarity as an indication of potential cross-reactivity; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of ≥ 35% sequence similarity as an indication of potential cross-reactivity; and (iii) 8 mer sequence exact match[22]. No sequence alerts for potential allergenicity were identified.

[22] It is herein acknowledged that isolated identity matches of 8 contiguous amino acids have poor clinical correlation with the likelihood of allergenic cross-reactivity (see AllergenOnline.org homepage for statement); however, this search algorithm was performed as a precautionary measure and regulatory due diligence.

6.7 General Recognition

In 2016, 2'-FL manufactured using microbial fermentation was concluded, by Glycom, to have GRAS status for use in non-exempt infant formula and specified conventional food and beverage products. This GRAS conclusion was based on scientific procedures using generally available data and information obtained from the peer-reviewed literature, and on consensus among a panel of experts who were qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. Glycom's GRAS conclusion was subsequently notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). Subsequently, a mixture of 2'-FL and DFL manufactured under highly similar conditions was concluded to be GRAS for use in non-exempt infant formula and specified conventional food and beverage products and notified to the offices of the FDA and filed by the Agency without objection under GRN 815 (Glycom A/S, 2018; U.S. FDA, 2019).

Glycom has recently extended its GlyCare brand portfolio to include a non-crystallized version of 2'-FL. A revision of the GRAS specifications for 2'-FL described in GRN 650 (or GRN 815) (Glycom A/S, 2016, 2018; U.S. FDA, 2016, 2019) are required to accommodate the necessary modifications to the manufacturing process that entail replacement of the crystallization purification step with a combination of optimized unit operations including drying (*e.g.*, freeze or spray-drying); these changes will reduce the energy and environmental load of the 2'-FL manufacturing process and reduce the cost per unit produced. Specification changes include a decrease in the levels of 2'-FL from ≥ 94 to ≥ 85%, and corresponding increases in the levels of the minor saccharides present, namely an increase in the levels of D-lactose from ≤ 3.0% to ≤ 10.0%, and an increase in the levels of DFL from ≤ 1.0% to ≤ 5.0%. To ensure that the overall purity of the ingredient, Glycom has also included an additional purity specification requiring the sum of the levels of 2'-FL and of the minor saccharides (D-lactose, DFL, L-fucose, and 2'-fucosyl-D-lactulose) to be equal to or greater than 90.0%.

In accordance with scientific procedures, Glycom has presented data and information within this Notice to support the company's conclusion that the compositional changes imparted to the ingredient as a result of the manufacturing changes do not have material significant impact on the composition in a manner that would impact the original GRAS conclusion. General consensus among qualified experts that this conclusion is appropriate is supported by recent evaluation of these manufacturing changes and revised specifications conducted by the European Commission following submission of an application for amendment of the novel food ingredient specification for 2'-FL in accordance with the requirements of Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (EU, 2017). Glycom confirms that data and information presented to the European Commission in support of the specification change for 2'-FL are fully representative of data and information presented in this notification. Based on this information the Commission issued the following conclusion under implementing regulation (EU) 2019/388 of 11 March 2019:

> "*The proposed changes do not alter the safety considerations that supported the authorisation of the 2'-fucosyllactose produced with Escherichia coli strain K-12. Therefore, it is appropriate to amend the specifications of the novel food '2'-fucosyllactose' at the proposed levels of 2'-fucosyllactose, of D-lactose, of difucosyl-D-lactose, and of the overall levels of 2'-fucosyllactose together with the minor saccharides (D-lactose, L-fucose, difucosyl-D-lactose, and 2'-fucosyl-D-lactulose)*" (EU, 2019).

6.8 GRAS Panel Evaluation

Glycom has concluded that non-crystallized 2'-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.4, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of non-crystallized 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 concluded that non-crystallized 2'-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.4, 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 non-crystallized 2'-FL, is presented in Appendix A.

6.9 Conclusion

Based on the data and information presented herein, Glycom has concluded that the intended uses of non-crystallized 2'-FL in non-exempt term infant formula and specified conventional food products, as described in Section 1.4, 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 non-crystallized 2'-FL in infant formula and conventional food, who similarly concluded that the intended uses of non-crystallized 2'-FL in infant formula and conventional food as described herein is GRAS.

Non-crystallized 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* (U.S. FDA, 2021d).

Part 7. § 170.255 List of Supporting Data and Information

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GRAS Panel Evaluation of Non-Crystallized 2'-Fucosyllactose (2'-FL) for Use in Infant Formula and Conventional Food Products

06 August 2021

INTRODUCTION

Glycom A/S (Glycom)[1] 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 non-crystallized 2'-fucosyllactose (2'-FL), produced by fermentation using a modified strain of *Escherichia coli* K-12 DH1, and to determine whether the intended uses of non-crystallized 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 "*GRAS Status of Non-Crystallized 2'-Fucosyllactose (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 non-crystallized 2'-FL and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, the product specifications, the supporting analytical data, the intended conditions of use, the estimated exposure under the intended uses, the history of consumption from human milk, and the safety of non-crystallized 2'-FL.

Following its independent and collective critical evaluation, and on the basis of scientific procedures, the GRAS Panel unanimously concluded that non-crystallized 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. The GRAS Panel members were provided an honorarium that was not contingent on the outcome of deliberations.

[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.

SUMMARY AND BASIS FOR GRAS

Non-crystallized 2'-FL manufactured by Glycom is a purified ingredient containing primarily 2'-FL (min. 85%), difucosyl-D-lactose (DFL; max. 5%), D-lactose (max. 10%), L-fucose (max. 1%), and 2'-fucosyl-D-lactulose (max. 1.5%). Crystallized 2'-FL manufactured by Glycom using a microbial fermentation process has previously been determined to be GRAS for use in non-exempt infant formula at a use level of up to 2,400 mg/L of the ready-to-drink or reconstituted formula, as well as in select conventional food and beverage products (GRN 650 – Glycom A/S, 2016; U.S. FDA, 2016). Non-crystallized 2'-FL is manufactured using downstream manufacturing conditions modified from those described in GRN 650 to produce 2'-FL as a dried amorphous powder without a crystallization step. Additionally, non-crystallized 2'-FL is manufactured from an optimized variant of the production strain described in GRN 650, although from a highly similar variant derived from the same platform strain. Glycom intends to market non-crystallized 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). Food uses of non-crystallized 2'-FL will be fully substitutional on a wt/wt basis to all GRAS uses of 2'-FL described in GRN 650.

2'-FL is a naturally occurring trisaccharide detected in some mammalian milks with the highest concentrations present in human milk and is, therefore, typically referred to as a human milk oligosaccharide (HMO). 2'-FL is a chemically defined linear trisaccharide consisting of L-fucose, D-galactose, and D-glucose, which occurs as one specific constitutional isomer. Based on proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), carbon-13 nuclear magnetic resonance spectroscopy (^{13}C-NMR), mass spectrometry (MS), and high-performance liquid chromatography (HPLC) with corona charged aerosol detector data, it has been confirmed that 2'-FL produced by microbial fermentation is chemically and structurally identical to 2'-FL present in human breast milk. Therefore, 2'-FL has an established long history of safe consumption as a component of human milk in infants on the basis that 2'-FL manufactured by Glycom is chemically identical to 2'-FL naturally present in human milk.

Glycom's 2'-FL is produced by microbial fermentation using biosynthetic processes introduced into a recombinant strain of *E. coli* K-12 DH1. *Escherichia coli* K-12 DH1 is a safe laboratory strain with a well-characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). *E. coli* K-12 DH1 was optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various sugars. Further modifications to the production strain were introduced by genomic insertion of defined DNA sequences that enabled the efficient biosynthesis and export of 2'-FL from the production organism. The resulting strain was designated *Escherichia coli* K-12 DH1 MDO MAP1001h and it has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany.

The GRAS Panel critically reviewed details of the manufacturing process for non-crystallized 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 of the manufacturing process, D-lactose and D-glucose[2] are converted by the engineered metabolic pathway of the production microorganism into 2'-FL by cellular fermentation. The fermentation is maintained for several days until in-process controls indicate a favorable ratio of 2'-FL to other carbohydrates and high consumption of D-lactose. 2'-FL is excreted into the fermentation broth, and the microbial biomass containing the production organism is then removed from the culture supernatant

[2] Alternative options of raw materials for energy and carbon source are D-sucrose and glycerol

containing 2'-FL by ultrafiltration/diafiltration and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of in-process controls and then further purified by the second stage of the production process, the downstream processing. Detailed information on the fermentation procedures including descriptions of the raw materials are incorporated by reference to Sections II.B.2 through II.B.4 of GRN 650 and Sections 2.2.2 and 2.2.3 of GRN 815 (Glycom A/S, 2018, 2019; U.S. FDA, 2016, 2019).

Modifications to the production of 2'-FL as described in GRN 650 *vs*. non-crystallized 2'-FL relate to implementation of manufacturing changes to produce a dried amorphous product that does not require crystallization. Omission of the crystallization step has a significant impact on certain aspects of the production process since a crystallization operation comes at a significant premium on cost, waste management (solvents), and through-put of the manufacturing line. Glycom notes that omission of the crystallization step is feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improves the performance of each purification step) accompanied by a drying procedure (*e.g*., freeze- or spray-drying, but not limited to these), which will be used instead of crystallization to isolate 2'-FL during downstream processing. The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH) that do not change the overall process. The change eliminates the use of the acetic acid solvent in the production of 2'-FL and has not only an impact on the overall costs of the process, but also has a tremendous impact from an environmental perspective, as it eliminates the use of significant amounts of acetic acid per kg of produced 2'-FL (*ca*. 3.0 kg acetic acid per kg of final 2'-FL) and, therefore, significantly reduces waste associated with human-identical milk oligosaccharide (HiMO) manufacturing and also produces a finished product that is absent of solvent residues. The primary impact of refining the manufacturing process to enable the omission of the crystallization step is the production of a non-crystallized 2'-FL product with slightly higher levels of 2'-FL biosynthesis-related carbohydrates, namely D-lactose (≤ 10.0%) and DFL (≤ 5.0%); this is in comparison to 2'-FL that is crystallized, where lower levels of D-lactose (≤ 3.0%) and DFL (≤ 1.0%) are obtained. D-Lactose and DFL are naturally present in human milk; therefore, they are not considered as an "impurity" in the non-crystallized 2'-FL ingredient. Glycom has analytical data demonstrating that the use of its improved purification procedures in lieu of crystallization largely limits the changes to D-lactose and DFL and does not alter the levels of other potential production strain metabolites or process-related residues (*e.g.*, residual protein, DNA, endotoxins), a conclusion that is attributed to the fact that several purification steps for removal of these compounds (*e.g*., ion adsorption and de-colorization) are maintained.

Glycom has established food-grade specifications for non-crystallized 2'-FL. The specifications for non-crystallized 2'-FL include parameters related to physical properties, specified saccharides, and contaminants. The specifications are similar to those for 2'-FL described in GRN 650 but with increased levels of D-lactose and DFL, which consequently lead to a proportional decrease of 2'-FL. Microbiological specifications have been revised from those described in GRN 650 to bring the ingredient in alignment with updated specifications that consider the use of Glycom's HiMOs in wet blending of infant formula ingredients during the manufacture of finished powdered products. All analytical methods are internationally recognized or have been validated internally. The GRAS Panel reviewed the results from three batches of non-crystallized 2'-FL and concluded that the manufacturing process produces a consistent material in conformance with the established product specifications.

Aside from the minor changes in the levels of some of the existing saccharides in Glycom's non-crystallized 2'-FL (*e.g.*, an increase in D-lactose and DFL), the modification to the processing method does not affect the structural/chemical identity of 2'-FL itself. As such, no changes are expected with regard to the stability profile of non-crystallized 2'-FL obtained from a genetically modified strain of *E. coli* K-12, neither during

bulk storage nor when incorporated into food matrices. Bulk stability data on non-crystallized 2'-FL confirm the stability over its intended storage period.

Non-crystallized 2'-FL is intended to be added to non-exempt term infant formula and various conventional food and beverage products (see Table A-1). The use of non-crystallized 2'-FL will be completely substitutional to the GRAS uses of 2'-FL described in GRN 650; therefore, introduction of the non-crystallized form of 2'-FL to the U.S. marketplace will not change dietary exposures to 2'-FL.

The GRAS Panel critically evaluated published and unpublished data and information characterizing the safety of 2'-FL. This information included a discussion of the safety of the production strain, the metabolic fate of HMOs, the available toxicity studies on 2'-FL, and an allergenicity assessment of the ingredient. For the purposes of identifying any new data relevant to the safety of 2'-FL published since the most recent 2'-FL GRAS determination notified to the U.S. FDA with a no questions response (*i.e.*, GRN 897; DuPont Nutrition and Health, 2019; U.S. FDA, 2020a), a comprehensive search of the published scientific literature was conducted on 31 May 2021, spanning the period of September 2019 to May 2021. 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. Therefore, available toxicity studies of 2'-FL were considered corroborative in nature.

The GRAS Panel noted that all HiMOs manufactured by Glycom are produced from a production organism originating from the same optimized platform strain (*E. coli* K-12 DH1 MDO), and 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 D-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'-FL/DFL, lacto-*N*-tetraose (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.

In terms of absorption, distribution, metabolism, and excretion (ADME), the powder properties (amorphous *vs*. crystalline) of the 2'-FL ingredient are of no relevance to its use in infant nutrition products since the production of most infant beverage formulations includes dissolution, heat-treatment, and drying during manufacturing of the food/beverage, converting any crystalline component into an amorphous powder. Moreover, since any potential difference in dissolution properties of amorphous and crystalline 2'-FL is equalized through food formulation processes, there will be no difference in the ADME profile of the ingredients. Apart from powder properties, the proposed specification varies only slightly in the ratio between certain compositional constituents (*e.g.*, D-lactose), and the amount of D-lactose that would result from the intake of non-crystallized 2'-FL would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula). Therefore, the ADME profile of 2'-FL is incorporated by reference to Section IV.D of GRN 650.

The general recognition of safety of 2'-FL under the specified conditions of use in term infant formula and conventional food and beverage products is largely based on published studies characterizing the concentrations of 2'-FL in human milk (incorporated by reference to Section IV.B of GRN 650), the corresponding history of safe consumption of 2'-FL by breastfed infants, and data demonstrating that 2'-FL is of high purity and is structurally identical to naturally occurring 2'-FL in breast milk.

The results of published and unpublished toxicological studies in neonatal and mature rats further corroborate the safety of the ingredient. Comprehensive discussions of the published toxicity studies as they apply to the safety of 2'-FL for use in infant formula and foods are incorporated by reference to Section IV.B.5 of GRN 546, Section IV.E of GRN 650, Part 6 of GRN 815, and Part 6 of GRN 897. These studies included a 90-day oral toxicity study in neonatal rats, and *in vivo* and *in vitro* genotoxicity assays undertaken on high-purity 2'-FL preparations (or 2'-FL-containing mixtures). Findings from these studies demonstrated that 2'-FL is not genotoxic and is of low toxicity potential following gavage dosing in neonatal pups. Glycom identified recent published toxicological assessments of mixtures containing 2'-FL (including 2'-FL in combination with 3-fucosyllactose, LNT, 3'-SL, and 6'-SL[3]; and 2'-FL in combination with lacto-*N*-fucopentaose I[4]) (Parchat *et al.*, 2020 and Phipps *et al.*, 2021). These results confirm that these mixtures were not genotoxic and the no-observed-adverse-effect-levels (NOAELs) were established as the highest dose tested. The absence of adverse or toxicity effects reported in the literature on 2'-FL (and mixtures thereof) in animal models is consistent with its natural presence in human milk.

Glycom has also undertaken corroborative product-specific studies that re-affirm the safety of non-crystallized 2'-FL. Non-crystallized 2'-FL was subject to a bacterial reverse mutation assay conducted in accordance with the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471; an *in vitro* mammalian cell micronucleus assay in accordance with OECD Test Guideline 487; and a modified 90-day repeat dose toxicity study in neonatal rats in accordance with OECD Test Guideline 408 (OECD, 2016, 2018, 2020). The results of the genotoxicity studies confirmed that non-crystallized 2'-FL was non-mutagenic and not clastogenic or aneugenic under the conditions of the studies (unpublished). Gavage administration of non-crystallized 2'-FL to neonatal rats (7 days of age) for at least 90 days at doses up to 5,000 mg/kg body weight/day (5,935 mg/kg body weight/day total carbohydrates), was well tolerated and not associated with any test item-related adverse effects (unpublished). Therefore, the NOAEL was concluded by the study investigators to be 5,000 mg/kg body weight/day, the highest dose tested. These conclusions are consistent with those reached previously in GRAS Notices for 2'-FL submitted by Glycom and others.

Glycom performed a search of the scientific literature for new interventional clinical studies relevant to the safety of 2'-FL. Overall, the new clinical studies examining the effect of the administration of 2'-FL to infants, children, and adults have not identified any safety concerns (Czerkies *et al.*, 2019; Storm *et al.*, 2019; Alliet *et al.*, 2020 [abstract]; Corsello *et al.*, 2020 [abstract]; Iribarren *et al.*, 2020; Leung *et al.*, 2020; Palsson *et al.*, 2020; Román Riechmann *et al.*, 2020; Vandenplas *et al*., 2020; Ramirez-Farias *et al.*, 2021).

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 a 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 non-crystallized 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

[3] The test article described in Parchat *et al.* (2020) contained 47% 2'-FL, 16% 3-fucosyllactose, 24% LNT, 4% 3'-SL, and 4% 6'-SL.
[4] The test article described in Phipps *et al.* (2020) contained 59% lacto-*N*-fucopentaose I and 32% 2'-FL.

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 non-crystallized 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 non-crystallized 2'-fucosyllactose (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]

Joseph F. Borzelleca, Ph.D.
Professor Emeritus Virginia Commonwealth
University School of Medicine

8/10/21
Date

[redacted]

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

8/11/21
Date

[redacted]

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

8/10/201
Date

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ATTACHMENT A: INTENDED FOOD USES AND USE LEVELS FOR NON-CRYSTALLIZED 2'-FL IN THE UNITED STATES

Table A-1 Summary of the Individual GRAS Food-Uses and Use-Levels for Non-Crystallized 2'-FL in Conventional Food and Beverage Products and Infant Formula*

Food Category	Proposed Food-Uses	RACC	GRAS Use Level (g/RACC)	Maximum GRAS Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	240 mL	0.28	1.2
Dairy Product Analogs	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	170 g	0.90	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[a]	0.24	2.4
	Toddler Formulas	100 mL[a]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	1.6	40
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk[b]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavored Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.28	5
	Yogurt	170 g	0.90	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-fucosyllactose; RACC = Reference Amounts Customarily Consumed (21 CFR §101.12 – U.S. FDA, 2020b); U.S. = United States.

* Food-Uses and Use Levels have been adapted from GRN 650; with due updates in the RACC to reflect current reference amounts.

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

[b] Milk is a standardized food in the United States. When the milk is fortified with 2'-FL it will then be classified as a milk product.

From:	Miks, Marta
To:	Morissette, Rachel
Subject:	[EXTERNAL] RE: questions for GRN 001060
Date:	Friday, November 18, 2022 12:35:24 PM
Attachments:	image001.png image002.png image003.png image004.png image015.png image016.png image017.png image018.png image019.png image020.png image008.png GRN 001060 - Response to FDA Questions - 23Sept"22_Final.pdf
Importance:	High

For Internal Use Only

Dear Rachel,

Please find enclosed responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 23 September 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

Kindly acknowledge receipt of this email

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

Glycom, the leading HMO expert is part of DSM

Click here to catch me on Microsoft Teams

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, October 7, 2022 2:51 PM
To: Miks, Marta <Marta.Miks@dsm.com>

Subject: RE: questions for GRN 001060

Hi Marta,

That will be fine. However, Glycom should be aware that this extension will likely result in our having to extend the review clock by an additional 90 days. I will look for responses on or before Nov. 18. Have a good weekend.

Best,

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: Friday, September 23, 2022 2:47 PM
To: Marta Hanna Miks <mhm@glycom.com>
Subject: questions for GRN 001060

Dear Marta,

Please see attached the questions for GRN 001060.

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
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FDA U.S. FOOD & DRUG ADMINISTRATION

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18 November 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 001060

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 23 September 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

CHEMISTRY (FDA Q1- Q12):

FDA.1. *Please specify the protein base of the infant formula into which the 2'-FL would be added (e.g., cow milk, soy, etc).*

Glycom does not intend to restrict the use of 2'-FL according to the protein source included in the infant formula. 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 safety of the addition of 2'-FL to an infant formula product.

FDA.2. *Glycom states that 2'-FL is intended for use in fruit juices. Please provide a statement that the 2'-FL will not be used in juices for which a standard of identity may preclude its use.*

Glycom confirms that 2'-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169 (U.S. FDA, 2021a), do not permit its addition in standardized products. Therefore, 2'-FL will not be used in juices for which standards of identity do not permit its use.

FDA.3. *The use levels in Table 1.4-1 (p. 5 of the notice) are expressed in g/RACC, as well as g/kg or g/L. While some of the RACCs in Table 1.4-1 are updated from the use levels (g/RACC) presented in GRN 000650, the RACC in beverages (e.g., sports drinks) does not reflect the updated RACC. Please address if it is Glycom's intent to maintain the use level in beverages on a g/RACC basis or g/L basis.*

Glycom would like to maintain the use level for all proposed food uses of 2'-FL, including beverages (*i.e.*, sports, isotonic, and energy drinks), on a g/L or g/kg basis.

The proposed use levels of 2'-FL provided on a g/RACC basis were included for reference only. These were back-calculated from the proposed use level of 2'-FL on a g/kg or g/L basis and reference amounts customarily consumed (RACCs) per eating occasion established in 21 CFR §101.12 (U.S. FDA, 2021b). The proposed use level of 2'-FL in sports, isotonic, and energy drinks expressed on a g/RACC basis has been corrected according to the updated RACC for beverages in the revised Table 1.4-1 below. A statement on the intended use of 2'-FL in unstandardized products, and not in foods where standards of identity exist and do not permit its addition, has also been added to the revised table (see response to FDA Question No. 2).

Use levels expressed on a g/L or g/kg basis were applied in the dietary exposure assessment. Therefore, the updated RACC for beverages does not affect dietary exposure estimates.

Table 1.4-1 Summary of the Individual GRAS Food Uses and Use Levels for Non-Crystallized 2'-FL in Conventional Food and Beverage Products and Infant Formula* [REVISED]†

Food Category	Proposed Food Uses‡	RACC	GRAS Use Level (g/RACC)	Maximum GRAS Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	360 mL	0.43	1.2
Dairy Product Analogs	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	170 g	0.90	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[a]	0.24	2.4
	Toddler Formulas	100 mL[a]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	1.6	40
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk[b]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavored Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.28	5
	Yogurt	170 g	0.90	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-fucosyllactose; GRAS = Generally Recognized as Safe; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

* Food uses and use levels have been adapted from GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016); with due updates in the RACC to reflect current reference amounts (U.S. FDA, 2021a).

† Revised values are indicated in green.

‡ 2'-FL is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

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

[b] Milk is a standardized food in the United States. When the milk is fortified with 2'-FL it will then be classified as a milk product.

FDA.4. *In the description of "downstream manufacturing process" (pp. 10-11 of the notice), the optimized unit operations, referred to as "adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH," are not fully characterized. Please provide additional information about the optimization of these steps to support Glycom's statements regarding changes/improvements in the process and the elimination of the crystallization step.*

Detailed description of the optimized unit operations of downstream manufacturing process of non-crystallized 2'-FL described in GRN 1060, have been compared with the purification sequence of crystallized 2'-FL described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016) in Table 1.

Table 1 Overview of the Downstream Manufacturing Process for Crystallized 2'-FL (Glycom A/S, 2016; U.S. FDA, 2016) and non-Crystallized 2'-FL (GRN 1060), highlighting the modifications in a purification process.

	GRN 650			GRN 1060	
	Crystallized 2'-FL			Non-crystallized 2'-FL	
Step No	PROCESS STEP	PURIFICATION	Step No	PROCESS STEP	PURIFICATION
01	Media Preparation		01	Media Preparation	
02	Propagation		02	Propagation	
03	Seed Fermentation		03	Seed Fermentation	
04	Fermentation	*Production of 2'-FL*	04	Fermentation	*Production of 2'-FL*
05	Ultrafiltration (UF/DF)	*Removal of cells and large biomolecules (e.g., protein, nucleic acids, and lipopolysaccharides)*	05	Removal of Microorganism	*Removal of cells and large biomolecules (e.g., protein, nucleic acids and lipopolysaccharides)*
06	Nanofiltration (NF)	*Concentration. Reduction water, minerals, and very small biomolecules*	06	Nanofiltration (NF) + Optional Diafiltration (DF)	*Concentration. Reduction of minerals, small biomolecules like amino acids, small organic acids, small carbohydrate-type metabolites*
06a	Optional Microfiltration	*Removal of potential microbiological contamination*	*06a*	Optional Microfiltration	*Removal of potential microbiological contamination*
06b	Optional Ion Removal (e.g., ion-exchange resin or electrodialysis)	*Removal of small, charged molecules and salts (e.g., trace metals)*	*07*	Ion Removal/Exchange (e.g., ion-exchange resin or electrodialysis)	*Removal/exchange of small and large charged and ionizable molecules (e.g., proteins, amino acids, organic acids, salts, e.g., trace metals)*
06c	Optional Pre-concentration (e.g., evaporation or nanofiltration)		*07a*	Optional Pre-concentration (e.g., evaporation or nanofiltration)	
07	Decoloration (e.g., charcoal filtration)	*Removal of color and impurities by adsorbent*	08	Decoloration (*e.g.*, active charcoal / adsorbent filtration)	*Removal of color and traces of residual impurities (e.g., residual protein, hydrophobic contaminants) by adsorbent*
08	Microfiltration	*Removal of potential microbiological contamination*	08a	Optional Microfiltration	*Removal of potential microbiological contamination*

09	Pre-concentration (*e.g.*, evaporation or nanofiltration)		09	Pre-concentration (*e.g.*, evaporation <u>and/or nanofiltration + optional diafiltration</u>)	*<u>Concentration and removal of remained residual charged molecules, if any</u>*
	Not applicable	*Not applicable*	09a	<u>Optional Microfiltration and/or pasteurization</u>	*<u>Removal of potential microbiological contamination</u>*
10	Crystallization (from water with acetic acid)	*Highly efficient removal of micro-impurities (traces of protein and DNA, amino acids, carbohydrate-type impurities, trace elements, etc.)*		Step removed	*Not applicable*
11	Solid-Liquid-Separation (SLS)			Step removed	*Not applicable*
12	Washing			Step removed	*Not applicable*
13	Drying	*Removal of water and acetic acid*	10	Drying	*Removal of water*
14	Sampling and Packaging		11	Sampling and Packaging	
15	Quality Control	*Parameters of specifications are tested, and CoA issued*	12	Quality Control	*Parameters of specifications are tested, and CoA issued*
16	Batch Release		12a	Batch Release	

2'-FL = 2'-Fucosyllactose; CoA = certificate of analysis; DF = Diafiltration, DSP = downstream processing; GRN = GRAS Notice, NF = Nanofiltration, SLS = solid-liquid-separation; UF = ultrafiltration; USP = upstream processing
Up-stream process (USP) steps are highlighted in green. Down-stream process (DSP) steps are highlighted in blue.
The purification steps of an improved performance are highlighted in yellow. The underlined modifications are the improvements in purification process compared to production workflow of the crystallized 2'-FL ingredient.
The purification steps which have been removed or are not applicable are highlighted in grey.

FDA.5. *Please confirm that all Glycom internal methods are validated for their intended use and provide method citations where applicable.*

Glycom confirms that all internal analytical methods are validated to analyze batches for conformance with the stated specification. Method citations for all internal methods are provided in Table 2.

Table 2 Overview of Glycom Internal Analytical Methods for Non-Crystallized 2'-FL (GRN 1060)

Parameter	Glycom Internal Analytical Method
Assay (water-free) specified saccharides	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015, HPLC-2-003
Assay (water-free) 2'-Fucosyllactose	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015
Difucosyl-D-lactose	Glycom method HPAEC-HMO-015
D-Lactose	Glycom method HPAEC-HMO-015
L-Fucose	Glycom method HPLC-2-003
2'-Fucosyl-D-lactulose	Glycom method HPLC-2-003
Water	Glycom method KF-001
Residual protein by Bradford assay	Glycom method UV-001

2'-FL = 2'-Fucosyllactose; HMO = human milk oligosaccharide, HPAEC = high-performance anion-exchange chromatography, HPLC = high-performance liquid chromatography, KF = Karl Fisher, UV = Ultraviolet

FDA.6. *On p. 12 of the notice, Glycom notes that it has "analytical data demonstrating that the use of its improved purification procedures in lieu of crystallization largely limits the changes to D-lactose and DFL and does not alter the levels of other potential production strain metabolites or process-related residues (e.g., residual protein, DNA, endotoxins)…".*

a. Please provide the results of at least three non-consecutive batch analyses to support this statement.

b. For the process-related residues (e.g., residual protein by Bradford assay, endotoxins), the results of batch analyses are shown as "complies". Please clarify the limits of detection of these parameters and provide the results in numerical value.

Glycom has presented in Table 3 (below) a side-by-side comparison for three non-consecutive batch results for crystallized 2'-FL produced by strain *E. coli* K-12 DH1 MDO MAP1001d (supplement to GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and non-crystallized 2'-FL (GRN 1060) to support the abovementioned statement on p. 12 of the GRAS Notice.

As demonstrated from batch analyses, the level of D-Lactose and DFL is higher in batches of non-crystallized 2'-FL (mean of 1.7 and 1.9 w/w %, respectively) compared to batches of crystallized 2'-FL (mean of 0.4 and 0.3 w/w %, respectively), while levels of L-Fucose and 2'-Fucosyl-D-lactulose are similar between batches.

The numerical values have been provided for residual proteins by Bradford assay and residual endotoxins. The limits of quantification for Bradford assay and residual endotoxin methods are < 0.0017% and < 0.00025 EU/mg, respectively. As demonstrated from batch analyses, residual proteins and endotoxins are generally below the limits of quantification for both crystallized and non-crystallized 2'-FL. Additionally, the absence of residual DNA from the production organisms confirmed by 3 different validated quantitative polymerase chain reaction (qPCR) methods has been demonstrated at the limit of quantification of 4 µg/kg in analyses for both crystallized and non-crystallized 2'-FL.

Table 3 **Comparison of the Batch Results for Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and Non-Crystallized 2'-FL (GRN 1060).**

Parameters	Unit	Batch Analyses (GRN 650)†			Batch Analyses (GRN 1060)†		
		CRYSTALLIZED 2'-FL			NON-CRYSTALLIZED 2'-FL		
		18233001	18312001	18355001	19481101	19501101	D_2FL_EP_ 2021_2_FD_1
Assay (water-free) HiMS / specified saccharides[a]	w/w %	98.9	97.6	97.9	92.3	92.8	94.4
Assay (water-free) 2'-fucosyllactose	w/w %	98.0	96.7	97.4	87.7	88.9	90.3
Difucosyl-D-lactose	w/w %	0.36	0.26	0.25	1.90	1.89	1.78
D-Lactose	w/w %	0.53	0.51	0.19	2.03	1.16	2.00
L-Fucose	w/w %	< 0.03	< 0.03	< 0.03	0.06	0.06	< 0.03
2'-Fucosyl-D-lactulose	w/w %	0.51	0.17	0.25	0.30	0.55	0.24
Acetic acid	w/w %	0.33	< 1.0	0.20	NA	NA	NA
Residual proteins by Bradford assay	w/w %	< LOQ[a]	0.002	< LOQ[a]	< LOQ[a]	< LOQ[a]	< LOQ[a]
Residual endotoxins	E.U./mg	< LOQ[b]	< LOQ[b]	< LOQ[b]	0.00892	< LOQ[b]	< LOQ[b]
Lead	mg/kg	< 0.05	< 0.1	< 0.05	0.011	< 0.05	< 0.01
Residual DNA							
Residual DNA by qPCR (FutC assay)	µg/kg	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]
Residual DNA by qPCR (melA assay)	µg/kg	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]
Residual DNA by qPCR (23S assay)	µg/kg	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]	< LOQ[c]

2'-FL = 2'-fucosyllactose; EU = endotoxin units; GRAS = Generally Recognized as Safe; HiMS = human-identical milk saccharides; 23S = ribosomal subunit of *E. coli*; DNA = deoxyribonucleic acid; *FutC* = fucosyltransferase; LOQ = limit of quantitation; *melA* = α-galactosidase gene; NA = not applicable; qPCR = quantitative polymerase chain reaction.

[a] LOQ < 0.0017 %.

[b] LOQ < 0.00025 EU/mg.

[c] LOQ < 4 µg/kg (parts per billion).

† Revised values are indicated in green.

FDA.7. *The revised method described in GRN 001060 results in an amorphous powder instead of a crystalline powder. Please clarify if the particle size of the amorphous powder (described as "agglomerates") is similar to that of the 2'-FL described in GRN 000650. Alternatively, is the ingredient added only at the wet-blend stage for infant formula as suggested on p. 22 of the notice for foods in general?*

The appearance of both 2'-FL ingredients in crystalline (GRN 650, Glycom A/S, 2016; U.S. FDA, 2016) and amorphous (non-crystalline) form (GRN 1060) can be described as: powders, agglomerates, or powder with agglomerates. Glycom confirms that the particle size of crystallized and non-crystallized 2'-FL are comparable based on results from analyses of particle size distribution. Therefore, no significant change to bulk, flow and solubility properties of the 2'-FL ingredient is expected. Similar to crystallized 2'-FL, the amorphous (non-crystalline) 2'-FL ingredient that is the subject of the current GRAS Notice is intended to be added at either the wet-blend or dry-blend stage of infant formula manufacture. As such, Glycom has established separate specification parameters for 2'-FL depending on whether the ingredient is utilized in wet-blending or dry-blending of the infant formula production process, as described in Section 2.3 of the GRAS Notice.

FDA.8. *The specification for pH (4.0-6.0) in GRN 001060 has changed from what was cited in GRN 000650 (pH 3.2-5.0). Please comment on what accounts for this difference in pH.*

The proposed pH range of HiMO ingredients in general is linked to the stability of the ingredient in given storage conditions, including *e.g.*, temperature, pH and relative humidity, and is thus specific to the properties of each HiMO ingredient. Glycom would like to extend the pH range for non-crystallized 2'-FL from 4.0-6.0 to 3.5-7.0, as stability data confirm that the amorphous ingredient is stable at a wider pH range than originally expected.

FDA.9. *The specification for D-lactose in Table 2.3-1 on p. 14 of the notice is ≤10.0 w/w %, while the batch analysis results indicate actual D-lactose levels are much lower (~1-3%). We recommend that Glycom reduces the D-lactose specification to reflect the results of the batch data. Alternatively, please address the variation in lactose levels (from 1% to possibly 10%) with the revised method of manufacture, even though Glycom notes that the lactose level is not a safety issue (p. 20 of the notice).*

The lactose substrate is utilized by the manufacturing strain during fermentation where it is converted to 2'-FL *via* the addition of a fucose monosaccharide. The fermentation is maintained until in-process controls indicate a favorable ratio of 2'-FL to other carbohydrates and high consumption of lactose. During fermentation, the lactose substrate, and subsequently the 2'-FL product, undergo continuous enzymatic reactions of fucosylation as a result of the highly productive manufacturing strain. Therefore, optimization of the reaction for enhanced 2'-FL production but limited DFL production (*via* a second fucosylation of 2'-FL) is dependent on the amount of available lactose substrate. As such, it can be a complex technical challenge to stop the fermentation process at a low lactose level when trying to also limit DFL concentration levels. Furthermore, crystallization is one of the few purification techniques that allows the removal structurally and physico-chemically highly similar carbohydrates. Therefore, due to the modified downstream processing for non-crystallized 2'-FL, the compositional changes between non-crystallized 2'-FL and crystallized 2'-FL are largely limited to higher potential levels of lactose and DFL.

In the absence of safety and quality concerns, Glycom prefers to keep the lactose specification at ≤ 10 %, not only to keep the specifications in the notice aligned with the existing approval of non-crystallized 2'-FL in the European Union (Commission Implementing Regulation (EU) 2019/388[1]) and to accommodate for variation of measurement and fermentation outcome. Lactose levels at ≤10% of non-crystalized 2'-FL will not have an impact on its safety, since lactose is a major component of milk/dairy, breast milk, and infant formula, and intakes from its presence in 2'-FL would be negligible in comparison. Food products that possess a defined lactose content, like infant formula, can be adjusted at formulation to accommodate for the incremental lactose amount derived from the 2'-FL ingredient.

FDA.10. *The batch analyses for lead range from <0.01 to <0.05 mg/kg. Please clarify the method of analysis and limit of detection for lead.*

Lead is analyzed using inductively coupled plasma mass spectrometry (ICP-MS) according to the most recent version of EPA 6020A (currently, EPA 6020A:2007). The sensitivity of the ICP-MS method was improved recently, resulting in the reduction of the limit of quantification (LOQ) for lead from 0.05 to 0.01 mg/kg for newer batches (*e.g.*, batch number D_2FL_EP_ 2021_2_FD_1).

FDA.11. *In the dietary exposure discussion, Glycom notes that 2'-FL will be substitutional for crystallized 2'-FL described in GRN 000650. Please provide the numerical dietary exposure estimates that Glycom considered in this current GRAS conclusion.*

In the GRAS conclusion of GRN 1060, Glycom had considered dietary exposures to crystallized 2'-FL from uses in infant formula and conventional food products referenced in Section IV.A of GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016). Estimated daily intakes (EDIs) of 2'-FL were reported under three different scenarios.

The first scenario considered the dietary intake of 2'-FL among infants and toddlers from the intended conditions of use of 2'-FL in infant formula only. As the intended condition of use of 2'-FL in infant formulas notified as GRAS in GRN 650 remained the same as that notified in GRN 546 (Glycom A/S, 2014; U.S. FDA, 2015), EDIs remained unchanged and are reproduced for consumers-only in Table 4 below from Tables IV.A.1-1 and IV.A.1-2 of GRN 546.

[1] Commission Implementing Regulation (EU) 2019/388: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R0388#:~:text=Commission%20Implementing%20Regulation%20(EU)%202019,Implementing%20Regulation%20(EU)%202017%2F.

Table 4 **Summary of the Estimated Daily Intake of 2'-FL from Infant Formulas and from All Proposed Food Uses in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data) (Reproduced from GRN 546 – Glycom A/S, 2014; U.S. FDA, 2015)**

Population Group	Age Group	% Users	n	All-Users Consumption (g/day)		All-Users Consumption (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 months	74.8	161	2.02	2.91	332.8	535.6
Infants	7 to 12 months	73.6	128	1.70	2.63	188.9	295.8
Toddlers	1 to 3 years	1.1	7	1.08[a]	1.41[a]	89.3[a]	117.1[a]

2'-FL = 2'-fucosyllactose; bw = body weight; n = sample size; na = not available; NHANES = National Health and Nutrition Examination Survey, U.S. = United States.
[a] Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

The second scenario considered the dietary intake of 2'-FL among infants and toddlers from the intended conditions of use of 2'-FL in infant formula and in foods (including baby foods). As the intended conditions of use of 2'-FL notified as GRAS in GRN 650 for these food categories remained the same as those notified in GRN 546, EDIs remained unchanged and are reproduced for consumers-only in Table 5 below from Tables IV.A.2-1 and IV.A.2-2 of GRN 546.

Table 5 **Summary of the Estimated Daily Intake of 2'-FL from Infant Formulas and from All Proposed Food Uses in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data) (Reproduced from GRN 546 – Glycom A/S, 2014; U.S. FDA, 2015)**

Population Group	Age Group	% Users	n	All-Users Consumption (g/day)		All-Users Consumption (mg/kg bw/day)	
				% Users	n	Mean	90th Percentile
Infants	0 to 6 months	80.5	168	2.93	5.29	449.7	712.4
Infants	7 to 12 months	100	161	4.63	8.36	520.2	987.1
Toddlers	1 to 3 years	99.7	644	1.39	2.59	107.1	200.0

2'-FL = 2'-fucosyllactose; bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey, U.S. = United States.

The third scenario considered the dietary intake of 2'-FL in the general U.S. population from all proposed food uses of 2'-FL. As the intended conditions of use of 2'-FL in conventional foods notified as GRAS in GRN 650 had been revised, EDIs for consumers-only are reproduced in Table 6 below from Tables IV.A-1 and IV.A-2 of GRN 650.

Table 6 **Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2011-2012 NHANES Data) (Reproduced from GRN 650 – Glycom A/S, 2016; U.S. FDA, 2016)**

Population Group	Age Group (Years)	All-Users Consumption (g/day)				All-Users Consumption (mg/kg bw/day)			
		% Users	n	Mean	90th Percentile	% Users[a]	n[a]	Mean	90th Percentile
Toddlers	1 to 3	99.3	561	1.12	1.97	99.3	558	84.9	146.0
Children	4 to 10	98.5	1,161	0.69	1.25	98.5	1,161	27.4	54.5

Table 6 **Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2011-2012 NHANES Data) (Reproduced from GRN 650 – Glycom A/S, 2016; U.S. FDA, 2016)**

Population Group	Age Group (Years)	All-Users Consumption (g/day)				All-Users Consumption (mg/kg bw/day)			
		% Users	n	Mean	90th Percentile	% Users[a]	n[a]	Mean	90th Percentile
Female Teenagers	11 to 18	90.0	513	0.50	1.12	89.9	506	9.2	21.1
Male Teenagers	11 to 18	90.2	474	0.65	1.29	90.1	471	10.9	22.4
Female Adults of Childbearing Age	19 to 40	84.7	698	0.50	1.05	84.6	688	7.2	15.8
Female Adults	19 to 64	81.3	1,438	0.49	1.10	81.4	1,419	7.1	15.3
Male Adults	19 to 64	79.8	1,313	0.64	1.38	79.7	1,304	7.6	16.0
Elderly Adults	65 and up	87.9	816	0.49	1.12	87.9	804	6.8	15.4
Total Population	All Ages	85.0	6,574	0.64	1.31	85.0	6,523	20.0	33.1

2'-FL = 2'-fucosyllactose; bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey, U.S. = United States.
[a] Consumers with available body weight data only.

Glycom acknowledges that 2'-FL has since been notified as GRAS for use in additional food categories and at higher maximum levels of use in certain food categories. Since GRN 650, the following GRAS Notices for 2'-FL have received no questions letters from the U.S. FDA: GRNs 735,749, 815, 852, 897, 929, 932, and 1014. Therefore, cumulative estimated daily intakes of 2'-FL were calculated considering Glycom's proposed conditions of use (revised Table 1.4-1 of the GRAS Notice) and those previously notified as GRAS to the U.S. FDA that received a no questions letter from the Agency. Food uses and maximum use levels evaluated in the cumulative dietary exposure assessment of 2'-FL are presented in Table 7.

Table 7 **Summary of Individual Food Uses and Maximum Use levels Proposed or Previously Notified as GRAS for 2'-FL in the U.S.[a]**

Food Category (21 CFR §170.3) (U.S. FDA, 2021c)	Proposed Food Use[b]	Maximum Use Level (g/kg or g/L)	
		Notified in GRN 650 or GRN 1060	Other GRAS Notices with a "No Questions" letter
Beverages and Beverage Bases	**Soft Drinks**	-	**1.5 (GRN 815[d])**
	Non-Milk Meal Replacement and Nutritional Beverages[e]	5.0	**12.0 (GRN 1014)**
	Sports, Isotonic, and Energy Drinks	1.2	**6.0 (GRN 1014)**
	Enhanced or Fortified Waters	-	**1.5 (GRN 815[d])**
Breakfast Cereals	**Hot Cereals**	-	**31.0 (GRN 897)**
	RTE Cereals	-	**80.0 (puffed) 30.0 (high fiber) 20 (biscuit type) (GRN 735)**
Dairy Product Analogs	Imitation milks	1.2	1.2
	Non-Dairy Yogurt	5.3	**12.0 (GRN 897)**

Table 7 **Summary of Individual Food Uses and Maximum Use levels Proposed or Previously Notified as GRAS for 2'-FL in the U.S.[a]**

Food Category (21 CFR §170.3) (U.S. FDA, 2021c)	Proposed Food Use[b]	Maximum Use Level (g/kg or g/L)	
		Notified in GRN 650 or GRN 1060	Other GRAS Notices with a "No Questions" letter
Frozen Dairy Desserts and Mixes	**Frozen desserts including ice creams and frozen yogurts, frozen novelties**	-	**17.0 (GRN 735)**
Gelatins, Puddings, and Fillings	**Dairy-based puddings, custards, and mousses**	-	**17.0 (GRN 735)**
	Fruit pie filling	-	**14.1 (GRN 735)**
	Fruit filling in bars, cookies, yogurt, and cakes	-	**30.0 (GRN 735)**
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40.0	40.0
	Cereal and Nutrition Bars	-	**30.0 (GRN 897)**
Infant and Toddler Foods	Term Infant Formulas	2.4	2.4
	Toddler Formulas[f]	2.4	2.4
	Hypoallergenic Toddler Formula[f]	-	**2.0 (GRN 929)**
	Other Baby Foods for Infants and Young Children	12.0	12.0
	Other Drinks for Young Children	1.2	**12.0 (GRN 1014)**
	Baby crackers, pretzels, cookies, and snack items	-	**57.0 (GRN 735)**
Jams and Jellies	**Jellies and jams, fruit preserves, and fruit butters**	-	**60.0 (GRN 735)**
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	1.2	**1.5 (GRN 815[d])**
Milk Products	Buttermilk	1.2	**1.5 (GRN 815[d])**
	Flavored Milk	1.2	**1.5 (GRN 815[d])**
	Milk-Based Meal Replacement Drinks, for Weight Reduction[e]	5.0	**12.0 (GRN 1014)**
	Smoothies (Dairy and Non-Dairy)	-	**5.0 (GRN 897)**
	Yogurt	5.3	**15.0 (GRN 815[d])**
Processed Fruits and Fruit Juices	**Fruit Drinks and Ades**	-	**1.5 (GRN 815[d])**
	Fruit Juices and Nectars	1.2	1.2
Processed Vegetables and Vegetable Juices	**Vegetable Juice**	-	**1.2 (GRN 897)**
Sweet Sauces, Toppings, and Syrups	**Syrups used to flavor milk beverages**	-	**7.0 (GRN 735)**

2'-FL = 2'-fucosyllactose; '-' = not applicable; CFR = Code of Federal Regulations; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; RTE = Ready-to-Eat; U.S. = United States.

[a] Food uses that have been notified as GRAS to the U.S. FDA (which were not included in GRN 650 or GRN 001060) and received a "no questions" letters from the Agency are **bolded**.

[b] 2'-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169 (U.S. FDA, 2021a), do not permit its addition in standardized products.

[c] Use level expressed on a 2'-FL basis in the final food, as consumed.

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

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

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

Estimated daily intakes from the cumulative dietary exposure assessment of 2'-FL are presented in Table 8 on an absolute basis and in Table 9 on a body weight basis.

Table 8 Summary of the Cumulative Estimated Daily Intake of 2'-FL 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	1.56	3.47	72.1	133	2.16	3.73
Infants	7 to < 12 m	3.63	6.28	100	124	3.63	6.28
Toddlers	1 to 2 y	2.84	5.24	99.9	306	2.84	5.24
Children	3 to 11 y	2.65	5.04	99.5	992	2.66	5.04
Female Teenagers	12 to 19 y	2.50	5.55	97.1	434	2.57	5.71
Male Teenagers	12 to 19 y	2.87	5.48	97.6	426	2.94	5.49
Female Adults of Childbearing Age	20 to 40 y	2.40	5.69	94.1	656	2.56	6.06
Female Adults	20 to 64 y	2.45	5.67	93.7	1,540	2.61	5.76
Male Adults	20 to 64 y	3.20	7.65	94.5	1,356	3.39	7.96
Elderly	65 y and older	3.40	7.71	96.3	1,011	3.53	7.77
Total Population	2 y and older	2.87	6.34	95.5	5,912	3.01	6.51

2'-FL = 2'-fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

Table 9 Summary of the Cumulative Estimated Daily Per Kilogram Body Weight Intake of 2'-FL 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	236	495	72.1	133	328	499
Infants	7 to < 12 m	401	678	100	124	401	678
Toddlers	1 to 2 y	230	469	99.9	297	230	469
Children	3 to 11 y	103	205	99.5	989	104	205
Female Teenagers	12 to 19 y	41	91	97.0	427	43	91
Male Teenagers	12 to 19 y	44	85	97.6	423	45	87
Female Adults of Childbearing Age	20 to 40 y	34	78	94.1	655	36	81
Female Adults	20 to 64 y	34	77	93.7	1,533	36	79
Male Adults	20 to 64 y	37	86	94.5	1,348	39	89
Elderly	65 y and older	44	98	96.5	994	46	102
Total Population	2 y and older	48	110	95.5	5,860	50	112

2'-FL = 2'-fucosyllactose; bw = body weight; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

Among the total population (2 years and older), the mean and 90th percentile consumer-only intakes of 2'-FL were determined to be 3.01 g/person/day (50 mg/kg body weight/day) at the mean and 6.51 g/person/day (112 mg/kg body weight/day) at the 90th percentile. Cumulative dietary intake estimates of 2'-FL among the total U.S. population (2 years and older) reported herein are slightly higher than those calculated in the most recent GRAS Notice of 2'-FL that received a no questions letter from the U.S. FDA (Table 9 of GRN 1014 – Chr. Hansen A/S, 2021, U.S. FDA, 2022). As the intended conditions of use of non-crystallized 2'-FL are the same as those for Glycom's crystallized 2'-FL (GRN 650), the results of the cumulative dietary exposure assessment described herein would have been expected to be similar to those reported in GRN 1014, though the current assessment used consumption data from a more recent cycle of the National Health and Nutrition Examination Survey (2107-2018 *versus* 2015-2016). Nevertheless, there were a number of assumptions included in the cumulative dietary exposure assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in that all food products within a food category that has been notified as GRAS to the U.S. FDA (and received a no questions letter from the Agency) contain 2'-FL at the maximum notified 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 2'-FL will have 100% market penetration in all GRAS notified food categories.

FDA.12. *Please confirm that all food contact materials (e.g., filtration materials, membranes) are used in accordance with an existing regulation or effective food contact notification.*

Glycom confirms that all food contact materials are GRAS and/or conform to the specifications stated in 21 CFR and/or the Food Chemicals Codex (FCC).

MICROBIOLOGY (FDA Q13- Q18):

FDA.13. *Please state whether E. coli "K-12 DH1 MDO MAP1001h" is non-pathogenic and non-toxigenic.*

The 2-FL production strain, *Escherichia coli* K-12 DH1 MDO MAP1001h, is non-pathogenic and non-toxigenic.

FDA.14. *Please describe the in-process controls you have in place during your fermentation process and clarify how you monitor the fermentation process for potential contaminants.*

In Stage 1 of the manufacturing process (Upstream Processing), the substrate (D-lactose) and a carbon/energy source (*e.g.*, D-glucose) are converted by the production microorganism into 2'-FL by fermentation (Step 4). A schematic overview of Stage 1 of the manufacturing process (Upstream Processing), accompanied by applied processing aids, raw materials and in-process quality controls (IPC), is depicted in Table 10.

Table 10 Schematic Overview of the Upstream Processing (STAGE 1) of 2'-FL Product

STAGE 1		*Upstream Processing (USP)*	*USP Processing Aids*	*USP In-Process Control (IPC)*
STEPS	*1*	*Media Preparation*	*Raw materials*	
	2	*Propagation*	*Working Cell Bank (WCB) or Master Cell Bank (MCB)*	*T, OD, pH, rpm*
	3	*Seed Fermentation*	*Fermentation medium, inoculum*	*T, OD, pH, pO_2, rpm*
	4	*Fermentation Phases:*		
	4A	*Growth (Batch) Phase[a]*	*Fermentation medium, inoculum*	*T, OD, pH, pO_2, BWM, NH4+*
	4B	*Feeding (Fed-Batch) Phase*	*Fermentation medium ingredients*	*T, OD, pH, pO_2, BWM, NH4+, feed rate, HPLC/HPAEC*
	4C	Harvest/storage of culture broth	Buffering agent	T, pH
	5	*Removal of Microorganism*	*e.g., Ultrafiltration / Diafiltration (UF/DF)*	*T, pH, HPLC/HPAEC, Brix, Flux, Protein*
		Sterile Filtration	*e.g., Microfiltration membranes*	

2'-FL = 2'-fucosyllactose; Brix = endpoint control of concentration; BWM = bio wet cell mass; feed rate = control of feed rate accuracy; Flux = mass flow determination during addition to fermenter; HPLC = high-performance liquid chromatography and/or HPAEC = high-performance anion-exchange chromatography to monitor carbohydrate composition; NH4+ = ammonium; OD = Optical density of cell culture, pH = monitoring of pH; pO2 = partial pressure of oxygen to track oxygen levels during fermentation; rpm = revolutions per minute; T = temperature; USP = upstream processing.
[a] The batch phase of fermentation is optional.

The IPC parameters applied during fermentation (Step 4) include measurements of temperature (T), pH, optical density of cell culture (OD), bio wet cell mass (BWM) to monitor the growth of the production strain and to achieve high biomass levels, as well as the control of feed rate accuracy of feeding solution containing the substrate, and ammonium feed (NH4+) to avoid nitrogen-limitation. Fermentation is maintained for several days until in-process quality controls indicate a favorable ratio of 2'-FL to other carbohydrates and high consumption of D-lactose. The fermentation samples are analyzed with high-performance liquid chromatography (HPLC) and/or high-performance anion-exchange chromatography (HPAEC) to monitor carbohydrate composition, the levels of selected organic acids and elements.

Moreover, over the course of the fermentation, the microbiological analyses are performed to control and identify any microbial or phage contamination using plating assays and/or microscopic examination.

FDA.15. *Please confirm that the starting materials for fermentation are food-grade and/or are safe and suitable for their intended use.*

Glycom confirms that all starting materials are either food grade or are evaluated to be safe and suitable for their intended use.

FDA.16. *In Table 2.3-1 on p. 14 of the notice, the specification parameter for Enterobacteriaceae is listed as 10 g/CFU/g. Please provide clarification regarding the units of this specification, as well as the analytical method used to test for Enterobacteriaceae.*

Glycom would like to thank the FDA and apologize for the typo in the unit of specification parameter for Enterobacteriaceae in Table 2.3-1 on p. 14 of the GRAS Notice. For the regulatory batches of non-crystallized 2'-FL presented in the current GRAS Notice (GRN 1060), the enumeration of Enterobacteriaceae was performed using plating methods ISO 21528-2 or NMKL 144, with units expressed as colony forming units per g of the 2'-FL ingredient (CFU/g). Alternatively, Glycom may use plating method ISO 21528-1 to confirm the absence of Enterobacteriaceae in 10 g of the final ingredient.

FDA.17. *The notice states on p. 13 that "Glycom has established separate specification parameters for 2'-FL depending on whether the ingredient is utilized in wet-blending or dry-blending of the infant formula production process." We note that on p. 14, Table 2.3-1 only includes batch analysis data for wet blended 2'-FL.*
Additionally, footnote c on p. 15 of the notice states, "The minimum microbial requirements for 2'-FL that is added during the dry-blending stage of infant formula manufacturing include the following additional parameters: Cronobacter (Enterobacter) sakazakii (Absent in 10 g), Listeria monocytogenes (Absent in 25 g), and Bacillus cereus (not more than 50 CFU/g)." Please clarify whether 2'-FL is intended for use in powdered infant formula and what is meant by "minimum microbial requirements" in footnote c. If 2'-FL is intended for use in powdered infant formula, please provide the analytical methods and data from three non-consecutive batches for C. sakazakii, L. monocytogenes, and B. cereus.

Table 2.3-1 of the GRAS Notice has been revised below according to updated specifications for the non-crystallized 2'-FL ingredient. Revisions include:

- Extension of the pH range specification from 4.0–6.0 to **3.5-7.0.** The change in the pH range specification has been explained in Glycom's response to the FDA Question No. 8 above.
- A stricter specification for water from 9.0 to **≤ 7.0 w/w %.**
- Addition of ***Cronobacter* spp., *Listeria monocytogenes,*** and ***Bacillus cereus*** specification parameters for 2'-FL intended for addition at the dry blending stage of infant formula manufacture. The results for *Cronobacter spp.*, *Listeria monocytogenes*, and *Bacillus cereus* are provided for the same three independent production batches of non-crystallized 2'-FL (19481101, 19501101 and D_2FL_EP_ 2021_2_FD_1) as those from the GRAS Notice.
- Numerical values for batch analysis results (where applicable).

Table 2.3-1 Updated Specifications and Batch Analyses for Non-Crystallized 2'-FL in Comparison to Specifications for Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and Non-Crystallized 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019) [REVISED]†

Parameter		GRAS Specification			Batch Analyses		
	Unit	2'-FL GRN 650 (crystallized)	2'-FL/DFL GRN 815 (non-crystallized)	2'-FL GRN 1060 (non-crystallized)	19481101	19501101	D_2FL_EP_ 2021_2_FD_1
Appearance		**Powder, agglomerates, powder with agglomerates**			Complies	Complies	Complies
Color		**White, white to off-white, off-white**			Complies	Complies	Complies
Assay (water-free) specified saccharides[a]	w/w %	≥ 96.0	≥ 92.0	**≥ 90.0**	92.3	92.8	94.4
Assay (water-free) 2'-fucosyllactose	w/w %	≥ 94.0	≥ 75.0	**≥ 85.0**	87.7	88.9	90.3
Difucosyl-D-lactose	w/w %	≤ 1.0	≤ 20.0	**≤ 5.0**	1.90	1.89	1.78
D-Lactose	w/w %	≤ 3.0	**≤ 10.0**		2.03	1.16	2.00
L-Fucose	w/w %	≤ 1.0	**≤ 1.0**		0.06	0.06	< 0.03
2'-Fucosyl-D-lactulose	w/w %	≤ 1.0	≤ 2.0	**≤ 1.5**	0.30	0.55	0.24
pH in 5% solution (20°C)		3.2–5.0	3.5–5.4	**3.5-7.0**	4.7	4.8	5.3
Water	w/w %	≤ 5.0	≤ 6.0	**≤ 7.0**	5.38	5.24	2.3
Ash, sulphated	w/w %	≤ 1.5	≤ 0.8	**≤ 0.8**	< 0.05	0.1	< 0.01
Acetic acid	w/w %	≤ 1.0	NA[b]		NA[b]	NA[b]	NA[b]
Residual proteins by Bradford assay	w/w %	**≤ 0.01**			< LOQ[c]	< LOQ[c]	< LOQ[c]
Residual endotoxins	E.U./mg	**≤ 10**			0.00892	< LOQ[d]	< LOQ[d]
Lead	mg/kg	≤ 0.1		**≤ 0.05**	0.011	< 0.05	< 0.01
Microbiological Criteria[c]							
Aerobic mesophilic bacteria total count	CFU/g	≤ 500	**≤ 1,000**		<10	<10	<10
Enterobacteriaceae	CFU/g or absent in 10 g	Absent	**≤ 10**		<10	<10	<10
Salmonella	Absent in 25 g	**Absent**			Absent	Absent	Absent
Cronobacter spp.[e]	Absent in 10 g	Absent	**Absent**		Absent	Absent	Absent

Table 2.3-1 Updated Specifications and Batch Analyses for Non-Crystallized 2'-FL in Comparison to Specifications for Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and Non-Crystallized 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019) [REVISED]†

Parameter		GRAS Specification			Batch Analyses		
	Unit	2'-FL GRN 650 (crystallized)	2'-FL/DFL GRN 815 (non-crystallized)	2'-FL GRN 1060 (non-crystallized)	19481101	19501101	D_2FL_EP_ 2021_2_FD_1
Listeria monocytogenes[e]	Absent in 25 g	Absent	Absent		Absent	Absent	Absent
Bacillus cereus[e]	CFU/g	≤ 5	≤ 50		<10	<10	<10
Yeasts	CFU/g	≤ 10	≤ 100		<10	<10	<10
Molds	CFU/g	≤ 10	≤ 100		<10	<10	<10

2'-FL = 2'-fucosyllactose; CFU = colony forming units; DFL = difucosyllactose; E.U. = endotoxin units; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; NA = not applicable.

[a] Assay (water-free) specified saccharides includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose and 2'-fucosyl-D-lactulose. In GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016) the parameter HiMS (human-identical milk oligosaccharides) is used and includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose.

[b] Acetic acid is used as a processing aid during the crystallization step of the production process described in GRN 650 (Glycom A/S, 2016; U.S. FDA, 2016).

[c] LOQ < 0.0017 %.

[d] LOQ < 0.00025 EU/mg.

[c] Not applicable when 2'-FL is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior to drying.

† Revised values are indicated in green.

FDA.18. *Cronobacter sakazakii has been isolated from foods intended for young children and can cause infection in infants and young children. The intended use of 2'-FL as an ingredient in formula and drinks intended for young children (>12 months of age) and in foods intended for infants and young children provides a potential risk to these vulnerable populations if C. sakazakii is not controlled for during the production of 2'-FL or if foods formulated with this ingredient are not treated with an inactivation step (e.g., retort) before consumption by infants or young children. We note the following publications that discuss the prevalence and potential concerns of C. sakazakii presence in foods intended for infants and young children:*

1. *Chen, Q., Zhu, Y., Qin, Z., Qiu, Y., & Zhao, L. (2018). Cronobacter spp., foodborne pathogens threatening neonates and infants. Frontiers of Agricultural Science and Engineering, 5(3), 330-339.*
2. *Forsythe, S. J. (2015). New insights into the emergent bacterial pathogen Cronobacter. In Food Safety (pp. 265-308). Academic Press.*

Please provide a specification for C. sakazakii along with at least three non-consecutive batch analyses of 2'-FL for use in formula and drinks intended for young children (>12 months of age) and in foods intended for infants and young children.

As reviewed by Chen *et al*. (2018), contaminated powdered infant formula has been epidemiologically associated with *Cronobacter* infection in infants. *Cronobacter* are resistant to desiccation, and heat treatment can change the organoleptic, nutritional, and functional properties of powdered infant formula. Accordingly, a specification limit for *Cronobacter* spp. (absent in 10 g) has been established as part of microbiological quality standards for powdered infant formula in the U.S. under 21 CFR §106.55 (U.S. FDA, 2021d). Hence, as part of the system of process controls to prevent adulteration of powdered infant formula from microorganisms, the specifications for 2'-FL added during the dry blending stage of infant formula manufacture include this specification limit for *Cronobacter* spp. Results from the analysis of three non-consecutive batches of non-crystallized 2'-FL demonstrate compliance of the ingredient with this specification limit (see revised Table 2.3-1 presented above in Glycom's response to the FDA Question No. 17).

Enterobacteriaceae including *C. sakazakii* are highly sensitive to heat processing and can be effectively controlled via thermal killing steps (WHO, 2006). Infant and toddler formula with 2-FL added during the wet-blending stage of the manufacturing process are subject to heat-treatment[2] microbial kill steps. Heat-treatment during wet blending can achieve reductions in excess of 8 to 12 log units for vegetative microorganisms such as *C. sakazakii*, and therefore heat-treatment is a highly effective process control for microbial contamination during wet blending (Cordier, 2006).

The FAO/WHO expert meetings have identified infants (<12 months of age) as the population at risk for *Cronobacter* infections, especially infants less than 2 months of age (*i.e.*, including neonates) and those that are pre-term, low-birth weight (<2,500 g), or immunocompromised (CAC/RCP, 2008). According to the Codex Alimentarius Commission (CAC) criteria within the *Code of Hygienic Practice for Powdered Formulae for Infants and Young Children*, there is no criterion for *Cronobacter* spp. for follow-up formula

[2] *E.g.*, heat-treatments at temperatures above 75°C for 30 seconds will provide a reduction in excess of 10 log units of vegetative microorganisms such as *Enterobocteriaceae*, including *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*); heat-treatments above 100°C will lead to reductions in excess of several hundred log units (WHO, 2006). Microbial specifications used for wet-blending applications will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55 (U.S. FDA, 2021d).

and formula for special medical purposes for young children due to decreased susceptibility in older infants and young children (CAC/RCP, 2008; Buchanan and Oni, 2012).

The proposed microbiological criteria for uses of 2'-FL in foods intended for infants and young children, which may not be subjected to thermal processing, are aligned with international standards for microbiological examination of ready-to-eat foods (NRC, 1985; EU, 2005; FSANZ, 2022). Both the World Health Organization (WHO) and American Academy of Pediatrics (AAP) recommend the introduction of complementary foods at 6 months (Meek *et al*., 2022; WHO, 2021), at which point older infants and young children have decreased susceptibility to *Cronobacter* infection (CAC/RCP, 2008; Buchanan and Oni, 2012).

Nevertheless, *Cronobacter* spp. (absent in 10 g) is an established internal specification for Glycom's 2'-FL ingredient. Furthermore, the specifications for 2'-FL under all intended conditions of use include a specification limit for the *Enterobacteriaceae* family, which encompasses the *Cronobacter* genus.

TOXICOLOGY (FDA Q19- Q27):

FDA.19. *On p. 13 of the notice, Glycom notes that D-lactose is a raw material used in the manufacturing of non-crystallized 2'-FL and that DFL is a carbohydrate formed during its fermentation. However, on p. 11 of the notice, Glycom states that D-lactose and DFL are not considered "impurities" in the non-crystallized 2'-FL ingredient due to their naturally occurring presence in human milk. We note that Glycom's definition of "impurities" differs from how FDA views impurities for food ingredients. We view D-lactose and DFL as either: (1) residual starting materials, substances formed as a result of manufacturing processes (i.e., fermentation) or degradation by-products which are found in the finished ingredient or (2) they are being intentionally formed or added to the final ingredient. If D-lactose and DFL are not impurities, please clarify why the article of commerce should not be considered a mixture consisting of non-crystallized 2'-FL, DFL, and D-lactose.*

Glycom acknowledges the ambiguity around the terminology used to define other carbohydrates in the final non-crystallized 2'-FL ingredient as a result of the biosynthesis of 2'-FL. The lactose substrate is a residual starting material from the fermentation process, while DFL is formed as a result of the fermentation process by the addition of a second fucose unit to 2'-FL by the same fucosylating enzyme. They are not being intentionally formed or added to the final non-crystallized 2'-FL ingredient.

Glycom's non-crystallized 2-FL obtained by microbial fermentation using modified downstream processing methods (*i.e.*, spray-drying in place of the crystallization step) continues to contain 2'-FL as the primary constituent. However, the levels of DFL and lactose resulting from the fermentation process in the final non-crystallized 2-FL ingredient may be higher (up to 5% and 10% w/w, respectively) compared to crystallized 2'-FL due to the modified downstream processing. For Glycom's 2'-FL/DFL, the production strain is optimized for DFL production, *i.e.*, DFL is being intentionally formed.

Specification parameters for defined carbohydrates between crystallized 2'-FL (GRN 650, Glycom A/S, 2016; U.S. FDA, 2016), non-crystallized 2'-FL (GRN 1060) and 2'-FL/DFL (GRN 815, Glycom A/S, 2018; U.S. FDA, 2019) are compared in the Table 11.

Table 11 Comparison of GRAS Notified Specifications for 2'-FL, DFL and D-Lactose Between Non-Crystallized 2'-FL (GRN 1060), Crystallized 2'-FL (GRN 650) (Glycom A/S, 2016; U.S. FDA, 2016) and 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019)

Parameters	Unit	GRN 650 2'-FL Crystallized	GRN 1060 2'-FL Non-Crystallized	GRN 815 2'-FL/DFL
Assay (water-free) 2' fucosyllactose	w/w %	≥ 94.0	≥ 85.0	≥ 75.0
Difucosyl-D-lactose	w/w %	≤ 1.0	≤ 5.0	≤ 20.0
D-Lactose	w/w %	≤ 3.0	≤ 10.0	≤ 10.0

2'-FL = 2'-fucosyllactose; DFL = difucosyllactose; GRAS = Generally Recognized as Safe; GRN = GRAS Notice.

FDA.20. *On p. 20 of the notice, Glycom states, "The increase in the specification limit for DFL from ≤ 1.0 to ≤ 5.0% will not have an impact on the safety of 2'-FL as it has already been concluded to be GRAS in the case of GRN 815 of 2'-FL/DFL with levels of DFL ≤ 20%...". However, we note that GRN 000815 specified a use level for the 2'-FL/DFL ingredient in infant and "toddler" formula that is different than the use level proposed in this notice for non-crystallized 2'-FL. Thus, a direct comparison of the specifications for these two ingredients requires additional context. Please provide additional information that clarifies how levels of DFL specified in GRN 000815 can support the safety of DFL in the subject of this notice.*

The Agency is correct. Direct comparison of the specification limit for DFL in non-crystallized 2'-FL (GRN 1060) and 2'-FL/DFL (GRN 815, Glycom A/S, 2018; U.S. FDA, 2019) without taking into account the different conditions of use in term infant formula or toddler formula has limited translation to maximum potential exposure to DFL from non-crystallized 2'-FL. To provide additional context, as demonstrated in Table 12, maximum potential levels of DFL added to term infant formula or toddler formula have been calculated for both ingredients taking into consideration the ingredient specifications and GRAS notified or proposed levels of use in these food categories. In numerical values, dietary exposure to DFL from term infant and toddler formulas is two to three times higher from 2'-FL/DFL (0.32 and 0.24 g/L, respectively) than from non-crystallized 2'-FL (0.12 g/L in both cases).

Table 12 Comparison of the Maximum Potential Level of DFL Added to Term Infant Formula and Toddler Formula According to Specifications and the Conditions of Use of Non-Crystallized 2'-FL (GRN 1060) and 2'-FL/DFL (GRN 815) (Glycom A/S, 2018; U.S. FDA, 2019)

Human Milk Oligosaccharide	GRN 1060 2'-FL Non-Crystallized	GRN 815 2'-FL/DFL
Max DFL content allowed by specification	5.0 %	20.0 %
Max GRAS notified or proposed use level in term infant formulas	2.4 g/L	1.6 g/L
Max GRAS notified or proposed use level in toddler formulas	2.4 g/L	1.2 g/L
Calculated max DFL content in term infant formulas	**0.12 g/L**	**0.32 g/L**
Calculated max DFL content in toddler formulas	**0.12 g/L**	**0.24 g/L**

2'-FL = 2'-fucosyllactose; DFL = difucosyllactose, IF = infant formula, GRN = GRAS Notice, GRAS = Generally recognized as safe, Max = maximum .

FDA.21. *Please provide a brief discussion regarding the formation and safety of 2'-fucosyl-D-lactulose.*

An isomer of 2'-FL, 2'-fucosyl-D-lactulose, is generated by the isomerization of the terminal glucose moiety of 2'-FL to fructose. This type of isomerization is pH and temperature dependent and has been commonly reported for the closely related conversion of D-lactose into lactulose during heat treatment [i.e., ultra-high temperature (UHT) processing and pasteurization] of milk, including human donor milk (Beach and Menzies, 1983; Schuster-Wolff-Bühring *et al.,* 2010; Gómez de Segura *et al.,* 2012). This isomerization reaction of carbohydrates is also known as the Lobry de Bruyn–van Ekenstein transformation (Angyal, 2001; Wang, 2010). Various infant formulas have been reported to contain lactulose at relative levels between 1 and 7% of their D-lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Although the isomerization product of 2'-FL has not been specifically evaluated in heat treated human donor milk, lactulose has also been detected at significant proportions of D-lactose (Gómez de

Segura *et al.*, 2012), and it can thus be reasonably assumed that 2'-fucosyl-D-lactulose is present at comparable ratios and can thereby be equally regarded to have a history of safe use from heat treated human donor milk. In any case, at the low levels of this isomerization product detected in the batches of 2'-FL (not more than 1.0%), its exposure is expected to be negligible and not biologically or nutritionally relevant.

FDA.22. *On p. 23 of the notice, Glycom discusses the results of the unpublished 90-day repeated dose toxicity study performed using non-crystallized 2'-FL as the test article. Specifically, Glycom notes, "There were 9 deaths during the course of the study, but none of the deaths were considered to be test item-related." Without additional information beyond what is currently provided in the notice, this observation could appear counter to a GRAS conclusion for Glycom's non-crystallized 2'-FL ingredient. Please provide additional details regarding these nine deaths, as well as a brief explanation of the main statistically significant changes, if any, observed during the study.*

The early decedents were primarily in the mid-dose group (5 in total), with 1 in the low-dose group and 3 in the high-dose group. Two of the deaths in the high-dose group were clearly due to dosing errors (confirmed by macroscopic examination). Autolysis of the large intestine precluded pathological confirmation of the cause of death for the other high-dose decedent, but since it was euthanized due to observations of labored breathing and a cream discharge from the mouth, it is possible that a dosing error was also the cause of death for this animal. The cause of the other seven deaths was unclear. Autolysis of the large intestine precluded pathological examination of 2 of the mid-dose decedents so the cause of death could not be confirmed for those animals, and only inflammatory changes in the large intestine were noted for the other 3 decedents in that group. There were no noteworthy pathological findings in the low-dose decedent. Overall, there were no findings in the study to suggest that any of these deaths were caused by the test item, especially due to the absence of any clear test item-related deaths in the high-dose group, which makes it highly unlikely that the deaths in the lower dose groups were due to the test item.

FDA.23. *Beginning on p. 24 of the notice, Glycom discusses newly identified clinical studies in infants that utilize test formulas containing 2'-FL. We note that Storm et al., 2019; Czerkies et al., 2019; Román Riechmann et al., 2020; Corsello et al., 2020 [abstract]; Ramirez-Farias et al., 2021; and Vandenplas et al., 2020 report on test formulas that contain 2'-FL at levels below the proposed use level of 2.4 g/L. Please clarify how these studies support Glycom's GRAS conclusion for 2'-FL at a use level of 2.4 g/L. As part of this response, please also confirm that the Corsello et al., 2020 abstract has been recently published as Alliet et al., 2022.*[1]

The safety of the proposed use level of 2'-FL in non-exempt term infant formula of 2.4 g/L is based on the average concentration of 2'-FL naturally occurring in human milk, as HMOs including 2'-FL have an established history of safe consumption by breastfed infants. This same condition of use has been notified as GRAS for numerous other 2'-FL ingredients that have received no question letters from the U.S. FDA, including Glycom's crystallized 2'-FL ingredients manufactured by chemical synthesis (GRN 546, Glycom A/S, 2014; U.S. FDA, 2015) or by fermentation (GRN 650, Glycom A/S, 2016; U.S. FDA, 2016).

Part 6 of a GRAS Notice is required to demonstrate a complete and balanced evaluation of all applicable data and information (U.S. FDA, 2017). Glycom considers newly identified clinical studies in infants that utilize test formulas containing 2'-FL relevant in determining the GRAS status of 2'-FL. Although the newly

identified studies report on test formulas that contain 2′-FL at levels below the proposed use level of 2′-FL in the current GRAS Notice, these include safety-related endpoints, the results of which could still affect the GRAS conclusion. It also provides a means for monitoring the safety of the supplementation of 2′-FL in different types of infant formula and additional samples of the infant population. As there were no concerns related to safety reported in the newly identified clinical studies of 2′-FL in infants, Glycom considered these studies to support its GRAS conclusion.

Glycom confirms that the double-blind randomized controlled trial in infants provided formula with 2′-FL and *Lactobacillus reuteri* summarized from the Corsello *et al.* (2020) abstract has recently been published as Alliet *et al*. (2022). The safety conclusions of the study remain the same – infant formula supplemented with *Lactobacillus reuteri* and 1.0 g/L of 2′-FL support age-appropriate growth, is well-tolerated, and is safe. In addition to growth-, tolerability-, and safety-related parameters, the Alliet *et al*. (2022) publication reports results for gastrointestinal-related effects.

FDA.24. *On pp. 30-31 of the notice, Glycom discusses the study by Leung et al., 2020, which investigated the effects of formulas for young children on respiratory and gastrointestinal infections in Chinese children (1-2.5 years). Although a detailed summary is provided, Glycom does not specifically comment on the finding that one of the test formulas containing 2′-FL (YCF-C) prolonged upper respiratory tract infections by an average of 1.6 days. Please discuss why this finding is not expected to be a safety concern for Glycom's intended use.*

The randomized, controlled, double-blind, parallel-group clinical trial (described by Leung *et al.,* 2020) investigated the effects of new young child formulas (YCFs) containing 2′-FL at levels found in breast milk and/or bioactive proteins [immunoglobulins, lactoferrin, and transforming growth factor-β (TGF-β)], provided at a volume of 400 mL/day for a duration of 6 months, on respiratory and gastrointestinal infections in toddlers. Primary outcomes were the incidence of upper respiratory tract infection (URTI) and duration of gastrointestinal tract infections (GITI). A cohort of 456 healthy Chinese children aged 1 to 2.5 years were randomized to receive a reference milk formula (YCF-Ref) containing 0.4 g/100 mL galacto-oligosaccharides (GOS) or one of the following test YCFs:

1. **YCF-A** – milk formula containing added GOS (0.4 g/100 mL), **2′-FL** (0.3 g/100 mL), immunoglobulins (0.1 g/100 mL), lactoferrin (0.17 g/100 mL), and TGF-β (1.5 µg/100 mL), as well as higher milk fat (1.7 g/100 mL)
2. **YCF-B** - milk formula containing added GOS (0.4 g/100 mL), immunoglobulins (0.01 g/100 mL), lactoferrin (0.01 g/100 mL), and TGF-β (1.5 µg/100 mL)
3. **YCF-C** - milk formula containing added GOS (0.4 g/100 mL) and **2′-FL** (0.3 g/100 mL)

The mean duration of URTI, a secondary outcome, was reported to be significantly longer ($p < 0.05$) in infants receiving the test formula containing GOS plus 2′-FL (YCF-C; 8.9 ± 5.6 days) compared to those receiving the reference milk formula containing only GOS (YCF-Ref; 7.3 ± 4.6 days). Contrary, the mean duration of URTI was not significantly different between YCF-Ref and YCF-A, which also contained 2′-FL (8.1 ± 7.4 days), nor YCF-B (8.6 ± 6.0 days). As the reference YCF contains the prebiotic GOS it is not possible to make any conclusions on the effect of YCF containing 2′-FL on mean duration of URTI in the absence of GOS.

Furthermore, the incidence of URTI was low in all study groups (average of 1.7 – 2.0 episodes/subject), especially in comparison to the assumption on mean number of URTIs applied in the sample size

calculation for this primary outcome (3.5 episodes/subject). No differences were reported between the reference YCF and test YCF groups for URTI incidence and GITI duration even though the study was powered for these primary outcomes. However, statistically significant results were found for secondary and tertiary outcomes, for which p-values were not adjusted for multiple testing to control for false positives. As such, it is difficult to interpret the clinical relevance of the finding that URTI was prolonged in YCF-C compared to YCF-Ref.

FDA.25. *On p. 32 of the notice, Glycom discusses the clinical study by Iribarren et al., 2020. We note there is a typo in the high dose given to study volunteers. It is 10 g/d, not 19 g/d, at a 4:1 ratio of 2'-FL and LNnT. Please provide a correction to this statement.*

We would like to thank the FDA for recognizing this error and apologize for the discrepancy between the GRAS Notice and the dosages evaluated by Iribarren *et al.* (2020). Indeed, the high dose given to study volunteers in the Iribarren *et al.* (2020) clinical study was 10 g/day, at a 4:1 ratio of 2'-FL and LNnT, respectively.

FDA.26. *On p. 33 of the notice, Glycom discusses the Hascoët et al., 2022 clinical study in preterm infants; however, we note that several aspects of the study design are suggestive of a therapeutic intervention and may not be relevant for the safety assessment of 2'-FL in infant formula. Specifically, 2'-FL and LNnT were given as a liquid supplement with a "dosage" calculated each day according to the infant's daily weight; the liquid supplement was provided three times per day in a "sterile solution" (i.e., it is unclear if this solution was an infant formula matrix); and enrolled infants were permitted mixed feeding modalities, including human milk, human milk fortifier, and preterm formula. Please clarify how the Hascoët et al., 2022 clinical study supports Glycom's GRAS conclusion for 2'-FL in infant formula for term infants.*

The Hascoët *et al.* (2022) clinical study in preterm infants is relevant to Glycom's GRAS conclusion for 2'-FL in infant formula for term infants since it evaluated safety-related outcomes following the intake of 2'-FL in combination with another HiMO, LNnT, in a vulnerable infant population group. Specifically, the HMO supplement containing 2'-FL and LNnT was administered by syringe directly into the mouth or by enteral tube (*i.e.*, directly into the stomach or small intestine) before feeding. As a result, 2'-FL administered in the study would be subject to the same metabolic fate as described in the GRAS Notice, given that human milk oligosaccharides do not undergo any significant digestion in the upper gastrointestinal tract. Safety-related outcomes evaluated included anthropometric measures (growth), gastrointestinal tolerance, and incidence and severity of adverse events. The study authors concluded that 2'-FL and LNnT supplementation was safe and well-tolerated in preterm infants. Thus, the absence of unfavorable effects in a preterm population group that have an immature gastrointestinal tract reinforces the safety of 2'-FL in term infants whose gastrointestinal tract is more mature.

FDA.27. *On p. 43 of the notice, Glycom states that the GRAS Panel, "independently and critically evaluated all data and information presented herein…". However, we note that the end dates of the literature searches differ between the notice and the GRAS Panel statement (December 2021 vs. May 2021, respectively). As such, it does not appear that the GRAS Panel evaluated all of the published studies presented in the notice (e.g., Parschat et al., 2021; Fonvig et al., 2021). Please clarify which studies discussed in the notice were not evaluated by Glycom's GRAS Panel and whether the existence of these newer studies impacts the GRAS Panel's conclusion.*

Glycom would like to thank the FDA and apologize for the discrepancy between the GRAS Notice and the GRAS Panel evaluation. There were three additional studies included in the GRAS Notice that were not evaluated by the GRAS Panel (Fonvig *et al.,* 2021; Hascoët *et al.,* 2021; Parschat *et al.,* 2021). For all three studies, the study investigators concluded that 2'-FL supplementation, alone or in combination with other HiMOs, was safe and well tolerated in infant and child population groups under the conditions of the study. As such, these studies do not impact the GRAS Panel's conclusion.

--

We hope this information adequately addresses the Agency's questions on GRN 001060, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Marta H. Mikš, DSc, PhD
Senior Regulatory & Scientific Affairs Manager
Glycom A/S

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From:	Miks, Marta
To:	Morissette, Rachel
Subject:	[EXTERNAL] RE: GRN 001060 follow-up questions
Date:	Monday, January 23, 2023 10:21:04 AM
Attachments:	image008.png image009.png image010.png image011.png image015.png image016.png image017.png image018.png image019.png image020.png image002.png GRN 001060 - Response to FDA Questions - 06Jan"23_Final.pdf
Importance:	High

For Internal Use Only

Dear Rachel,

Please find enclosed responses to the United States (U.S.) Food and Drug Administration (FDA)'s follow-up questions received by email on 06 January 2023 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

Kindly acknowledge receipt of this email

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

Glycom, the leading HMO expert is part of DSM

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, January 6, 2023 7:55 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Subject: GRN 001060 follow-up questions

Hi Marta,

We had a couple follow-up questions.

1. For the internal methods listed in Table 2 of Glycom's response to FDA's question 5 from the November 18, 2022 amendment, we request that the detection method be indicated for the HPLC methods described for 2'-FL, DFL, D-lactose, L-fucose, and 2'-Fucosyl-D-lactose. While the separation methods are indicated (e.g., HPLC, HPAEC), the methods of detection are not.

2. In question 17 of the November 18, 2022 amendment, we asked, "If 2'-FL is intended for use in powdered infant formula, please provide the analytical methods and data from three non-consecutive batches for *C. sakazakii*, *L. monocytogenes*, and *B. cereus*." We note that the complete citations for the analytical methods used for these specifications were not provided. Please provide the complete citations for the analytical methods used for the microbial specifications listed in Table 2.3-1 on page 16 of the November 18, 2022 amendment. Further, please state whether these analytical methods were validated for their intended purpose.

   Additionally, we note that the analytical method often used to test for *Cronobacter* spp. is ISO/TS 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of *Cronobacter spp*." Please clarify whether presumptive positives resulting from this test are further analyzed to determine if the isolate is *C. sakazakii*.

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

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23 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: GRAS Notice No. GRN 001060

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 06 January 2023 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

FDA.1. *For the internal methods listed in Table 2 of Glycom's response to FDA's question 5 from the November 18, 2022 amendment, we request that the detection method be indicated for the HPLC methods described for 2'-FL, DFL, D-lactose, L-fucose, and 2'-Fucosyl-D-lactose. While the separation methods are indicated (e.g., HPLC, HPAEC), the methods of detection are not.*

The corresponding detection methods to each of Glycom's internal analytical methods for carbohydrate specification parameters are provided in Table 1 below.

Table 1 Glycom Internal Analytical Methods for Carbohydrate Specification Parameters of Non-Crystallized 2'-FL (GRN 1060), with Corresponding Detection Methods

Glycom Internal Analytical Method	Detection Method
HPLC-202-2C4-002	Refractive index detector (RID)
HPAEC-HMO-015	Pulsed amperometric detector (PAD)
HPLC-2-003	Charged corona aerosol detector (cCAD)

HMO = human milk oligosaccharide; HPAEC = high-performance anion-exchange chromatography; HPLC = high-performance liquid chromatography.

FDA.2. *In question 17 of the November 18, 2022 amendment, we asked, "If 2'-FL is intended for use in powdered infant formula, please provide the analytical methods and data from three non-consecutive batches for C. sakazakii, L. monocytogenes, and B. cereus." We note that the complete citations for the analytical methods used for these specifications were not provided. Please provide the complete citations for the analytical methods used for the microbial specifications listed in Table 2.3-1 on page 16 of the November 18, 2022 amendment. Further, please state whether these analytical methods were validated for their intended purpose.*

Additionally, we note that the analytical method often used to test for Cronobacter spp. is ISO/TS 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp." Please clarify whether presumptive positives resulting from this test are further analyzed to determine if the isolate is C. sakazakii.

Analytical methods used to evaluate microbiological specifications for non-crystallized 2'-FL (added during the wet-blending or dry-blending stage of infant formula manufacture) are provided in Table 2 below. All methods for microbiological specifications are standardized methods that are internationally recognized and widely accepted for microbiological testing of foodstuffs.

Table 2 Microbiological Specifications and Analytical Methods for Non-Crystallized 2'-FL (GRN 1060)

Parameter	Specification	Method
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	< 10 CFU/g[a]	ISO 21528-2[a]
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter spp.[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 Française de Normalisation; CFU = colony forming units; ISO = International Organization for Standardization.

[a] Alternatively, *Enterobacteriaceae* may be evaluated using ISO 21528-1, resulting in the stricter specification 'Absent in 10 g'.

[a] Not applicable when 2'-FL is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior to drying.

Indeed, Glycom tests for the presence of *Cronobacter* spp. for non-crystallized 2'-FL that is added during the dry blend stage of infant formula manufacture using ISO 22964, which corresponds to *"Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp."*.

As per the current ISO 22964:2017 version of the method, presumptive positives would be isolated (Chapter 10.4) and biochemically confirmed (Chapter 10.5), from which *Cronobacter* spp. can be phenotypically distinguished from closely related species based on specific biochemical characteristics (Annex C).

Historically at Glycom, batch analysis results have never identified a presumptive positive for *Cronobacter* spp. Furthermore, the specifications for non-crystallized 2'-FL that is added during the dry-blending stage of infant formula manufacture require for *Cronobacter* spp. to be absent in 10 g.

--

We hope this information adequately addresses the Agency's questions on GRN 001060, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.
Sincerely,

Marta H. Mikš, DSc, PhD
Senior Regulatory & Scientific Affairs Manager
Glycom A/S

From: Miks, Marta
To: Morissette, Rachel
Subject: RE: [EXTERNAL] RE: GRN 001060 follow-up questions
Date: Friday, February 10, 2023 9:10:52 AM
Attachments: image001.png
image003.png
image004.png
image005.png
image009.png
image010.png
image011.png
image012.png
image014.png
image015.png
image017.png
image018.png
image002.png
Importance: High

For Internal Use Only

Dear Rachel,

Glycom confirms that each microbiological parameter is measured using a fit-for-purpose compendial method or validated alternative method for their intended purpose.

Kindly acknowledge the receipt of our response

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

Glycom, the leading HMO expert is part of DSM

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, January 25, 2023 8:03 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Subject: RE: [EXTERNAL] RE: GRN 001060 follow-up questions

Hi Marta,

Please see below:

In our January 6, 2023 questions, we asked,

"In question 17 of the November 18, 2022 amendment, we asked, "If 2′-FL is intended for use in powdered infant formula, please provide the analytical methods and data from three nonconsecutive batches for *C. sakazakii*, *L. monocytogenes*, and *B. cereus*." We note that the complete citations for the analytical methods used for these specifications were not provided. Please provide the complete citations for the analytical methods used for the microbial specifications listed in Table 2.3-1 on page 16 of the November 18, 2022 amendment. Further, please state whether these analytical methods were validated for their intended purpose."

We note that in your response from January 23, 2023, you provided the analytical methods used for the microbial specifications but you did not state whether these methods have been validated for their intended purpose. Please confirm whether the analytical methods you provided for the microbial specifications have been validated for their intended purpose.

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: Miks, Marta <Marta.Miks@dsm.com>
Sent: Monday, January 23, 2023 10:21 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: [EXTERNAL] RE: GRN 001060 follow-up questions
Importance: High

For Internal Use Only

Dear Rachel,

Please find enclosed responses to the United States (U.S.) Food and Drug Administration (FDA)'s follow-up questions received by email on 06 January 2023 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

Kindly acknowledge receipt of this email

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, January 6, 2023 7:55 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Subject: GRN 001060 follow-up questions

Hi Marta,

We had a couple follow-up questions.

1. For the internal methods listed in Table 2 of Glycom's response to FDA's question 5 from the November 18, 2022 amendment, we request that the detection method be indicated for the HPLC methods described for 2'-FL, DFL, D-lactose, L-fucose, and 2'-Fucosyl-D-lactose. While the separation methods are indicated (e.g., HPLC, HPAEC), the methods of detection are not.

2. In question 17 of the November 18, 2022 amendment, we asked, "If 2'-FL is intended for use in powdered infant formula, please provide the analytical methods and data from three non-consecutive batches for *C. sakazakii*, *L. monocytogenes*, and *B. cereus*." We note that the complete citations for the analytical methods used for these specifications were not provided. Please provide the complete citations for the analytical methods used for the microbial specifications listed in Table 2.3-1 on page 16 of the November 18, 2022 amendment. Further, please state whether these analytical methods were validated for their intended purpose.

   Additionally, we note that the analytical method often used to test for *Cronobacter* spp. is ISO/TS 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of *Cronobacter spp*." Please clarify whether presumptive positives resulting from this test are further analyzed to determine if the isolate is *C. sakazakii*.

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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From:	Miks, Marta
To:	Morissette, Rachel
Cc:	Roehrig, Christoph
Subject:	RE: [EXTERNAL] RE: additional questions for GRN 001060
Date:	Thursday, March 16, 2023 10:00:58 AM
Attachments:	image001.png image002.png image003.png image004.png image015.png image016.png image017.png image018.png image021.png image022.png image025.png image006.png Responses to FDA additional questions-GRN 1060 - Final - 16Mar"23.pdf
Importance:	High

For Internal Use Only

Dear Rachel,

Please find enclosed responses to the United States (U.S.) Food and Drug Administration (FDA)'s follow-up questions received by email on 16 February 2023 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

Kindly acknowledge receipt of this email

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, March 10, 2023 5:37 PM

To: Miks, Marta <Marta.Miks@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: additional questions for GRN 001060

Thanks, Marta. That will be fine.

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: Miks, Marta <Marta.Miks@dsm.com>
Sent: Friday, March 10, 2023 11:26 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: additional questions for GRN 001060

For Internal Use Only

Dear Rachel,

Thank you for you kind inquiry.

DSM's responses to the FDA questions sent on 16th Feb 2023 have been prepared. However, together with a response letter we are planning to submit a full version of revised specification for non-crystalized 2'-FL ingredient (GRN 1060), since during the review process and Q&A period, multiple parameters and limits have been amended. We believe it would be supportive and well received by FDA.

Since, the draft specification is currently under review, my ambition is to complete submission mid next week. Hopefully, it meets your expectations.

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, March 10, 2023 2:49 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: additional questions for GRN 001060

Hi Marta,

We wanted to check on the status of our questions sent Feb. 16 and when you anticipated providing a response. We are unable to move your response letter along any further until we receive those responses.

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: Miks, Marta <Marta.Miks@dsm.com>
Sent: Thursday, February 16, 2023 4:06 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: additional questions for GRN 001060

For Internal Use Only

Dear Rachel,

This is to acknowledge receipt of your email and FDA questions to 2'-FL GRAS Notice No. 1060. Glycom would provide the responses as soon as possible.

Thank you for your kind understanding

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:



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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, February 16, 2023 4:32 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: additional questions for GRN 001060

Dear Marta,

We apologize for not including these questions in an earlier round, but please see some additional questions from chemistry below for GRN 001060. We are continuing to move your response letter along in the meantime, but will need responses to these questions as soon as possible before we can issue a no questions letter.

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

1. In the November 18, 2022 amendment, Glycom states in response to question 9 that "compositional changes between non-crystallized 2'-FL and crystallized 2'-FL are largely limited to higher potential levels of lactose and DFL." However, in the batch analyses provided in the notice, Glycom reports D-lactose levels between 1.2-2% and 3,2'-difucosyl-D-lactose (DFL) levels between 1.8 and 1.9%. In contrast, the unspecified carbohydrates, by calculation (using Table 2.3-1 values for "assay (water-free), specified saccharides"), are between 5.6 and 7.7%, suggesting that the unspecified carbohydrates comprise a greater portion of the ingredient than the sum of lactose and DFL. While we have seen "other" carbohydrates represent up to 7% of 2'-FL ingredients that have been the subjects of GRNs that received "No Questions" letters, the components that comprise the "other" carbohydrates fraction were described in these prior notices (e.g., glucose, galactose, fucosyl-galactose, 3-FL, lactitol, and lactitol derivatives). Please clarify the identity of the unspecified carbohydrates in the 2'-FL ingredient that is the subject of GRN 001060.

2. We request that Glycom considers reducing the limit for non-specified carbohydrates in its 2'-FL ingredient. We note that since GRN 001060 was submitted, the Food Chemicals Codex (FCC, 2022) issued a monograph effective December 1, 2022 for 2'-FL. In that monograph, 2'-FL is described as an ingredient that is dried or crystallized and contains, on an anhydrous weight basis, not less than (NLT) 92% 2'-FL (where 2'-FL is calculated as the sum of 2'-FL, L-fucose, D-lactose, and DFL); where D-lactose is not more than (NMT) 8.0%; DFL is NMT 7.0%; L-fucose is NMT 3.0%, and 2'-fucosyl-D-lactulose is NMT 2.0%. In GRN 001060, the specification for "specified saccharides" is ≥ 90% on a dry basis, where specified saccharides include 2'-FL, DFL, D-lactose, L-fucose, and 2'-fucosyl-D-lactulose. By inference, unspecified carbohydrates may comprise up to 10% of the ingredient dry matter. We suggest that, in accordance with the FCC monograph, Glycom proposes a specification of ≥ 92% of specified carbohydrates, where the specified carbohydrates include 2'-FL, D-lactose, DFL, and L-fucose. If Glycom's specifications do not agree with the FCC specifications, please discuss how the reduction in 2'-FL and increase in the proportion of unspecified components do not affect the safety conclusion for 2'-FL. Please also discuss these impurities in the context of the safety of the ingredient for its intended use. While lactose and fucose were addressed, we request that Glycom includes a discussion of the other minor components considered in the safety evaluation.

3. Please clarify if the dietary exposure estimates provided in the November 18, 2022 amendment are based on the level of 2'-FL in the ingredient, so that proportionally more of the whole ingredient would be added to provide the stated maximum use level of 2'-FL, or if they are based on a 1:1 weight basis for the total ingredient containing ~88-90% 2'-FL that would replace current uses of 2'-FL described in GRN 000650. For the dietary exposure estimates described in GRN 000650, and cited in Tables 4, 5, and 6 of the amendment, it appears that the estimates are based on the 2'-FL component (i.e., the ingredient purity was assumed to be 100%), but this is not explicitly stated. For the cumulative uses of 2'-FL described in Table 7, footnote c below the table indicates "Use level expressed on a 2'-FL basis in the final food, as consumed," although footnote c does not appear in the table itself. Please clearly state the basis of the estimates provided in the amendment.

4. Please correct the heading for Table 4 in the November 18, 2022 amendment, which appears to include uses other than infant formula in the description.

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

16th March 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: GRAS Notice No. GRN 001060

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s additional follow-up questions received by email on 16th February 2023 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of non-crystallized 2'-fucosyllactose (2'-FL) filed by the Agency under GRN 001060.

FDA.1. *In the November 18, 2022 amendment, Glycom states in response to question 9 that "compositional changes between non-crystallized 2'-FL and crystallized 2'-FL are largely limited to higher potential levels of lactose and DFL." However, in the batch analyses provided in the notice, Glycom reports D-lactose levels between 1.2-2% and 3,2'-difucosyl-D-lactose (DFL) levels between 1.8 and 1.9%. In contrast, the unspecified carbohydrates, by calculation (using Table 2.3-1 values for "assay (water-free), specified saccharides"), are between 5.6 and 7.7%, suggesting that the unspecified carbohydrates comprise a greater portion of the ingredient than the sum of lactose and DFL. While we have seen "other" carbohydrates represent up to 7% of 2'-FL ingredients that have been the subjects of GRNs that received "No Questions" letters, the components that comprise the "other" carbohydrates fraction were described in these prior notices (e.g., glucose, galactose, fucosyl-galactose, 3-FL, lactitol, and lactitol derivatives). Please clarify the identity of the unspecified carbohydrates in the 2'-FL ingredient that is the subject of GRN 001060.*

The "unspecified carbohydrates" fraction in the non-crystallized 2'-FL ingredient is comprised of glucose (raw material), 2-fucosyl-galactose, 3-fucosyllactose, lactitol, 2'-fucosyl-lactitol, sorbitol, mannitol and galactitol (fermentation by-products), as well as thermal degradation products of glucose (typical components of caramel), such as levoglucosan, 1,6-anhydro-glucofuranose, isomaltose, kojibiose, gentiobiose, 6-α-glucofuranosyl-glucose and 6-β-glucofuranosyl-glucose.

Glycom suggests adding new parameter to the non-crystallized 2'-FL ingredient specifications - Sum of other carbohydrates – with a limit of ⩽ 6.0 w/w% to cover these unspecified carbohydrates. The analytical results for regulatory batches of non-crystallized 2'-FL ingredient for the new parameter - Sum of other carbohydrates – have been provided in Table 1 below. This parameter is assessed using the same Glycom method indicated for other carbohydrate parameters (Glycom method HPAEC-HMO-015), which has been validated for the analysis of the sum of other carbohydrates in batches of non-crystallized 2'-FL for conformance with the above specification. The updated specification for the non-crystallized 2'-FL ingredient is provided in Attachment 1.

Table 1 **The Batch Results for Parameter Sum of Other Carbohydrates for Non-Crystallized 2'-FL (GRN 1060)**

Parameter		Batch Analyses (GRN 1060)		
		NON-CRYSTALLIZED 2'-FL		
	Unit	19481101	19501101	D_2FL_EP_ 2021_2_FD_1
Sum of other carbohydrates	w/w %	2.21	2.61	2.25

FDA.2. *We request that Glycom considers reducing the limit for non-specified carbohydrates in its 2'-FL ingredient. We note that since GRN 001060 was submitted, the Food Chemicals Codex (FCC, 2022) issued a monograph effective December 1, 2022 for 2'-FL. In that monograph, 2'-FL is described as an ingredient that is dried or crystallized and contains, on an anhydrous weight basis, not less than (NLT) 92% 2'-FL (where 2'-FL is calculated as the sum of 2'-FL, L-fucose, D-lactose, and DFL); where D-lactose is not more than (NMT) 8.0%; DFL is NMT 7.0%; L-fucose is NMT 3.0%, and 2'-fucosyl-D-lactulose is NMT 2.0%. In GRN 001060, the specification for "specified saccharides" is ≥ 90% on a dry basis, where specified saccharides include 2'-FL, DFL, D-lactose, L-fucose, and 2'-fucosyl-D-lactulose. By inference, unspecified carbohydrates may comprise up to 10% of the ingredient dry matter. We suggest that, in accordance with the FCC monograph, Glycom proposes a specification of ≥ 92% of specified carbohydrates, where the specified carbohydrates include 2'-FL, D-lactose, DFL, and L-fucose. If Glycom's specifications do not agree with the FCC specifications, please discuss how the reduction in 2'-FL and increase in the proportion of unspecified components do not affect the safety conclusion for 2'-FL. Please also discuss these impurities in the context of the safety of the ingredient for its intended use. While lactose and fucose were addressed, we request that Glycom includes a discussion of the other minor components considered in the safety evaluation.*

Glycom is agreeable to increasing the specification limit for the "Assay (water-free) specified saccharides" parameter of the non-crystallized 2'-FL ingredient to ≥ 92%, calculated as the sum of 2'-FL, L-fucose, D-lactose, DFL and 2'-fucosyl-D-lactulose.

As indicated above, the updated specification for the non-crystallized 2'-FL ingredient is provided in Attachment 1.

FDA.3. *Please clarify if the dietary exposure estimates provided in the November 18, 2022 amendment are based on the level of 2'-FL in the ingredient, so that proportionally more of the whole ingredient would be added to provide the stated maximum use level of 2'-FL, or if they are based on a 1:1 weight basis for the total ingredient containing ~88-90% 2'-FL that would replace current uses of 2'-FL described in GRN 000650. For the dietary exposure estimates described in GRN 000650, and cited in Tables 4, 5, and 6 of the amendment, it appears that the estimates are based on the 2'-FL component (i.e., the ingredient purity was assumed to be 100%), but this is not explicitly stated. For the cumulative uses of 2'-FL described in Table 7, footnote c below the table indicates "Use level expressed on a 2'-FL basis in the final food, as consumed," although footnote c does not appear in the table itself. Please clearly state the basis of the estimates provided in the amendment.*

We acknowledge that footnote c is missing from Table 7 of the November 18, 2022, amendment. Maximum use levels indicated in Table 7 of the November 18, 2022, amendment used to calculate the cumulative estimated daily intake of 2'-FL are expressed on a 2'-FL basis, meaning that addition levels of the ingredient are dependent on the 2'-FL content of the ingredient. Therefore, dietary exposure

estimates provided in the November 18, 2022, amendment are for the 2'-FL component. This means that proportionally more of the non-crystallized 2'-FL ingredient (containing at least 85 w/w% of 2'-FL on a water-free basis) would be added compared to crystallized 2'-FL (containing at least 94 w/w% of 2'-FL on a water-free basis) to achieve the stated maximum use level of 2'-FL.

FDA.4. *Please correct the heading for Table 4 in the November 18, 2022 amendment, which appears to include uses other than infant formula in the description.*

Indeed, there is an error in the description of the evaluated conditions of use in Table 4 of the November 18, 2022 amendment. The estimated daily intakes of 2'-FL in Table 4 of the November 18, 2022 amendment are representative of the intended conditions of use in infant formulas only. Please find the corrected table below.

Table 4 **Summary of the Estimated Daily Intake of 2'-FL from Infant Formulas in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data) (Reproduced from GRN 546 – Glycom A/S, 2014; U.S. FDA, 2015) [Corrected from the November 18, 2022 amendment]**

Population Group	Age Group	% Users	n	All-Users Consumption (g/day)		All-Users Consumption (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 months	74.8	161	2.02	2.91	332.8	535.6
Infants	7 to 12 months	73.6	128	1.70	2.63	188.9	295.8
Toddlers	1 to 3 years	1.1	7	1.08[a]	1.41[a]	89.3[a]	117.1[a]

2'-FL = 2'-fucosyllactose; bw = body weight; n = sample size; na = not available; NHANES = National Health and Nutrition Examination Survey, U.S. = United States.

[a] Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

--

We hope this information adequately addresses the Agency's questions on GRN 001060, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Marta H. Mikš, DSc, PhD
Senior Regulatory & Scientific Affairs Manager
Glycom A/S

Attachment 1

Table 1 Updated Regulatory Specifications for Non-Crystallized 2'-FL (GRN 1060)

Parameter	Unit	Specification	Methods
Appearance	NA	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	NA	White, white to off-white, off-white	ISO 6658
Assay (water-free) specified saccharides[a]	w/w %	≥ 92.0	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015, HPLC-2-003
Assay (water-free) 2'-fucosyllactose	w/w %	≥ 85.0	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015
Difucosyl-D-lactose	w/w %	≤ 5.0	Glycom method HPAEC-HMO-015
D-Lactose	w/w %	≤ 10.0	Glycom method HPAEC-HMO-015
L-Fucose	w/w %	≤ 1.0	Glycom method HPLC-2-003
2'-Fucosyl-D-lactulose	w/w %	≤ 1.5	Glycom method HPLC-2-003
Sum of other carbohydrates	w/w %	≤ 6.0	Glycom method HPAEC-HMO-015
pH in 5% solution (20°C)		3.5 –7.0	Ph. Eur. 2.2.3
Water	w/w %	≤ 7.0	Glycom method KF-001
Ash, sulphated	w/w %	≤ 0.8	Ph. Eur. 2.4.14
Residual proteins by Bradford assay	w/w %	≤ 0.01	Glycom method UV-001
Residual endotoxins	E.U./mg	≤ 10	Ph. Eur. 2.6.14
Lead	mg/kg	≤ 0.05	EN 13805 and EPA-6020A or EN ISO 17294 or EN 15763
Microbiological Criteria[c]			
Aerobic mesophilic bacteria total count	CFU/g	≤ 1,000	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae[b]	CFU/g	≤ 10[a]	ISO 21528-2[b]
Salmonella	25 g	Absent	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter spp.[c]	10 g	Absent	ISO 22964
Listeria monocytogenes[c]	25 g	Absent	ISO 11290-1
Bacillus cereus[c]	CFU/g	≤ 50	ISO 7932
Yeasts	CFU/g	≤ 100	ISO 21527-2
Molds	CFU/g	≤ 100	ISO 21527-2

2'-FL = 2'-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EPA = Environmental Protection Agency, EU = endotoxin units, HMO = human milk oligosaccharide, HPAEC = high-performance anion-exchange chromatography, HPLC = high-performance liquid chromatography, ISO = International Organization for Standardization, KF = Karl Fisher, Ph. Eur. = European Pharmacopoeia, UV = Ultraviolet

[a] Specified saccharides includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose and 2'-fucosyl-D-lactulose.

[b] Alternatively, *Enterobacteriaceae* may be evaluated using ISO 21528-1, resulting in the stricter specification 'Absent in 10 g'.

[c] Not applicable when 2'-FL is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior to drying.

From:	Miks, Marta
To:	Morissette, Rachel
Cc:	Roehrig, Christoph
Subject:	[EXTERNAL] RE: requested info for GRN 1060 as soon as possible
Date:	Monday, April 3, 2023 9:03:23 AM
Attachments:	image008.png image009.png image010.png image011.png image015.png image016.png image017.png image018.png image019.png image020.png image001.png Non-crystalized 2FL Specification_US_Final_Revised.pdf
Importance:	High

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Dear Rachel,

Thank you for your inquiry.

I am pleased to share the following clarification:

- DSM/Glycom's crystalized 2'-FL ingredient specification in US comprises parameter - **HiMS = Human-identical milk saccharides** that includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose and L-fucose (4 saccharides) with a limit of NMT 96.0 w/w %
- DSM/Glycom's non-crystalized 2'-FL ingredient specification in EU comprises parameter - **Assay (water-free) Specified saccharides** that includes 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, L-fucose and 2'-fucosyl-D-lactulose (5 saccharides) with a limit of NMT 90.0 w/w %

Therefore, for the alignment with the 13th Edition of the Food Chemicals Codex (FCC 13) monograph specification for 2'-FL, DSM would like to propose following amendments to DSM/Glycom's non-crystalized 2'-FL ingredient specification in US (GRN 1060):

1. Exchange the parameter '"Assay (water-free) Specified saccharides" (covering 5 saccharides, including 2'-fucosyl-D-lactulose) with the parameter "HiMS = Human-identical milk saccharides" (covering 4 saccharides, excluding 2'-fucosyl-D-lactulose) with the limit of NLT 92.0 w/w %. Kindly note that the proposed change in the parameter name is essential from DSM/Glycom's QC perspective, since the parameter '"Assay (water-free) Specified saccharides" including 5 saccharides is defined and used for EU market, as explained above.
2. Establish a stand-alone parameter for 2'-fucosyl-D-lactulose impurity with a limit of NMT 1.5 w/w %.

The analytical methods and other parameters of the DSM/Glycom's non-crystalized 2'-FL ingredient specification are not affected. The revised Attachment 1 - Table 1 Updated Regulatory Specifications

for Non-Crystallized 2'-FL (GRN 1060) compared to DSM's response dated March 16th is attached for your records.

We hope this information adequately addresses the Agency's question on GRN 001060, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Kindest Regards,

Marta Hanna Miks | Regulatory Depatment – Senior Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Horsholm | Denmark | T +45 88309500 | M +45 50372222 | marta.miks@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, March 30, 2023 7:00 PM
To: Miks, Marta <Marta.Miks@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: requested info for GRN 1060 as soon as possible

Dear Marta,

Our chemist noted an apparent typo in the response to our question 2 in your March 16th amendment. Glycom's response states that the "Assay (water-free) specified saccharides" specification includes 2'-fucosyl-D-lactulose, which is not one of the human milk oligosaccharide components included in the 13th Edition of the Food Chemicals Codex (FCC 13) monograph specification for 2'-FL. In the FCC monograph, the specification is 2'-FL not less than 92%, where 2'-FL is calculated as the sum of 2'-FL, L-fucose, D-lactose, and difucosyllactose only. Please clarify that the specification for 2'-FL is calculated as the sum of 2'-FL, L-fucose, D-lactose, and difucosyllactose, which does not include 2'-fucosyl-D-lactulose. We want to note that if Glycom intends to keep the 2'-fucosyl-D-lactulose impurity as part of the specification, we will not be able to move forward with a no questions letter.

The draft no questions letter for this notice is at the final stages of clearance. However, we cannot finalize this letter without the requested information above. Please provide us a response as soon as possible, but no later than COB Monday April 3.

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
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FDA U.S. FOOD & DRUG ADMINISTRATION

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Attachment 1 [REVISED]†

Table 1 Updated Regulatory Specifications for Non-Crystallized 2'-FL (GRN 1060)

Parameter	Unit	Specification	Methods
Appearance	NA	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	NA	White, white to off-white, off-white	ISO 6658
Assay (water-free) HiMS[a]	w/w %	≥ 92.0	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015, HPLC-2-003
Assay (water-free) 2'-fucosyllactose	w/w %	≥ 85.0	Glycom methods HPLC-202-2C4-002, HPAEC-HMO-015
Difucosyl-D-lactose	w/w %	≤ 5.0	Glycom method HPAEC-HMO-015
D-Lactose	w/w %	≤ 10.0	Glycom method HPAEC-HMO-015
L-Fucose	w/w %	≤ 1.0	Glycom method HPLC-2-003
2'-Fucosyl-D-lactulose	w/w %	≤ 1.5	Glycom method HPLC-2-003
Sum of other carbohydrates	w/w %	≤ 6.0	Glycom method HPAEC-HMO-015
pH in 5% solution (20°C)		3.5 –7.0	Ph. Eur. 2.2.3
Water	w/w %	≤ 7.0	Glycom method KF-001
Ash, sulphated	w/w %	≤ 0.8	Ph. Eur. 2.4.14
Residual proteins by Bradford assay	w/w %	≤ 0.01	Glycom method UV-001
Residual endotoxins	E.U./mg	≤ 10	Ph. Eur. 2.6.14
Lead	mg/kg	≤ 0.05	EN 13805 and EPA-6020A or EN ISO 17294 or EN 15763
Microbiological Criteria[c]			
Aerobic mesophilic bacteria total count	CFU/g	≤ 1,000	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae[b]	CFU/g	≤ 10[a]	ISO 21528-2[b]
Salmonella	25 g	Absent	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter spp.[c]	10 g	Absent	ISO 22964
Listeria monocytogenes[c]	25 g	Absent	ISO 11290-1
Bacillus cereus[c]	CFU/g	≤ 50	ISO 7932
Yeasts	CFU/g	≤ 100	ISO 21527-2
Molds	CFU/g	≤ 100	ISO 21527-2

2'-FL = 2'-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EPA = Environmental Protection Agency, EU = endotoxin units, HiMS = Human-identical milk saccharides, HMO = human milk oligosaccharide, HPAEC = high-performance anion-exchange chromatography, HPLC = high-performance liquid chromatography, ISO = International Organization for Standardization, KF = Karl Fisher, Ph. Eur. = European Pharmacopoeia, UV = Ultraviolet

[a] HiMS = Human-identical milk saccharides include 2'-fucosyllactose, difucosyl-D-lactose, D-lactose, and L-fucose.

[b] Alternatively, *Enterobacteriaceae* may be evaluated using ISO 21528-1, resulting in the stricter specification 'Absent in 10 g'.

[c] Not applicable when 2'-FL is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior to drying.

† Revised values are indicated in green.