Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

04 November 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for 3-fucosyllactose (3-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 3-fucosyllactose (3-FL) produced by an *E. coli* K-12 DH1 MDO-derived strain, is GRAS on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products across multiple categories; these food uses of 3-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,

[redacted]

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

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

GRAS NOTICE FOR 3-FUCOSYLLACTOSE (3-FL)

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

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

DATE:
4 November 2021

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 3-Fucosyllactose (3-FL)

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GRAS Notice for 3-Fucosyllactose (3-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 3-fucosyllactose (3-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 1.3 below. In addition, as a responsible official of Glycom, the undersigned hereby certifies that all data and information presented in this Notice represents a complete, representative, and balanced submission, and considered all unfavorable, as well as favorable, information known to Glycom and pertinent to the evaluation of the safety and GRAS status of 3-FL as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

05 Nov 2021

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

Date

1.1 Name and Address of Notifier

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

1.2 Common Name of Notified Substance

3-Fucosyllactose; 3-FL

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

1.3 Conditions of Use

3-FL is intended to be added to non-exempt term infant formula, foods targeted to young children, as well as uses in specific conventional food and beverage products used by the general population (Table 1.3-1). Use of this ingredient in infant formula (*i.e.*, infants up to 12 months), toddler formulas (*i.e.*, young children older than 12 months) and beverages targeted to young children will provide a use level of 3-FL of 0.75 g/L in infant formula and 2.0 g/L in ready-to-drink and reconstituted products, and up to 6.25 g/kg for products other than beverages for infants and young children (*e.g.*, baby foods). 3-FL is also intended for use in food and beverages targeted towards the general U.S. population (up to 2.0 g/L or 25 g/kg), and foods for special dietary use (*e.g.*, meal replacement bars) at levels up to 2.5 g/L or 25 g/kg. The maximum use levels are proposed on the basis of providing similar levels of 3-FL, on a body weight basis, as those consumed by breast-fed infants (see Section 3.1). A summary of the food categories and use levels in which 3-FL is intended for use is provided in Table 1.3-1 below. Use levels are expressed on a 3-FL basis.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 3-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk Meal Replacement and Nutritional Beverages[c]	240	0.48	2.00
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.45	1.25
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.075	0.75
	Toddler Formulas[e]	100[d]	0.20	2.00
	Other Baby Foods for Infants and Young Children	7 to 170	0.04 to 1.06	6.25
	Other Drinks for Young Children	120	0.24	2.00
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	1.00	25.00
	Cereal and Nutrition Bars	40	1.00	25.00
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk*	240	0.48	2.00
Milk Products	Buttermilk*	240	0.48	2.00
	Flavored Milk	240	0.48	2.00
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.60	2.50
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.16 to 0.41	2.00
	Yogurt*	170	2.13	12.50
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.30	1.25

3-FL = 3-fucosyllactose; CFR = *Code of Federal Regulations*; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

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

[b] Use level expressed on a 3-FL basis in the final food, as consumed.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 3-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)

[c] Includes ready-to-drink and powder forms.
[d] RACC not available, 100 mL employed as an approximation.
[e] Formula products targeted toward young children (> 12 months of age).
[f] Portion sizes are based on representative products on the U.S. market.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020a), Glycom has concluded, on the basis of scientific procedures, that 3-FL is GRAS for addition to non-exempt term infant formula and specified conventional food and beverage products, as described in Table 1.3-1.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification 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 Notification, Glycom will supply these data and information upon request.

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

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

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

2.1 Identity

3-FL manufactured by Glycom consists of ≥ 87% 3-FL and contains small residual quantities of D-lactose and L-fucose, and minor amounts of other related and fully characterized carbohydrates originating from the fermentation process resulting in a total specified saccharide concentration of ≥ 92%.

3-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. Using a combination of 1D ^{1}H and ^{13}C and 2D double-quantum filtered correlation spectroscopy (DQFCOSY), heteronuclear single quantum coherence (gHSQC), and heteronuclear multiple bond coherence (gHMBC) nuclear magnetic resonance (NMR) spectra and mass spectrometry (MS)/MS mass spectra, it has been demonstrated that the structure of 3-FL as manufactured by Glycom is β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucose. It has been also shown by ^{1}H and ^{13}C and 2D nuclear Overhauser effect spectroscopy (NOESY) spectra that 3-FL manufactured by Glycom is chemically and structurally identical to 3-FL that is naturally present in human milk. Further description of the structural and chemical identity of 3-FL is presented below in Table 2.1-1.

Table 2.1-1 Identity of 3-FL

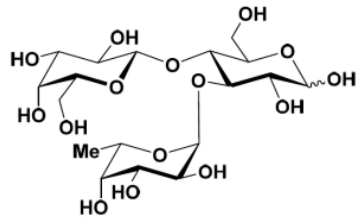
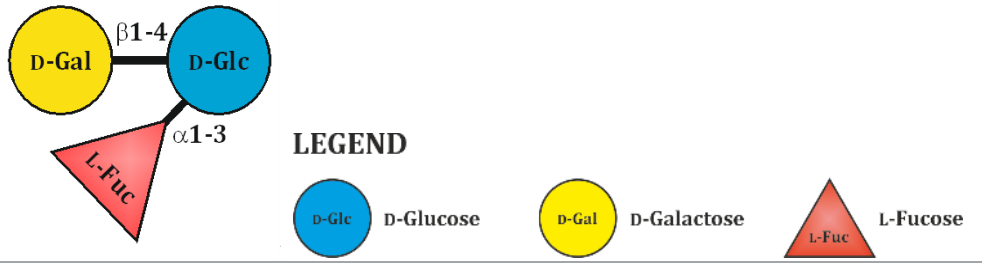
Generic Product Name	3-Fucosyllactose
Common Abbreviations	3-FL; 3FL
Trade Name	GlyCare™ 3FL 9000 and 9001
Synonyms	3-*O*-Fucosyllactose, 3-*O*-L-Fucosyl-D-lactose, 3-Fucosidolactose; “LewisX-2g” (= glucose analog of histo-blood group LewisX antigen)
IUPAC Name	β-D-Galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucose
IUPAC Abbreviation (extended)	β-D-Gal*p*-(1-4)-[α-L-Fuc*p*-(1-3)] D-Glc*p*
IUPAC Abbreviation (condensed)	Gal-(β1-4)-[Fuc-(α1-3)]-Glc
Molecular Structure	
Symbol Nomenclature	
Molecular Formula	$C_{18}H_{32}O_{15}$
Molecular Mass	488.44

Table 2.1-1	Identity of 3-FL
	41312-47-4
	O-6-Deoxy-α-L-galactopyranosyl-(1→3)-*O*-[β-D-galactopyranosyl-(1→4)]-D-glucose

3-FL = 3-fucosyllactose; CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

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

2.2.1.1 Parental (Host) Strain

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

[2] www.dsmz.de.

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

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

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

2.2.1.2 Production Strain for 3-FL

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

The MDO strain is further modified to generate the production strain, *E. coli* K-12 DH1 MDO MAP1834, to biosynthesize 3-FL. The production strain is a genomically stable microorganism that provides high titers of 3-FL. The strain has been deposited in the DSMZ in Braunschweig, Germany. To enable the MDO strain to biosynthesize 3-FL, the strain has been modified by replacement of a promoter element, insertion of four gene cassettes encoding enzymes for biosynthesis and export of 3-FL and resulting in the deletion of four genes at each targeted insertion site, and subsequently the deletion of four additional genes to optimize 3-FL production. These modifications are described as follows:

1. The P*lac* promoter upstream of the intrinsic GDP-mannose-4,6-dehydratase encoding gene *gmd*, which is part of the GDP-fucose biosynthetic pathway, was replaced by the P*glpF* expression element.

2. Chromosomal integration of three expression cassettes, containing a codon-optimized DNA sequence derived from *Helicobacter pylori,* encoding the enzyme α-1,3-fucosyl-transferase that catalyzes the transfer of fucose (Fuc) from its activated sugar nucleotide form, guanosine 5'-diphosphate fucose (GDP-Fuc), to the 3-position of lactose, resulting in the formation of 3-FL.

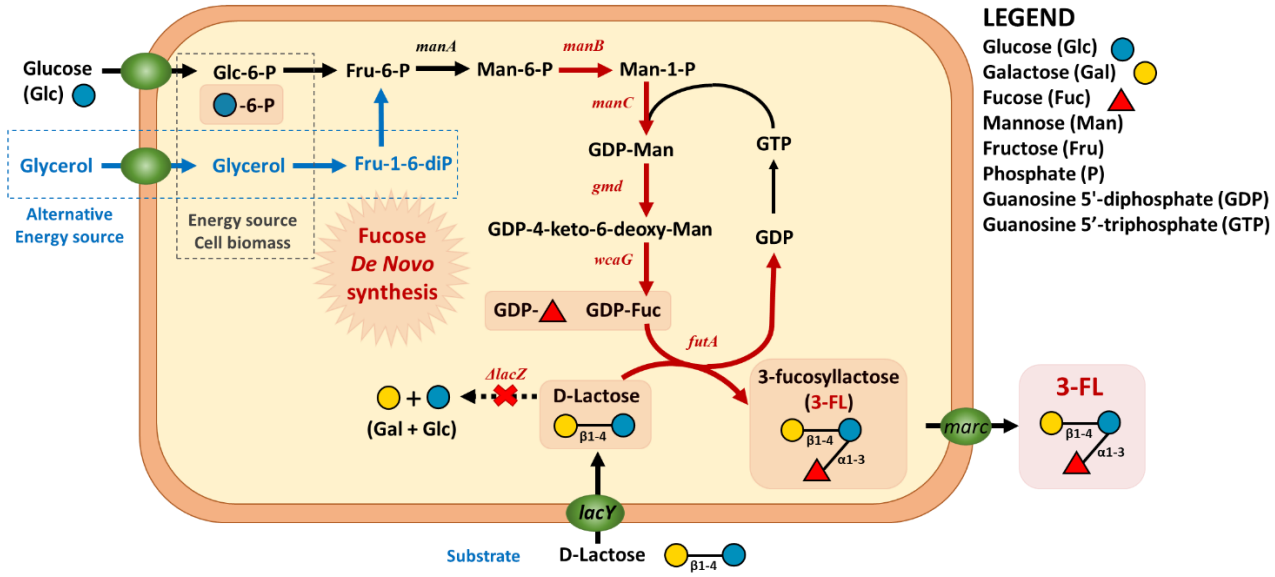
3. Chromosomal integration of one expression cassette, containing a DNA sequence derived from *Serratia marcescens,* encoding a major facilitator superfamily (MFS) transporter that enhances the efflux of newly formed 3-FL out of the cell.

4. In addition, the *lacI* gene, which encodes a transcriptional repressor of the P*lac* promoter has been deleted. An additional three gene deletions have been implemented to inhibit synthesis of unwanted mixed-acid metabolites and to improve the safety of the final product.

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

Production of 3-FL is generated in a biosynthetic reaction involving D-lactose (substrate) and D-glucose[4] (carbon source) as shown Figure 2.2.1.2-1 below. During the fermentation process of 3-FL neither antibiotics nor inducer molecules are used. During manufacture, the production strain excretes 3-FL extracellularly, and then is entirely removed through a series of purification steps (as described in Section 2.2.2). Therefore, in this process the production strain is used exclusively as a processing aid.

Figure 2.2.1.2-1 Pathway for 3-FL Biosynthesis by the *Escherichia coli* K-12 DH1 MDO-derived Production Strain



3-FL = 3-fucosyllactose; futA = α-1,3-fucosyl-transferase.

[4] Alternatively, D-sucrose or glycerol.

The inserted enzyme is well characterized and based on its enzymatic function it was concluded that the introduced gene would not confer toxicogenic or pathogenic properties to the host organism. The genetic modifications made to the production strain result in the expression of proteins that are involved in the normal carbohydrate processing within their donor sources. These proteins are "carbohydrate-active enzymes" ("CAZy"), a panel of enzymes that can degrade, modify, or create glycosidic bonds, and accordingly are involved in the metabolism of complex carbohydrates. When expressed together in the recipient strains, these proteins work in concert to convert the starting carbohydrates (D-lactose and D-glucose[5]) into oligosaccharides that are identical to those in human milk. In contrast, bacterial protein toxins (exotoxins) are known to mediate their pathogenic effects by disrupting cellular processes through various mechanisms such as proteolysis (*e.g.*, tetanus and botulinum), ADP-ribosylation (*e.g.*, cholera, pertussis, and diphtheria), or membrane disruptions through pore formation (Finlay and Falkow, 1997; Wilson *et al.*, 2002; Popoff, 2018). Bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the *E. coli* K-12 DH1 MAP1834 strain by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods.

2.2.2 Description of the Production Process

Glycom's 3-FL is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacture of 3-FL is largely comparable to the production processes previously evaluated for other HiMOs with GRAS status [see GRNs 650, 659, 815, 833, 880, 881, and 895 (U.S. FDA, 2016a,b, 2019a,b, 2020b,c,e)]. All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously determined to be GRAS for their respective uses, or have been the subject of an effective Food Contact Notification. The manufacturing process can be broadly divided into two stages.

In Stage 1 [upstream processing (USP)], D-lactose and D-glucose[7] are converted to 3-FL by the adapted cellular metabolism of the production microorganism, which uses D-glucose as an energy and carbon source and D-lactose as a substrate for 3-FL biosynthesis. The production microorganism is removed from the fermentation medium at the end of the fermentation process.

In Stage 2 [downstream processing (DSP)], a series of purification, isolation, and concentration steps are used to generate the final high-purity 3-FL product. Production of 3-FL may include an optional crystallization step (with acetic acid) to generate a higher-grade ingredient with further minimization of certain carbohydrate-type impurities (discussed in Section 2.3.2.2).

A schematic overview of the manufacturing process for 3-FL is presented in Table 2.2.2-1 below.

Table 2.2.2-1 Overview of the Manufacturing Process for 3-FL

STAGE 1		**Upstream Processing (USP)**
STEPS	**1**	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase[a]

[5] Alternatively, D-sucrose or glycerol.

Table 2.2.2-1		Overview of the Manufacturing Process for 3-FL
	4B	Feeding (Fed-Batch) Phase
	4C	Harvest/Storage of Culture Broth
	5	Removal of Microorganism*
STAGE 2		**Downstream Processing (DSP)**
STEPS	**6**	Purification/Concentration 1*
	7[b]	Ion Removal
	8[b]	Decolorization
	9	Purification/Concentration 2*
	OP	Crystallization
	10	Drying
	11	Sampling and Packaging
	12	Quality Control and Batch Release

3-FL = 3-fucosyllactose, OP = Optional Step.

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

[a] The batch phase of fermentation is optional.

[b] The order of steps is interchangeable.

2.2.3 Quality Control

The manufacture of 3-FL by microbial fermentation is conducted in accordance with cGMP and HACCP principles. Considering the chemically well-characterized principal raw materials and final products, the whole production process can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by Certificate of Analysis) to allow full control of the production process (refer to Table 2.2.2-1).

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

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

Incorporation of sterile filtration units throughout the manufacturing process of the HiMOs ensures high microbiological purity, while the presence of the production microorganism is devoid in the final product. The product microorganism is efficiently removed in the ultrafiltration step, which is applied directly following fermentation. In addition, several additional purification steps are carried out in the down-stream processing stage to help achieve a highly purified 3-FL, which is free from bacterial cells and residual fermentation by-products. The absence of the microorganisms can be measured by analysis for *Enterobacteriaceae* in the final product according to an internationally recognized method (ISO 21528-2). This specification for *Enterobacteriaceae* is set at "≤10 colony-forming units per gram" of test article, which also ensures absence of enumerable production microorganism as *E. coli* belong to the *Enterobacteriaceae*

family. As further assurance of the absence of viable production organism in the finished products, batches of 3-FL have been tested for *E. coli*, specifically, in accordance with ISO 16649-2. The results have confirmed the absence of enumerable *E. coli* in all tested batches of 3-FL (results available upon request).

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

The absence of traces of residual DNA of the production organism in the product following fermentation and purification of 3-FL is confirmed by three different validated quantitative polymerase chain reaction (qPCR) methods. These methods target short subsequences of the inserted genes *marc* and *futA*[6] as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. All three methods are validated to detect traces of DNA down to 4 pg/mg (parts per billion). The qPCR tests were applied to all analyzed batches and the results were below the limit of quantification (LOQ) in all tested batches (see Section 2.3.3).

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

The specifications for 3-FL are presented in Table 2.3.1-1. The parameters include 3-FL, L-fucose, D-lactose, and 3-fucosyl-lactulose. Limits for the sum of other carbohydrates and acetic acid (optional antisolvent when crystallization is used) have been established. The determination of these carbohydrates is conducted using high-performance liquid chromatography coupled with corona charged aerosol detection (HPLC-cCAD) and high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) analysis. Upper limits have also been established for microbiological parameters. All methods of analysis are either internationally recognized or developed internally by Glycom.

Table 2.3.1-1 Specifications for 3-FL

Description		
GlyCare™ 3-FL 9000 and 9001 is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 87% of 3-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	White, white to off-white, off-white	ISO 6658

[6] The *marc* gene encodes the Major Facilitator Superfamily transporter and the *futA* gene encodes an α-1,3-*O*-fucosyltransferase enzyme.

Table 2.3.1-1 Specifications for 3-FL

Description		
GlyCare™ 3-FL 9000 and 9001 is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 87% of 3-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Identification by Retention Time	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-402-4C4-001
Assay (water-free) – Specified saccharides[a]	≥ 92.0 w/w %	Glycom method HPLC-402-4C4-001, HPAEC-HMO-021, HPLC-3FL-002 or HPLC-3FL-003
Assay (water-free) – 3-Fucosyllactose	≥ 87.0 w/w %	Glycom method HPLC-402-4C4-001
L-Fucose	≤ 1.0 w/w %	Glycom method HPLC-3FL-002 or HPLC-3FL-003
D-Lactose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021
3-Fucosyl-lactulose	≤ 1.5 w/w %	Glycom method HPAEC-HMO-021
Sum of other carbohydrates	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021 + HPLC-3FL-003
pH in 5% solution (20°C)	3.2 – 6.0	Ph. Eur. 9.2 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-001
Ash, sulphated	≤ 0.5 w/w %	Ph. Eur. 9.2 2.4.14
Acetic acid[b]	≤ 1.0 w/w %	Megazyme K-ACETTRM 07/12
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Ph. Eur 2.6.14
Lead	≤ 0.1 mg/kg	EN 13805; EPA-6020A
Microbiological specifications[c]		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	≤ 10 CFU/g	ISO 21528-2 or NMKL 144
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Molds	≤ 100 CFU/g	ISO 21527-2

3-FL = 3-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EPA = Environmental Protection Agency; E.U. = endotoxin units; HMO = human milk oligosaccharide HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; NMKL = Nordic Committee on Food Analysis; Ph. Eur. = European Pharmacopeia; RT = retention time; UV = ultraviolet.

[a] Specified saccharides include 3-fucosyllactose, D-lactose, L-fucose, and 3-fucosyl-lactulose.

[b] Relevant only for GlyCare™ 3FL 9000 crystallized with acetic acid.

[c] The microbiological specifications in the table represent limits for 3-FL that is added to infant formula and toddler formula products during the wet-mix stage of the formula manufacturing process (*i.e.*, prior to retort) and is also suitable for conventional food products. Additional microbiological limits are established for 3-FL that is added during the dry blend stage and include the following (in addition to the parameters listed above): *Cronobacter* spp.: absent in 10 g; *Listeria monocytogenes:* absent in 25 g; *Bacillus cereus*: ≤ 50 CFU/g.

2.3.2 Product Analyses

The analytical results of eight independent production batches of 3-FL are summarized in Table 2.3.2-1 and are discussed in further details in the following subsections. Four of the included batches (GEMO2020_17_1, GEMO2020_18_2, GEMO2020_19_4, and GEMO2020_20_4) were manufactured with an optional crystallization step and presented in parallel with the results for four 3-FL products produced without a crystallization step (GEMO2020_9_1, GEMO2020_13_1, GEMO2020_14_1, and 20193201). The optional crystallization step was employed for the elective removal of select carbohydrate by-products; however, as shown by the following data, both manufacturing options yield a consistent, high-purity product.

Table 2.3.2-1 Summary of Product Analyses of 3-FL

Parameters	Specification	Manufacturing Batch No.							
		Non-Crystallized 3-FL				Crystallized 3-FL			
		GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Appearance	Powder, agglomerates, powder with agglomerates	Powder or agglomerates							
Color	White, white to off-white, off-white	White to off-white							
Identification by Retention Time	RT of main component corresponds to RT of standard ± 3%	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Assay (water-free) – Specified saccharides[a]	≥ 92.0 w/w %	96.3%	98.5%	96.4%	95.4%	93.8%	95.9%	97.4%	96.5%
Assay (water-free) – 3-FL	≥ 87.0 w/w %	93.4%	97.5%	92.1%	94.6%	93.2%	95.8%	97.4%	96.5%
L-Fucose	≤ 1.0 w/w %	0.13%	0.16%	0.39%	0.23%	0.11%	0.03%	< 0.03%	< 0.03%
D-Lactose	≤ 5.0 w/w %	2.15%	0.29%	3.40%	0.36%	0.30%	< 0.03%	< 0.03%	< 0.03%
3-Fucosyl-lactulose	≤ 1.5 w/w %	0.57%	0.56%	0.39%	0.18%	0.12%	0.11%	< 0.03%	< 0.03%
Sum of other carbohydrates	≤ 5.0 w/w %	0.46%	0.73%	0.82%	1.76%	2.70%	1.52%	0.75%	0.98%
pH in 5% solution (20°C)	3.2 – 6.0	4.6	5.7	5.7	5.5	3.7	3.8	3.9	3.9
Water	≤ 6.0 w/w %	3.60	3.23	3.49	2.41	0.14	0.18	0.04	0.01
Ash, sulphated	≤ 0.5 w/w %	< 0.01	< 0.01	< 0.01	0.10	< 0.01	< 0.01	< 0.01	< 0.01
Acetic acid[b]	≤ 1.0 w/w %	NA	NA	NA	NA	0.5	0.3	0.1	0.1
Residual protein by Bradford assay	≤ 0.01 w/w %	< 0.0017	< 0.0017	< 0.0017	< 0.001	< 0.0017	< 0.0017	0.004	< 0.0017
Residual endotoxins	≤ 10 E.U./mg	0.0025	0.0066	0.0122	0.0016	< 0.00025	0.0498	0.011	0.0007
Lead	≤ 0.1 mg/kg	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01	< 0.01	< 0.01	< 0.01
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	≤ 10 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

Table 2.3.2-1 Summary of Product Analyses of 3-FL

Parameters	Specification	Manufacturing Batch No.							
		Non-Crystallized 3-FL				Crystallized 3-FL			
		GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Salmonella	Absent in 25 g	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Yeasts	≤ 100 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Molds	≤ 100 CFU/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units; E.U. = endotoxin units; NA = not applicable; RT = retention time.

[a] Specified saccharides include 3-fucosyllactose, D-lactose, L-fucose and 3-fucosyl-lactulose.

[b] Relevant only for GlyCare™ 3FL 9000 crystallized with acetic acid.

2.3.2.1 Physicochemical Properties

The physical properties of 3-FL, as manufactured by Glycom, may be described as white to off-white powder or agglomerates. 3-FL is readily soluble in aqueous solutions (maximum 400 mg/mL, 25°C) with poor solubility in any organic solvents. The pH of crystalline 3-FL is lower than non-crystalline 3-FL due to the presence of some residual acetic acid, the antisolvent used during crystallization. A summary of the batch analyses corresponding to the selected physicochemical properties of 3-FL is presented in Table 2.3.2.1-1.

Table 2.3.2.1-1 Batch Results for Selected Physicochemical Properties of 3-FL

Parameters	**Manufacturing Batch No.**							
	Non-Crystallized 3-FL				**Crystallized 3-FL**			
	GEMO2020_9_1	**GEMO2020_13_1**	**GEMO2020_14_1**	**20193201**	**GEMO2020_17_1**	**GEMO2020_18_2**	**GEMO2020_19_4**	**GEMO2020_20_4**
Appearance	Powder or agglomerates							
Color	White to off-white							
pH (20°C, 5% solution)	4.6	5.7	5.7	5.5	3.7	3.8	3.9	3.9

3-FL = 3-fucosyllactose.

2.3.2.2 Human-Identical Milk Saccharides and Other Carbohydrates

3-FL is a purified carbohydrate ingredient (purity *ca.* 95.1 w/w % of dry matter) which contains minor amounts of related and fully characterized carbohydrates (D-lactose, L-fucose, 3-fucosyl-lactulose) (Table 2.3.2.2-1). Besides 3-FL, the predominant carbohydrates in the ingredient are D-lactose (≤ 5.0%) and L-fucose (≤ 1.0%) which are naturally occurring compounds that are found in human milk.

Collectively, the sum of 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose comprises not less than 92.0% of the total dry batch weight (average results from eight batches is 96.3% on a water-free basis). The remaining portion of the product is made up of other carbohydrate-type compounds and moisture.

Table 2.3.2.2-1 Batch Results for Fermentation Metabolites and Other Carbohydrate By-Products in 3-FL

Parameters	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Assay (water-free) Specified saccharides[a]	96.3%	98.5%	96.4%	95.4%	93.8%	95.9%	97.4%	96.5%
Assay (water free) – 3-FL	93.4%	97.5%	92.1%	94.6%	93.2%	95.8%	97.4%	96.5%
L-Fucose	0.13%	0.16%	0.39%	0.23%	0.11%	0.03%	< 0.03%	< 0.03%
D-Lactose	2.15%	0.29%	3.40%	0.36%	0.30%	< 0.03%	< 0.03%	< 0.03%
3-Fucosyl-lactulose	0.57%	0.56%	0.39%	0.18%	0.12%	0.11%	< 0.03%	< 0.03%
Sum of other carbohydrates	0.46%	0.73%	0.82%	1.76%	2.70%	1.52%	0.75%	0.98%

3-FL = 3-fucosyllactose.
[a] Sum of specified saccharides includes 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

As previously mentioned, 3-FL is a trisaccharide comprised of L-fucose, D-galactose, and D-glucose. Alternatively, the molecular constitution can be described as consisting of the monosaccharide fucose and the disaccharide D-lactose, which are linked by an α(1→3) bond from fucose to glucose to form the trisaccharide. 3-FL is a chemically defined trisaccharide and the structural isomer of 2'-fucosyllactose (2'-FL). Both human milk oligosaccharides (HMOs), 3-FL and 2'-FL, are naturally present in human and other mammalian milks (see Section 3.1).

Also as previously mentioned, the structure of Glycom's 3-FL has been confirmed by 1D ^{1}H and ^{13}C and 2D DQFCOSY, gHSQC, and gHMBC NMR spectra and MS/MS mass spectra, and the results of ^{1}H and ^{13}C and 2D NOESY spectra demonstrate that 3-FL manufactured by Glycom is chemically and structurally identical to 3-FL that is naturally present in human milk.

D-Lactose and L-fucose are the principal raw materials and by-product of the production of 3-FL but also are themselves natural constituents of the carbohydrate fraction in human milk. It is noted that levels of D-lactose and L-fucose in 3-FL are similar to those detected in the production of other HiMOs including 2'-FL and 2'-FL/DFL (see GRNs 650 and 815) (U.S. FDA, 2016a, 2019a).

3-Fucosyl-lactulose is an isomer of 3-FL, arising from the isomerization of the glucose moiety at the reducing end of 3-FL to fructose. This type of isomerization is pH- and temperature-dependent and has been commonly reported for the closely related conversion of 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). Different infant formulas have been reported to contain lactulose at levels between 1 and 7% relative of their lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Although the isomerization product of 3-FL has not been specifically evaluated in heat-treated human donor milk, lactulose has also been detected at significant proportions of lactose (Gómez de Segura *et al.*, 2012), and it can thus be reasonably assumed that 3-fucosyl-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. The low levels of this isomerization product (not more than 1.5%) in 3-FL batches are

negligible and not biologically/nutritionally relevant. This type of impurity is common to all lactose-containing HiMOs (*e.g.*, 2'-FL, LNnT, DFL, LNT, 6'-SL, 3'-SL).

2.3.2.3 Non-Carbohydrate Residues

Batches of 3-FL were tested for residual water (moisture, by modified Karl Fisher method) and the presence of macro- and micro-elements. The results indicate no detectable levels of calcium, phosphate, ammonium, magnesium, manganese, selenium, or molybdenum but trace levels of sulphate, chloride, iron, sodium, potassium, and zinc in only select batches (see Table 2.3.2.3-1). These levels do not pose any safety issues given the small amount of these minerals relative to allowable quantities in infant formula (see Table 2.3.2.3-2).

Table 2.3.2.3-1 Batch Results for Non-Carbohydrate Residues of 3-FL

Parameter	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Water (w/w %)	3.60	3.23	3.49	2.41	0.14	0.18	0.04	0.01
Ammonium (w/w %)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Calcium (w/w %)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Chloride (w/w %)	0.010	0.001	0.001	0.012	0.009	0.005	< 0.004	< 0.004
Iron (mg/kg)	0.5	< 0.1	< 0.5	< 10	0.6	< 2	< 0.5	< 0.5
Magnesium (w/w %)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.0005
Manganese (mg/kg)	< 0.1	< 0.1	< 0.1	< 1	< 0.1	< 0.1	< 0.1	< 0.1
Phosphate (w/w %)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0006	< 0.0005	< 0.0005	< 0.0006
Potassium (w/w %)	< 0.001	0.001	< 0.001	< 0.001	0.002	0.001	< 0.001	< 0.001
Sodium (w/w %)	0.010	0.006	0.007	0.011	0.003	0.001	< 0.001	< 0.001
Sulphate (w/w %)	< 0.0024	0.0004	< 0.004	0.0239	< 0.0051	< 0.0043	< 0.004	< 0.005
Zinc (mg/kg)	0.4	0.1	< 0.1	< 0.5	< 0.2	< 0.1	< 0.1	< 0.1
Selenium (mg/kg)	< 0.05	< 0.1	< 0.1	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Molybdenum (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	< 0.1

3-FL = 3-fucosyllactose.

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

Nutrient	Infant Formula Nutrient Specification (21 CFR 107.100) (U.S. FDA, 2020a)	Maximum Detected Content in 3-FL		Margin[b]
		As Measured	Equivalent in 100 kcal[a]	
Chloride	55 to 150 mg/100 kcal	0.012%	0.04 mg	**3,750**
Iron	0.15 to 3.0 mg/100 kcal	0.6 mg/kg	0.20 µg	**15,000**
Potassium	80 mg to 200 mg/100 kcal	0.002%	0.006 mg	**33,500**
Sodium	20 mg to 60 mg/100 kcal	0.011%	0.04 mg	**1,500**
Sulphate	Not specified	0.0239%	Not applicable	**Not applicable**
Zinc	Min. 0.5 mg/100 kcal	0.4 mg/kg	0.13 µg	**Not applicable**

3-FL = 3-fucosyllactose; CFR = *Code of Federal Regulations*; Min. = minimum.
[a] Assuming an inclusion level of 2 g 3-FL/L and a minimum caloric density of 60 kcal per 100 mL for the infant formula.
[b] Calculated as maximum theoretical level in formula containing 3-FL divided by the maximum allowable level in infant formula.

2.3.2.4 Microbial Contaminants

The microbiological purity of 3-FL production batches has been assessed for non-pathogenic microorganisms (bacteria, yeasts, and molds) as general hygiene indicators, and as well for selected food-borne pathogens and is summarized in Table 2.3.2.4-1 below.

Aerobic mesophilic total plate count, yeasts and molds levels, and the count of *Enterobacteriaceae* give an indication of a level of total contamination (bioburden) and the absence of the production strain in 3-FL. 3-FL was also tested for the absence of potentially pathogenic bacteria, namely *Salmonella* spp., *Cronobacter* spp., and *Listeria monocytogenes*. Spore-forming bacteria *Bacillus cereus*, which is a frequent contaminator of heat-treated or spray-dried foods, was also measured to control the number of surviving spores in the final product. The results of these analyses consistently indicate a low bioburden in the finished product and the absence of microbial contaminants. Accordingly, suitable specifications have been established for the ingredient when intended for inclusion at the wet blending stage of infant formula manufacture (*i.e.*, prior to retort) and also for conventional food products (see Table 2.3.1-1). More restrictive release specifications (including additional limits for *Cronobacter* spp*., L. monocytogenes,* and *B. cereus*) are established for 3-FL intended for addition at the dry blending stage of infant formula manufacture, where subsequent heat-treatment is not applied.

Table 2.3.2.4-1 Batch Results for Microbiological Analysis of 3-FL

Microbiological Parameters	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Yeasts [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Molds [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella spp. [in 25 g]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Cronobacter spp. [in 10 g]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes [in 25 g]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units.

2.3.3 Manufacturing By-Products, Impurities, and Contaminants

Carbohydrate-type by-products, as discussed in Section 2.3.2.2 are the main manufacturing impurities present in 3-FL. These compounds are detectable, and levels are limited by appropriate specifications. Glycom also has established quality control measures for compounds that include microbial endotoxins and residual proteins and precautionary analyses demonstrating the absence of deleterious levels of several other potential residual compounds and trace elements that may originate from fermentation. These include amino acids and biogenic amines, trace elements and the presence/absence of genes characteristic for the production microorganism. All of these by-products, impurities, and contaminants are confirmed to be absent at any safety-relevant levels and as such are not proposed for addition to the product specifications.

2.3.3.1 Amino Acids and Biogenic Amines

3-FL is efficiently released into the fermentation broth and the microorganism is removed. As a precautionary measure, production batches have been analyzed for secondary metabolites and cellular components that may potentially originate from the fermentation medium. Results of analyses of the ingredient for biogenic amines (histamine, cadaverine, and putrescine), and amino acids and their metabolites (glutamic acid and *gamma*-aminobutyric acid) did not identify detectable levels of these contaminants in any of the manufacturing batches of the finished product (data not shown). Therefore, these compounds do not contribute to the overall compositional data of 3-FL and they are not entered into the specification of 3-FL.

2.3.3.2 Microbial Endotoxins and Residual Proteins

As explained in Section, 2.2.3 the parental strain of the production organism, *E. coli* K-12, is a Gram-negative bacterium which contain complex glycolipids of high molecular weight in their cell walls, called either LPS or endotoxins (not to be confused with protein-type toxins). Specifications for endotoxin have been established [max. 10 endotoxin units (E.U.)/mg] as an additional quality control point to ensure that any microbial endotoxins are efficiently removed and/or not introduced during the production process. The endotoxin content in 3-FL, produced by fermentation, was analyzed/detected using the *Limulus* amoebocyte lysate kinetic chromogenic assay.

Similarly, a sensitive residual protein test (based on the Bradford assay) has been applied and a specification limit of ≤ 0.01 w/w % was established. Because batch analyses of 3-FL product demonstrated low endotoxin and residual protein concentrations, they were not considered as compositional or safety-related data of 3-FL (Table 2.3.3.2-1). However, the presence of residual endotoxins and protein are monitored during routine batch release as an element of HACCP that would allow to identify process deviations in a sensitive manner.

Table 2.3.3.2-1 Batch Results for Microbial Endotoxins and Residual Proteins in 3-FL

Parameters	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Residual endotoxins [E.U./mg]	0.0025	0.0066	0.0122	0.0016	< 0.00025	0.0498	0.011	0.0007
Residual protein by Bradford assay [%]	< 0.0017	< 0.0017	< 0.0017	< 0.001	< 0.0017	< 0.0017	0.004	< 0.0017

3-FL = 3-fucosyllactose; E.U. = endotoxin units.

2.3.3.3 Absence of Production Organism and its DNA

The production microorganism is efficiently removed by the ultrafiltration step during upstream processing, which is applied directly after the fermentation. During downstream processing, sequential filtration (including microfiltration to achieve retention of microorganisms and thus high microbial quality) and purification processes are carried out to ensure a high-purity 3-FL. The final product is tested for bacteria of the *Enterobacteriaceae* family according to an internationally recognized method (ISO 21528-2) to ensure the absence of the production microorganism. The results of specific tests for *E. coli* (ISO 16649-2) also confirm the absence of enumerable *E. coli* in all tested batches of 3-FL (results available upon request).

Analyses for residual DNA are also carried out to corroborate the absence of the production organism or its DNA in final production batches. Three different validated qPCR assays were carried out to confirm the absence of residual DNA from the production organism and is summarized in Table 2.3.3.3-1 below. Short subsequences of the inserted genes as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli* are targeted in the qPCR methods. Based on the analysis of the eight batches of 3-FL, no detectable levels of residual DNA (limit of quantification of 4 µg/kg or 4 ppb) were observed in the final product.

Table 2.3.3.3-1 Levels of Residual DNA in Representative Batches of 3-FL

Parameter	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	3FL_GEMO_2020_9_1	3FL_GEMO_2020_13_1	GEMO2020_14_1	20193201	3FL_GEMO_2020_17_1	3FL_GEMO_2020_18_2	3FL_GEMO_2020_19_4	3FL_GEMO_2020_20_4
Residual DNA by qPCR (*marc* assay)	< LoQ[a]	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Residual DNA by qPCR (*futA* assay)	< LoQ[a]	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Residual DNA by qPCR (23S assay)	< LoQ[a]	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

3-FL = 3-fucosyllactose; DNA = deoxyribonucleic acid; *futA* = α-1,3-*O*-fucosyltransferase enzyme; LOQ = limit of quantitation; *marc* = protein of the Major Facilitator Superfamily; qPCR = quantitative polymerase chain reaction.
[a] LOQ = 4 µg/kg (parts per billion).

2.3.3.4 Trace Elements and Heavy Metals

Trace levels of elements and minerals may be present in 3-FL as a result of the fermentation process (carry-over from the fermentation medium). These are used as cofactors for different enzymes. Examples of trace elements are iron, manganese, copper, zinc, molybdenum, and selenium added as inorganic salts. The use of trace elements in very low concentrations and sufficient quantities are required for the promotion of bacterial cell growth.

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

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

Table 2.3.3.4-1 Levels of Heavy Metals in Representative Batches of 3-FL

Parameter	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Arsenic (mg/kg)	< 0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1
Cadmium (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Mercury (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Copper (mg/kg)	0.2	0.3	0.5	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Lead (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01	< 0.01	< 0.01	< 0.01
Manganese (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Nickel (mg/kg)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

3-FL = 3-fucosyllactose.

2.3.3.5 Residual Antisolvent

The manufacture of 3-FL may include an optional crystallization resulting in the production of a non-hygroscopic crystalline anhydrate with low water content (GlyCare™ 3FL 9000). Crystallization of 3-FL is performed with food-grade acetic acid. Among 3-FL batches that are crystallized, the level of residual acetic acid is included as a specification parameter; a specification of < 1.0% is applied to the ingredient, the same residue specification established for crystalline 2'-FL notified under GRN 650[7] (U.S. FDA, 2016a). The results of analyses of crystallized batches of 3-FL is presented in Table 2.3.3.5-1 below and show low levels of residual acetic acid on a % w/w basis.

Table 2.3.3.5-1 Levels of Residual Antisolvent in Crystallized Batches of 3-FL

Acetic Acid (%)	≤ 1.0	0.5	0.3	0.1	0.1

3-FL = 3-fucosyllactose.

2.4 Stability

Storage (real-time and accelerated) stability studies on Glycom's pure ("bulk") powdered 3-FL have been initiated and are ongoing. Stressed (forced) stability studies on the purified bulk powder have also been completed. For Glycom's bulk 3-FL, experiments were performed both in solid state (crystallized powders) as well as in liquid form (in aqueous solutions). Studies were conducted in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Guidelines (ICH) (*Stability Testing of New Drug Substances and Products*) (ICH, 2003). The objectives of these studies were to:

1. Test the stability of 3-FL under normal and accelerated conditions of storage
2. Investigate degradation pathways when exposed to selected stress factors; and
3. Define the optimal storage conditions and corresponding re-test dates or shelf life.

[7] Acetic acid is not used at any point in the production of non-crystallized 3-FL and thus is not a specification parameter for those batches.

The stability of Glycom's 3-FL has also been investigated in its intended food matrix of powdered infant formula. In addition, the stability of 3-FL has been tested in a range of food matrices (Christensen *et al.*, 2020). These data are also presented in Section 2.4.2 below.

Overall, the results indicate that 3-FL is expected to be stable over its intended shelf life of 5 years and is stable under its intended conditions of use. These data are consistent with those of other HiMOs manufactured by Glycom and others, including 3-FL as described in GRN 925 (U.S. FDA, 2021a).

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of 3-FL produced from microbial fermentation, as described herein, is being subject to a 5-year real-time stability study [25°C, 60% relative humidity (RH)]. Studies are being conducted on one representative non-crystalline batch (20193201), and on a representative crystalline batch (D_3FL_GEMO_2020_20_4), with interim results available for up to 12 months (see Tables 2.4.1.1-1 and 2.4.1.1-2). These studies are ongoing.

Table 2.4.1.1-1 Interim Results of the 5-Year Real-Time Stability Study on the Non-Crystalline 3-FL (25°C, 60% Relative Humidity)

Manufacturing Batch No. 20193201

Parameter	Sample Time (Months)				
	0	3	6	9	12
Physical Properties					
Appearance	Powder	Powder	Powder	Powder	Powder
Color	White	White	White	White	White
Purity					
Assay (water free) – Specified saccharides[a] [% w/w]	95.4	95.3	98.6	94.9	93.0
Assay (water free) – 3-FL [% w/w]	94.6	94.3	97.5	94.1	92.1
D-Lactose [% w/w]	0.36	0.45	0.49	0.44	0.39
L-Fucose [% w/w]	0.23	0.24	0.25	0.20	0.25
3-Fucosyl-lactulose [% w/w]	0.18	0.29	0.32	0.15	0.19
Sum of other carbohydrates [% w/w]	1.76	2.24	2.27	2.18	1.75
Water [% w/w]	2.41	2.44	2.00	2.10	3.00
Microbiological Quality					
Aerobic mesophilic total plate count [CFU/g]	< 10	NT	NT	NT	< 10
Enterobacteriaceae [CFU/g]	< 10	NT	NT	NT	Absent in 10 g
Salmonella spp. [in 25 g]	Absent	NT	NT	NT	Absent
Yeasts [CFU/g]	< 10	NT	NT	NT	< 10
Molds [CFU/g]	< 10	NT	NT	NT	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units; NT = not tested.
[a] Specified saccharides includes 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

Table 2.4.1.1-2 Interim Results of the 5-Year Real-Time Stability Study on the Crystalline 3-FL (25°C, 60% Relative Humidity)

Manufacturing Batch No. D_3FL_GEMO_2020_20_4

Parameter	Sample Time (Months)				
	0	3	6	9	12
Physical Properties					
Appearance	Powder	Powder	Powder	Powder	Powder
Color	White	White	White	White	White
Purity					
Assay (water free) – Specified saccharides[a] [% w/w]	96.5	96.1	100.3	96.5	97.3
Assay (water free) – 3-FL [% w/w]	96.5	96.1	100.3	96.5	97.2
D-Lactose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
L-Fucose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
3-Fucosyl-lactulose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
Sum of other carbohydrates [% w/w]	0.98	0.93	0.91	1.04	0.98
Water [% w/w]	0.01	0.02	0.02	0.01	0.05
Microbiological Quality					
Aerobic mesophilic total plate count [CFU/g]	< 10	NT	NT	NT	< 10
Enterobacteriaceae [CFU/g]	< 10	NT	NT	NT	< 10
Salmonella spp. [in 25 g]	Absent	NT	NT	NT	Absent
Yeasts [CFU/g]	< 10	NT	NT	NT	< 10
Molds [CFU/g]	< 10	NT	NT	NT	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units; NT = not tested.
[a] Specified saccharides includes 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

It is further noted that 3-FL produced by Glycom using chemical synthesis technology was generated in 2012 and samples were retained (stored at ambient temperature and humidity conditions) and tested in July 2020. The results of high-performance liquid chromatography (HPLC) analyses revealed no significant degradation of 3-FL (produced by chemical synthesis) following eight years of storage. Results may be made available upon request.

2.4.1.2 Accelerated Stability

The stability of 3-FL produced from microbial fermentation, as described herein, is being investigated in a 2-year accelerated stability study (40°C, 75% RH). Studies are being conducted on one representative non-crystalline batch (20193201), and on a representative crystalline batch (D_3FL_GEMO_2020_20_4), with interim results indicating that there are no appreciable changes in the organoleptic properties of the ingredient, no appreciable degradation of 3-FL, no changes in the impurity profile, and no alterations in the microbiological quality of the ingredient under the timepoints tested thus far (see Tables 2.4.1.2-1 and 2.4.1.2-2). These studies are ongoing.

Table 2.4.1.2-1 Interim Results of the 2-Year Accelerated Stability Study on the Non-Crystalline 3-FL (40°C, 75% Relative Humidity)

Manufacturing Batch No. 20193201

Parameter	Sample Time (Months)					
	0	1	3	6	9	12
Physical Properties						
Appearance	Powder	Powder	Powder	Uniformly sized solid particles	Powder	Powder
Color	White	White	White	White	White	White
Purity						
Assay (water free) – Specified saccharides[a] [% w/w]	95.4	96.3	93.9	99.3	94.7	95.0
Assay (water free) – 3-FL [% w/w]	94.6	95.7	92.8	98.1	93.8	94.1
D-Lactose [% w/w]	0.36	0.41	0.45	0.50	0.45	0.40
L-Fucose [% w/w]	0.23	0.12	0.21	0.26	0.19	0.25
3-Fucosyl-lactulose [% w/w]	0.18	0.14	0.34	0.42	0.24	0.26
Sum of other carbohydrates [% w/w]	1.76	1.36	2.28	2.27	2.27	1.74
Water [% w/w]	2.41	2.46	2.30	2.00	2.00	2.50
Microbiological Quality						
Aerobic mesophilic total plate count [CFU/g]	< 10	NT	NT	< 10	NT	< 10
Enterobacteriaceae [CFU/g]	< 10	NT	NT	< 10	NT	Absent in 10 g
Salmonella spp. [in 25 g]	Absent	NT	NT	Absent	NT	Absent
Yeasts [CFU/g]	< 10	NT	NT	< 10	NT	< 10
Molds [CFU/g]	< 10	NT	NT	< 10	NT	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units; NT = not tested.
[a] Sum of specified saccharides includes 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

Table 2.4.1.2-2 Interim Results of the 2-Year Accelerated Stability Study on the Crystalline 3-FL (40°C, 75% Relative Humidity)

Manufacturing Batch No. D_3FL_GEMO_2020_20_4

Parameter	Sample Time (Months)						
	0	1	2	3	6	9	12
Physical Properties							
Appearance	Powder	Powder	Powder	Powder	Powder	Powder	Powder
Color	White	White	White	White	White	White	White
Purity							
Assay (water free) – Specified saccharides[a] [% w/w]	96.5	98.4	99.2	96.3	99.3	97.9	97.1
Assay (water free) - 3-FL [% w/w]	96.5	98.4	99.2	96.3	99.3	97.9	97.1
D-Lactose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
L-Fucose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
3-Fucosyl-lactulose [% w/w]	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03

Table 2.4.1.2-2 Interim Results of the 2-Year Accelerated Stability Study on the Crystalline 3-FL (40°C, 75% Relative Humidity)

Manufacturing Batch No. D_3FL_GEMO_2020_20_4

Parameter	Sample Time (Months)						
	0	1	2	3	6	9	12
Sum of other carbohydrates [% w/w]	0.98	0.87	0.95	0.91	0.88	0.97	0.96
Water [% w/w]	0.01	0.05	0.03	0.02	0.02	0.01	0.04
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	< 10	NT	NT	NT	< 10	NT	< 10
Enterobacteriaceae [CFU/g]	< 10	NT	NT	NT	< 10	NT	< 10
Salmonella spp. [in 25 g]	Absent	NT	NT	NT	Absent	NT	Absent
Yeasts [CFU/g]	< 10	NT	NT	NT	< 10	NT	< 10
Molds [CFU/g]	< 10	NT	NT	NT	< 10	NT	< 10

3-FL = 3-fucosyllactose; CFU = colony forming units; NT = not tested.
[a] Sum of specified saccharides includes 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

2.4.1.3 Stress Stability

The stress stability study described herein, was performed according to the ICH Guidelines (*Stability Testing of New Drug Substances and Products*) and aimed to identify the degradation pathways of 3-FL under harsh, stress conditions. The full study report can be made available upon request.

Stress Testing of 3-FL in Solid State

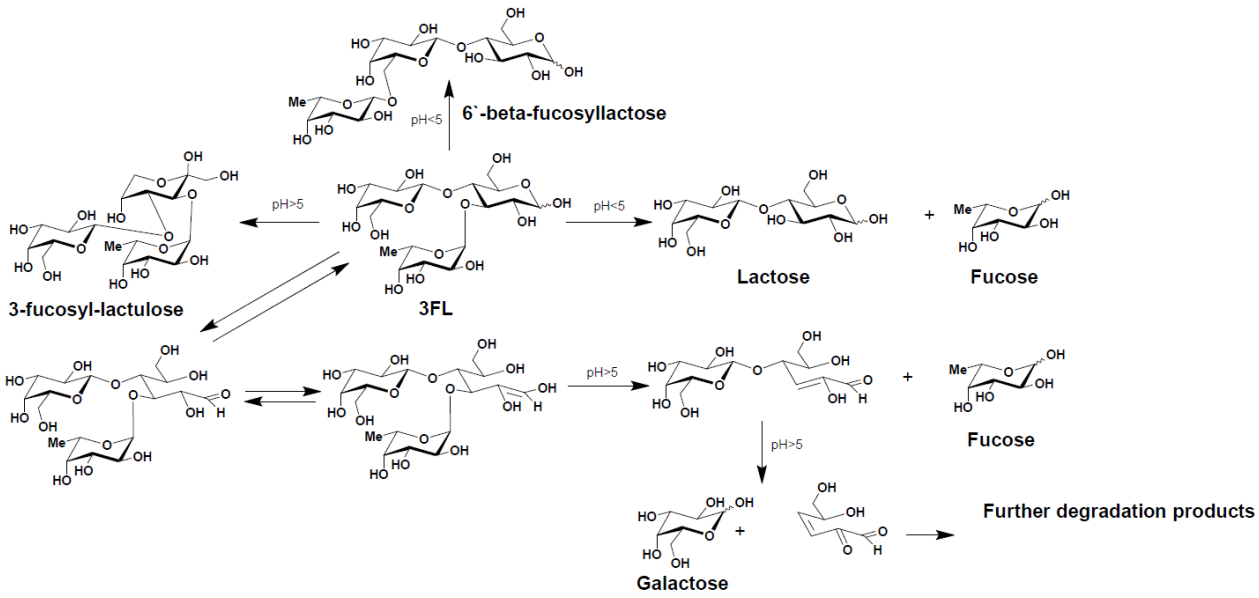
Forced, thermal stability tests of pure crystalline 3-FL in powdered solid state were performed at 80°C for 28 days of storage at two different humidity conditions (dry and humid). No degradation products were detected following a detailed HPLC assay of predicted degradation products.

Stress Testing 3-FL Stability in Aqueous Solution

Forced stability tests of aqueous solutions of 3-FL were performed at 60°C under seven conditions of varying pH (unbuffered; buffered at 3.0, 5.0, 6.8 and 9.0; in 0.1 N HCl and in 0.01 N NaOH). The solutions were stored until significant degradation was observed but maximum up to 28 days.

The results of this study showed the presence of four potential pH-dependent degradation pathways in the aqueous solutions of 3-FL. The first two pathways comprise of 3-FL hydrolysis to lactose and fucose and its isomerization to 6'-β-fucosyllactose—these are the main degradation pathways when the ingredient is in solution at pH below 5. Isomerization to 3-fucosyl-lactulose occurs when the pH is above 5. The fourth pathway is more complex; it corresponds to the “peeling reaction,” characteristic to carbohydrates substituted in position 3 related to the terminal anomeric carbon. The primary products detected are fucose and galactose in roughly equal amounts, with the glucose moiety going on to form further degradation products. The proposed degradation pathways are depicted in Figure 2.4.1.3-1 below. The stability data indicate that 3-FL is most stable at pH 5.

Figure 2.4.1.3-1 Degradation Pathways of 3-Fucosyllactose in Aqueous Solutions



3-FL was also challenged in solution at room temperature for 24 hours by oxidative stress using 0.1% hydrogen peroxide and 4,4'-azobis-(4-cyanovaleric acid) (ACVA) in 1:0.1 molar ratio. No degradation products were identified in this study.

2.4.2 Stability Under the Intended Conditions of Use

2.4.2.1 Stability in Infant Formula

The stability of 3-FL in powdered infant formula is currently undergoing investigation. Briefly, a commercially representative infant formula powder[8] was formulated with 3-FL at a final concentration of 1 g/100 g powder using wet blending techniques and processed using standard industry steps including pasteurization, homogenization, and spray-drying. A control infant formula was also included (not containing 3-FL). The powder was then packaged into nitrogen gas-flushed milk powder cans and stored at temperatures of 4, 20, 30, and 37°C. The powder was then sampled at regular timepoints up to 3 years and analyzed for 3-FL content by HPLC and select microbiological quality indicators (including total viable count, thermophilic count, Enterobacteriaceae, *E. coli, B. cereus, Clostridium perfringens, Salmonella* spp., *Staphylococcus aureus,* yeasts, and molds). The 3-year stability study is ongoing.

Interim results have thus far demonstrated that, similar to other HiMOs produced by Glycom, 3-FL is stable when formulated into infant formula and subject to industry-standard processing conditions (Table 2.4.2.1-1). This data is congruent to other stability studies initiated by Glycom which demonstrate that HiMOs produced by microbial fermentation are stable in infant formula for up to 5 years (2'-FL, LNnT, DFL, LNT, 3'-SL, and 6'-SL; reports available upon request).

[8] The base formulation of the infant formula included lactose, skimmed milk powder, whey protein concentrate, vegetable oil, lecithin, and a vitamin/mineral premix. The final infant formula powder macronutrient composition comprised of 1.9 g protein/100 kcal; 11.2 g carbohydrates/100 kcal; 5.3 g fat/100 kcal; and approximately 2.5% moisture.

Table 2.4.2.1-1 Interim Results of the 3-Year Stability Study in Infant Formula

Storage Condition	3-FL Concentration (g/100 g)				
	Target	Sample Time (Months)			
		0[a]	1	3	6
Infant Formula, 5°C	1.0	1.05	1.01	1.03	1.01
Infant Formula, 25°C / 60% Relative Humidity	1.0	1.05	1.06	1.06	1.05
Infant Formula, 30°C / 65% Relative Humidity	1.0	1.05	1.01	1.02	1.03
Infant Formula, 40°C / 75% Relative Humidity	1.0	1.05	1.06	1.07	1.04

3-FL = 3-fucosyllactose.
[a] Sample analyzed post-production.

2.4.2.2 Stability in Other Food Matrices

The stability of 3-FL in UHT-processed milk, whole milk, and yogurt have also been investigated by Christensen *et al.* (2020). Briefly, 3-FL (99.7% purity; provided by Danisco Sweeteners Oy, Kantvik, Finland; method of isolation/manufacture not specified) was formulated into whole and UHT-processed milk at 1 mg/mL and into yogurt at 10 mg/g. Samples were stored for up to 36 days (whole milk and yogurt) or 3 months (UHT milk), and the 3-FL content was evaluated using a validated method utilizing high-performance liquid chromatography with refractive index detection (HPLC-RI).

Over the course of the stability study, the 3-FL content in whole milk, UHT milk, and yogurt was stable. As the 3-FL described in this study is identical in chemical structure to Glycom's 3-FL and is similarly a highly purified ingredient produced by biotechnology, it can be assumed that Glycom's 3-FL is similarly stable in these food matrices.

It is further noted that the stability of a number of other HiMOs have been investigated in other food matrices including yogurts, ready-to-drink flavored milk, and citrus fruit beverages. These have been presented in GRAS Notifications for 2'-FL (GRN 546, 650, 735; U.S. FDA, 2015b, 2016a, 2018a), LNnT (GRN 547, 659; U.S. FDA, 2015a, 2016b), and sialic acid (GRN 602; U.S. FDA, 2016c).

Briefly, the results of these studies reveal no significant losses of the initial concentrations of these ingredients in these food matrices over the duration of the typical shelf-life of these foods, even when subject to pre-processing, pasteurization, and UHT heating. Based on the comparable method of manufacture and highly similar chemical structure, the conclusions on the stability of these other HiMOs in food matrices may be extended to support the stability of 3-FL in these food matrices.

Part 3. DIETARY EXPOSURE

3.1 History of Use of 3-FL in Food

3-FL as manufactured by Jennewein Biotechnologie GmbH[9] (Jennewein) was concluded to be GRAS for use as an ingredient in cow's milk-based, non-exempt term formula at a level of 0.44 g/L. On 23 March 2020, this GRAS conclusion was notified to the offices of the U.S. FDA under the Agency's voluntary GRAS Notification program and was subsequently filed by the agency without objection under GRN 925 on 08 February 2021 (U.S. FDA, 2021a).

On 04 January 2021, the GRAS uses of 3-FL (DuPont Nutrition Biosciences; DuPont) in non-exempt term infant formula (0.44 g/L), formula intended for young children aged 1 to 3 years (0.44 g/L), and foods for infants and toddlers (0.44 to 4.4 g/kg), and other foods at levels ranging from 0.03 to 0.09 g/serving were notified to the U.S. FDA under GRN 951 (U.S. FDA, 2021b). Uses of 3-FL as an ingredient in oral and enteral tube feeding formulas also were concluded by DuPont Nutrition and Biosciences to be GRAS under this Notice. The U.S. FDA subsequently filed GRN 951 without objection on 12 August 2021.

It is also noted that novel food applications for 3-FL produced by microbial fermentation have been submitted to the European Commission (European Commission, 2019, 2020). In the application submitted by DuPont Nutrition & Biosciences ApS which received a favorable opinion from the EFSA NDA Panel, 3-FL has been proposed for use in unflavored pasteurized and unflavored sterilized (including UHT) milk (0.85 g/L), unflavored fermented milk-based products (0.5 g/L), yogurt (5 g/kg), flavored fermented milk-based products including heat-treated products (1.2 g/L beverages and 12.0 g/kg products other than beverages), dairy analogues, non-dairy yogurts (0.85 g/L beverages and 0.85 g/kg products other than beverages), breakfast cereals, (21 g/kg), fine bakery wares, cereal bars only (30 g/kg), infant formula, follow-on formula, milk-based drinks and flavored drinks (0.85 g/L), processed cereal-based food and baby foods (0.3 g/kg and 0.3 g/L), foods for special medical purposes, total diet replacement (30 g/kg and 2.0 g/L) and food supplements (5.0 g/day for general population and 1.2 g/day for young children) (EFSA, 2021).

In the novel food application submitted by Jennewein, 3-FL is proposed only for use in the following food categories all with a use level of 1.2 g/L: infant formula, follow-on formula, dietary foods for special medical purposes (as defined by Directive 1999/21/EC) (EC, 1999).

3.1.1 History of Consumption of 3-FL in Human Milk

A comprehensive review of the scientific literature, as well as a thorough discussion on factors influencing 3-FL concentrations in human milk was conducted by Glycom. An overview of Glycom's findings and relevance of the published literature to the intended use level of 3-FL in infant formula are presented below.

3.1.1.1 The Oligosaccharide Fraction of Human Milk

3-FL is an important and significant component of the natural HMO fraction of human milk. Human milk contains, as its third largest solid component, a fraction consisting of a complex family of structurally related oligosaccharides (György *et al.*, 1952; Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013).

[9] Note Jennewein Biotechnologie GmbH was acquired by Chr. Hansen A/S on 22 Sept 2020; therefore, either entity may appear on the novel food applications.

These are known as HMOs because they were first discovered in human milk (Malpress and Hytten, 1958) and because they occur in human milk at much higher concentrations than in other mammalian milks (Urashima *et al.*, 2001).

More than 180 members of this family have been fully described on a structural basis (Chen, 2015; Remoroza *et al.*, 2020; Urashima *et al.*, 2021), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al.*, 1999; Wu *et al.*, 2010; Wu *et al.*, 2011). However, the contribution of individual HMOs to the total biomass of the HMO fraction is unequally distributed, with the smallest HMOs (degree of polymerization ≤ 5) contributing to the vast majority and most larger HMOs contributing minute amounts, such that their quantification has not been accomplished to date (Soyyilmaz *et al.*, 2021). The highest concentrations of total HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012), although high variations are observed on an individual level and in dependency on the lactation period and the genotype of the mother.

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

In reviewing the concentrations of HiMOs reported in the following sections, it should be emphasized that the components of breast milk are nutrients that were necessary to sustain the life of humans from an evolutionary perspective. It also can be surmised that a large margin of safety exists for concentrations of HiMOs in human milk, which is reflected in the wide range of concentrations of 3-FL that have been measured across lactational stages and across various demographic population groups. Although most HiMOs tend to decrease in composition over time, 3-FL is unique in that concentrations tend to increase across lactational (*i.e.*, increase across stages of infant development). The evolutionary basis for these temporal increases over time are unknown. The changes in HiMO concentrations over time simply reflect that the nutritional importance of HiMOs changes as infants grow and therefore maternal energy expenditure on synthesis of these complex molecules in breast tissue will change in a similar manner. Thus, the concentrations of nutrients in human milk over the lactation period are an evolutionary equilibrium reflecting a trade-off between mother and offspring and should not be mistaken as the perfectly optimized amount for the offspring. With respect to selecting a target concentration of 3-FL for use in infant formula, since no upper tolerable limit has been identified for either 3-FL alone or in combination with other non-digestible oligosaccharides, Glycom has selected a value that falls within the 95th percentile of mean values across all developmental stages, and has been demonstrated to be well tolerated and supports the growth and development of infants when formulated into infant formula (Parschat *et al*., 2021; Table 3.1.1.5-1). This approach ensures that the majority of infants will be provided with concentrations that fall within the population means and is well below the upper ranges of concentrations that have been reported for human milk samples, therefore ensuring that the intended uses levels are safe and well tolerated by all infants.

3.1.1.2 Genetic Polymorphisms Shaping Milk Composition: Secretor and Lewis Phenotypes

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

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

In consequence, four different milk phenotype groups can be characterized in the global population (corresponding to the combination of phenotypes $FUT2^{+}/FUT3^{+}$, $FUT2^{-}/FUT3^{+}$, $FUT2^{+}/FUT3^{-}$, and $FUT2^{-}/FUT3^{-}$). A person's *fut2*$^{+}$ genotype leads to the so-called ***Secretor*** phenotype, which has received its name historically from the observation that soluble blood group substances (carrying epitopes A, B, and 0) can be detected in their bodily secretions (*e.g.,* milk, blood, saliva, urine) (Grollman and Ginsburg, 1967; Shen *et al.*, 1968). The *fut3*$^{+}$ genotype acts phenotypically *in combination* with the *fut2*$^{+}$ genotype and leads either to the so-called ***Lewis a*** or ***Lewis b*** phenotypes (Thurl *et al.*, 1997). Please see Table 3.1.1.2-1 for a summary of this information, typical frequencies of the different milk phenotypes, and the information about which distinct HMO occurs in which milk phenotype group.

Table 3.1.1.2-1 Milk Phenotype Groups

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

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; DFL = difucosyllactose; DF-LNH = difucosyllacto-*N*-hexaose; DS = disialyl; DS-LNT = disialyllacto-*N*-tetraose; F = fucosyl; F-LNH = fucosyllacto-*N*-hexaose; FS-LNH = fucosyl-lacto-*N*-hexaose; FS-LNnH = fucosyl-lacto-*N*-neohexaose FSL = 3'-sialyl-3-fucosyllactose; HMO = human milk oligosaccharide; Le = Lewis; LNDFH = lacto-*N*-difucohexaose; LNFP = lacto-*N*-fucopentaose; LNH = lacto-*N*-hexaose; LNnH = lacto-*N*-neohexaose; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; LST = sialyl-lacto-*N*-tetraose; Se = Secretor; SL = sialyllactose; S-LNFP-II = 3'-sialyl-lacto-*N*-fucopentaose II; S-LNH = sialyl-lacto-*N*-hexaose; S-LNnH = sialyl-lacto-*N*-neohexaose; TF-LNH = Trifucosyllacto-*N*-hexaose.

The milk phenotype groups are oftentimes simply referred to as "milk groups." The key characteristic of each milk group can be expressed in words as follows (please note the order of Milk Groups 2 and 3 are inverted in Table 3.1.1.2-1 to allow for grouping of Secretor and non-Secretor phenotype HMOs):

- **Milk Group 1** is excreted by mothers who express both FUT2 and FUT3 in their mammary glands and thus synthesize both α-1,2-fucosylated HMO and α-1,4-fucosylated HMOs. This is one of the Secretor phenotype groups.

- **Milk Group 2** is excreted by mothers who express only FUT3 but not FUT2 in their mammary glands and thus synthesize α-1,4-fucosylated HMOs but not α-1,2-fucosylated HMOs. This is one of the non-Secretor phenotype groups[10].

- **Milk Group 3** is excreted by mothers who express only FUT2 but not FUT3 in their mammary glands and thus synthesize α-1,2-fucosylated HMOs but not α-1,4-fucosylated HMOs. This is one of the Secretor phenotype groups.

- **Milk Group 4** is excreted by mothers who express neither FUT2 nor FUT3 in their mammary glands and thus cannot produce α-1,2-fucosylated HMOs nor α-1,4-fucosylated HMOs. This is one of the non-Secretor phenotype groups.

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

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

3-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose, and occurs principally in the milk of all milk groups (phenotypes) since the α-(1,3)-fucose-bond to D-glucose of 3-FL can also be generated by fucosyltransferase enzymes that are not subject to populational genetic knock-out polymorphisms. However, it is found at highest concentration in the milk group 2, which is a loss-of-function *fut2* knock-out (*i.e.*, on both alleles), but possesses the functional *fut3* gene [encoding a α-(1,3/4)-fucosyltransferase that can also form the α-(1,3)-fucose bond to D-glucose of 3-FL]. 3-FL is one of few HMOs observed to increase in concentration over the course of lactation.

3.1.1.3 Basis for Quantitative Data of HMOs in Human Milk

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

Since 2016, many additional studies from around the world were reported increasing our understanding of global trends; hence, an update of the literature base was undertaken herein.

[10] Note that to express a non-Secretor phenotype a mother must have inherited the non-functional *fut2* gene through both parental alleles.

A detailed description of the literature search strategy, final scientific literature base, data extraction methods, and the data overview are presented in Appendix A.

3.1.1.4 Summary of 3-FL Concentrations in Human Milk

Of the 41 publications reporting the concentration of 3-FL in human milk, the lowest reported mean was 0.02 g 3-FL/L by Van Niekerk *et al.* (2014) (observed in 40 mothers participating at a larger clinical trial in Tygerberg Children's Hospital, Cape Town, South Africa, and who gave birth preterm and were sampled on Day 4 of lactation; all mother/infant dyads included in this study had been pre-selected for infants that had developed necrotizing enterocolitis) and the highest reported mean was 3.43 g 3-FL/L reported by Lefebvre *et al.* (2020) (observed in German women participating in the LIFE Child cohort in Leipzig, Germany, sampled at 12 months of lactation and belonging to the milk phenotype 2, *i.e.,* non-Secretor/Lewis^{+}).

The highest reported upper range was at 6.03 g 3-FL/L also by Lefebvre *et al.* (2020) in the same population as indicated above for the highest reported mean.

Using the reported standard deviations, values reported by Gabrielli *et al.* (2011) represented the highest extrapolated 95% confidence level (CL) at 4.72 g 3-FL/L.

3.1.1.5 3-FL Concentrations by Lactational Period

The concentration of 3-FL in human milk has been measured and reported to date in at least 41 independent publications.

The following table summarizes the levels of 3-FL that have been reported in human milk across these various studies over the course of lactation (Table 3.1.1.5-1). Please note that all studies that measured pooled samples from less than 10 donors were excluded.

Table 3.1.1.5-1 3-FL Concentrations in Human Milk over the Course of Lactation

Lactation Time	Parameter	g/L	References
Days 1 to 5 ("colostrum")	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	0.35 0.04 – 1.17 0.95 1.22 2.47	Coppa *et al.* (1999); Erney *et al.* (2000); Chaturvedi *et al.* (2001a); Sumiyoshi *et al.* (2003); Sjögren *et al.* (2007); Asakuma *et al.* (2008); Thurl *et al.* (2010); Gabrielli *et al.* (2011); Van Niekerk *et al.* (2014); Spevacek *et al.* (2015); Aakko *et al.* (2017); Ma *et al.* (2018); Huang *et al.* (2019); Samuel *et al.* (2019); Ferreira *et al.* (2020); Torres Roldan *et al.* (2020); Wu *et al.* (2020)
Days 6 to 14 ("transitional milk")	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	0.56 0.22 – 1.40 1.44 1.58 2.80	Coppa *et al.* (1999); Erney *et al.* (2000); Chaturvedi *et al.* (2001a); Sumiyoshi *et al.* (2003); Thurl *et al.* (2010); Gabrielli *et al.* (2011); Spevacek *et al.* (2015); Austin *et al.* (2016, 2019); Ma *et al.* (2018); Huang *et al.* (2019); McJarrow *et al.* (2019); Borewicz *et al.* (2020); Wu *et al.* (2020)
Days 15 to 90 ("mature milk")	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	0.69 0.04 – 1.87 1.60 3.00 5.72	Chaturvedi *et al.* (1997, 2001a); Coppa *et al.* (1999, 2011); Kunz *et al.* (1999); Erney *et al.* (2000); Sumiyoshi *et al.* (2003); Thurl *et al.* (2010); Gabrielli *et al.* (2011); Smilowitz *et al.* (2013); Van Niekerk *et al.* (2014); Alderete *et al.* (2015); Spevacek *et al.* (2015); Austin *et al.* (2016, 2019); Ma *et al.* (2018); Huang *et al.* (2019); Samuel *et al.* (2019); Tonon *et al.* (2019); Borewicz *et al.* (2020); Ferreira *et al.* (2020); Lefebvre *et al.* (2020); Lagström *et al.* (2020); Saben *et al.* (2020); Torres Roldan *et al.* (2020); Wu *et al.* (2020)

Table 3.1.1.5-1 3-FL Concentrations in Human Milk over the Course of Lactation

Lactation Time	Parameter	g/L	References
Days 90 to 180 ("mature milk")	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	0.92 0.1 – 2.26 1.87 3.14 6.03	Chaturvedi *et al.* (2001a); Sumiyoshi *et al.* (2003); Alderete *et al.* (2015); Williams *et al.* (2017); Azad *et al.* (2018); Ma *et al.* (2018); Austin *et al.* (2019); Larsson *et al.* (2019); McJarrow *et al.* (2019); Samuel *et al.* (2019); Ferreira *et al.* (2020); Lefebvre *et al.* (2020)
After 6 months ("late milk")	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	1.32 0.33 – 2.40 2.91 3.80 4.32	Chaturvedi *et al.* (2001a); Ma *et al.* (2018); Larsson *et al.* (2019); Lefebvre *et al.* (2020)

3-FL = 3-fucosyllactose; CL = confidence level.

3.1.1.6 3-FL: Data in Milk Phenotype 2

3-FL occurs at highest concentration in the milk phenotype 2 of mothers that are FUT2-negative (non-Secretor phenotype) but FUT3-positive (Lewis phenotype). These mothers represent approximately 20% of the female world population and it can therefore be argued that the levels of 3-FL in their breast milk can be considered safe also for infants of mothers of different milk phenotype, as one would assume that milk banks or the historical tradition of wet-nursing would have provided accounts of donor milk-infant incompatibility.

Table 3.1.1.6-1 summarizes the data reported. The mean of reported means is at 2.28 g/L and the range of reported means varies from 0.4 to 5.1 g/L. The mean of reported approximated 95% CLs is at 3.42 g/L and the mean of reported upper levels is at 4.72 g/L with the highest reported level being at 6.03 g/L reported by Lefebvre *et al.* (2020) and observed in German women participating in the LIFE Child cohort in Leipzig, Germany, sampled at 12 months of lactation and belonging to the milk phenotype 2, *i.e.*, non-Secretor/Lewis^{+}.

Table 3.1.1.6-1 3-FL Concentrations in Human Milk of Phenotype Group 2 [Se– / Le+ (a+b–)]

Lactation Time	Parameter	g/L	References
All lactational periods included	Mean of Means Range of Means Mean of 95% CL Mean of Upper Ranges Max of Upper Range	2.28 0.40-5.10 3.42 4.72 6.03	Thurl *et al*. (2010); Coppa *et al*. (2011); Gabrielli *et al*. (2011); Galeotti *et al*. (2012, 2014); Austin *et al*. (2019); Tonon *et al*. (2019); Lefebvre *et al*. (2020)

3-FL = 3-fucosyllactose; CL = confidence level.

3.2 Estimated Intake of 3-FL from Proposed Uses

The following section describes the estimated intake of the ingredient, expressed on a 3-FL basis (*i.e.*, water and other carbohydrate content are not included). 3-FL is not an appreciable component of the background diet outside of human milk (see Section 3.1.2) and thus was not considered in the exposure modelling herein.

3.2.1 Methods

An assessment of the anticipated intake of 3-FL as an ingredient under the intended conditions of use (see Table 1.2-1) was conducted using data available in the 2017-2018 cycle of the U.S. National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Survey (NHANES) (CDC, 2021a,b; USDA, 2021a). An abbreviated summary of the survey and methodology employed in the intake assessment of 3-FL along with the pertinent results is presented herein.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2017 to 2018. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on two non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (CDC, 2021a,b; USDA, 2021b). The NHANES data were employed to assess the mean and 90th percentile intake of 3-FL for each of the following population groups:

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

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of 3-FL by the U.S. population[11]. Estimates for the daily intake of 3-FL represent projected two-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018; these average amounts comprised the distribution from which mean, and percentile intake estimates were determined. Mean and percentile estimates were generated incorporating survey weights to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of 3-FL averaged over all individuals surveyed, regardless of whether they consumed food products in which 3-FL is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing 3-FL during the two survey days). "Consumer-only" intake refers to the estimated intake of 3-FL by those individuals who reported consuming food products in which the use of 3-FL is currently under consideration. Individuals were considered "consumers" if they reported consumption of one or more food products in which 3-FL is proposed for use on either Day 1 or Day 2 of the survey.

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

The estimates for the intake of 3-FL were generated using the maximum use level indicated for each intended food use, as presented in Table 1.2-1, together with food consumption data available from the 2017-2018 NHANES datasets. The results of this assessment are presented in Section 3.2.2.

3.2.2 Intake Estimates for 3-FL

A summary of the estimated daily intake of 3-FL from proposed food uses is provided in Table 3.2.2-1 on an absolute basis (g/person/day), and in Table 3.2.2-2 on a body weight basis (mg/kg body weight/day).

The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 76.2% of the population groups consisted of consumers of food products in which 3-FL is currently proposed for use (Table 3.2.2-1). Toddlers had the greatest proportion of consumers at 99%. The consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of 3-FL were determined to be 1.06 and 2.19 g/person/day, respectively. Of the individual population groups, infants aged 7 to < 12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of 3-FL on an absolute basis, at 2.02 and 3.74 g/person/day, respectively. Female teenagers had the lowest mean and 90th percentile consumer-only intakes of 0.79 and 1.68 g/person/day, respectively (Table 3.2.2-1).

Table 3.2.2-1 Summary of the Estimated Daily Intake of 3-FL from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	0.69	1.47	76.2	139	0.90	1.72
Infants	7 to < 12 m	1.96	3.74	97.5	122	2.02	3.74
Toddlers	1 to 2 y	1.23	2.02	99.0	300	1.24	2.03
Children	3 to 11 y	1.07	2.03	97.4	970	1.10	2.05
Female Teenagers	12 to 19 y	0.73	1.63	92.3	413	0.79	1.68
Male Teenagers	12 to 19 y	1.05	2.04	94.9	417	1.11	2.13
Female Adults of Childbearing Age	20 to 40 y	0.78	1.66	87.7	610	0.89	1.73
Female Adults	20 to 64 y	0.85	1.90	86.9	1,408	0.98	2.08
Male Adults	20 to 64 y	1.10	2.56	89.0	1,260	1.23	2.63
Elderly	65 y and older	0.82	1.85	87.7	904	0.93	1.93
Total Population	2 y and older	0.95	2.13	89.8	5,523	1.06	2.19

3-FL = 3-fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of 3-FL were determined to be 18 and 40 mg/kg body weight/day, respectively. Among the individual population groups, infants aged 7 to < 12 months were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 224 and 443 mg/kg body weight/day, respectively. The elderly and female adults of childbearing age had the lowest mean consumer-only intake

of 12 mg/kg body weight/day, while female adults of childbearing age had the lowest 90th percentile consumer-only intake of 23 mg/kg body weight/day (Table 3.2.2-2).

Table 3.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 3-FL from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	103	231	76.2	139	135	250
Infants	7 to < 12 m	219	443	97.5	122	224	443
Toddlers	1 to 2 y	100	176	98.9	291	101	176
Children	3 to 11 y	42	82	97.4	967	43	83
Female Teenagers	12 to 19 y	13	29	92.4	407	14	31
Male Teenagers	12 to 19 y	17	32	95.2	415	17	32
Female Adults of Childbearing Age	20 to 40 y	11	23	87.7	609	12	23
Female Adults	20 to 64 y	12	27	87.0	1,402	13	29
Male Adults	20 to 64 y	12	28	89.0	1,252	14	30
Elderly	65 y and older	11	23	88.0	891	12	24
Total Population	2 y and older	17	38	89.9	5,478	18	40

3-FL = 3-fucosyllactose; bw = body weight; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

3.2.3 Summary and Conclusions

Consumption data and information pertaining to the individual proposed food uses of 3-FL were used to estimate the *per capita* and consumer-only intakes of 3-FL (estimates performed on a 3-FL basis not including moisture or other carbohydrates) for specific demographic groups and for the total U.S. population. There were several assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in the exposure assessment that all food products within a food category contain 3-FL at the maximum specified level of use. In reality the levels added to specific foods will vary depending on the nature of the food product and it is unlikely that 3-FL will have 100% market penetration in all identified food categories.

In summary, on consumer-only basis, the resulting mean and 90th percentile intakes of 3-FL by the total U.S. population from all proposed food uses were estimated to be 1.06 g/person/day (18 mg/kg body weight/day) and 2.19 g/person/day (40 mg/kg body weight/day), respectively. Among the individual population groups, infants aged 7 to < 12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of 3-FL on an absolute basis and body weight basis, at 2.02 and 3.74 g/person/day, respectively, equivalent to 224 and 443 mg/kg body weight/day. The female teenagers had the lowest mean and 90th percentile consumer-only intake of 0.79 and 1.68 g/person/day, respectively.

Part 4. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with 3-FL.

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

Not applicable.

Part 6. NARRATIVE AND SAFETY INFORMATION

6.1 Introduction

Glycom has conducted a scientific procedures GRAS evaluation of 3-FL for use as an ingredient in infant formula at a use level of 0.75 g/L and specified conventional food applications marketed to the general population. 3-FL as manufactured by Glycom has been demonstrated to be identical in structure to its natural counterpart secreted into human milk and therefore can be referred to as an HiMO. The ingredient will be added to infant formula at levels that will result in concentrations that are within the 95th percentile range of reported mean concentrations that have been measured in human milk samples obtained from lactating women across all lactational stages, and therefore, the safety of adding 3-FL to infant formula is supported by pivotal information establishing its history of safe consumption by breast-feeding infants. As infants are a sensitive population group, the safety of dietary ingestion of HiMOs from human milk consumption also can be extended to adults consuming HiMOs at comparable ingestion levels in conventional food products.

Since conclusions on the GRAS use of 3-FL in infant formula are based on extrapolation of safe levels established from usual concentrations that have been measured in human milk samples, toxicological evaluations in animals, or tolerance studies in neonatal piglets, were not necessary to establish an appropriate margin of safety for dietary intakes of 3-FL from the intended food uses.

Notwithstanding conclusions on safety being supported by the history of safe consumption of HiMOs in the infant diet, published animal toxicity studies of 3-FL were identified during Glycom's comprehensive searches of the literature and are relevant to hazard characterization of the ingredient. Pitt *et al*. (2019) reported findings form a subchronic toxicity study of 3-FL produced by fermentation using a modified strain of *E. coli* K-12 and purified using similar downstream processes to a purity of 94.6%. A no-observed-adverse-effect level (NOAEL) of 10% 3-FL in the diet (equivalent to 5.98 g/kg body weight/day in males and 7.27 g/kg body weight/day in females) was established (Pitt *et al.*, 2019). Additionally, Pitt *et al*. (2019) reported that 3-FL was not mutagenic or genotoxic based on the results of a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration assay, and an *in vivo* mammalian micronucleus test (Pitt *et al.*, 2019). The findings reported by Pitt *et al*. (2019) corroborate the safety 3-FL.

Glycom also identified published studies evaluating the safety of an "HMO Mix" containing 3-FL (at 16.0% dry weight) as a blend with 2'-fucosyllactose, lacto-*N*-tetraose, 3'-sialyllactose, and 6'-sialyllactose (Parschat *et al.*, 2020). No genotoxicity was reported, and a NOAEL determination of 10% in the diet was reported by the authors (equivalent to 5.67 g HMO Mix/kg body weight/day for males and 6.97 g HMO Mix/kg body weight/day for females). Overall, there were no findings reported in the literature characterizing the hazard of 3-FL in animal studies to suggest that Glycom's conclusions on extrapolation of safety from the history of safe use would be inappropriate.

Product specific toxicological data that has been obtained on 3-FL manufactured by Glycom provides corroborating information on the safety of the company's production processes including the safety of the production strain (Unpublished). A NOAEL of 4,000 mg/kg body weight/day, the highest dose tested, was established based on the results of an adapted subchronic (90-day) oral toxicity study with neonatal rats. Additionally, a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test in human lymphocytes were without evidence of genotoxicity/mutagenicity.

A clinical infant study involving 3-FL was identified in the published literature and supportive of the safe use of Glycom's 3-FL at 0.75 g/L in infant formula. Parschat *et al*. (2021) reported that infant formula supplemented with a mixture of 5 HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL; 2.99, 0.75, 1.5, 0.23, and 0.28 g/L, respectively) and given to infants for the first four months of life was able to support normal infant growth and was safe and well-tolerated. As eligible subjects were healthy female and male infants ≤ 14 days of age at Visit 1, this study provides supporting safety information for the use of 3'-FL across all infant developmental stages.

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

Finally, Glycom evaluated the allergenicity risk of 3-FL. As a purified ingredient, 3-FL manufactured by Glycom is free of detectable levels of protein assayed using a modified Bradford method with a detection limit of 17 ppm. The amino acid sequences of heterologous genes introduced into the production organism were evaluated using Allergen Online (version 20) hosted by the University of Nebraska's Food Allergen Research and Resource Program (FARRP, 2020). No positive alignments ≥ 35% identity were identified between any of the recombinant proteins and known/putative allergen sequences within the database. 3-FL manufactured by Glycom was concluded to be of low allergenic risk. As milk-derived lactose is used as a substrate during fermentation, 3-FL would be labeled as "contains milk" in accordance with the requirements of the Food Allergen Labeling and Consumer Protection Act of 2004.

6.2 Literature Search

Glycom considered the totality of publicly available data and information relevant to the safety of 3-FL, and performed a literature search for studies relevant to the safety of 3-FL. A comprehensive and detailed search of the published scientific literature was conducted through August 2021 using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of 3-FL have considered all publicly available sources of information including favorable and potentially unfavorable information. Based on Glycom's updated search of the literature, the company did not identify any published studies to suggest 3-FL is unsafe for use as a food ingredient. The toxicological studies identified in the scientific literature examining the safety of a highly purified 3-FL and a "HMO Mix" are summarized in Section 6.4. A clinical infant study involving 3-FL is summarized in Section 6.5.

6.3 Absorption, Distribution, Metabolism, and Excretion

3-FL manufactured by Glycom has been demonstrated analytically to be structurally identical to its naturally occurring counterpart in human milk. The absorption, distribution, and metabolism of HMOs have been the subject of extensive investigation (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al.*, 2000; Chaturvedi *et al.*, 2001b; Rudloff and Kunz, 2012) and it can be concluded that HMOs, including 3-FL, do not undergo any significant digestion in the upper gastrointestinal tract. Very small quantities of ingested HMOs have been reported to be absorbed intact (approximately 1 to 2% of the total amount of HMO ingested) and are excreted unchanged in urine. The absorption of 3-FL added to infant formula would be no different to that occurring in infants consuming human milk.

6.4 Toxicological Studies

The risk assessment approach for 3-FL follows the same procedures used to support the safety of other HiMOs that have been concluded to be GRAS for use in infant formula by Glycom and others. The pivotal data and information supporting the safety of Glycom's HiMO ingredients are based on qualitative data establishing that HiMOs manufactured by Glycom are chemically and structurally identical to corresponding HMOs present in human milk, and the fact that the intended uses of 3-FL in infant formula are within ranges that have been quantitated in human milk samples across all lactational stages. Since all of Glycom's HiMOs are intended to be used alone or in combination with other HiMOs at levels that are individually and cumulatively within the range that has been reported in human milk samples, the risk assessment does not require derivation of a margin of safety for exposure to the ingredients from infant formula use relative to a NOAEL value from toxicological investigation.

As such, data from toxicological studies conducted on 3-FL produced by microbial fermentation largely corroborate the safety of Glycom's 3-FL and support that non-HiMO constituents originating from the production process and from the fermentation organism are not present at levels of toxicological concern.

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

A highly purified preparation of 3-FL (DuPont), as well as 3-FL as part of an "HMO Mix" (Jennewein) have been investigated in published genotoxicity studies, an acute oral toxicity studies in rat, two subchronic oral toxicology studies in rats, and one piglet feeding study. The results of two genotoxicity studies and a neonatal 90-day oral toxicology study in rats conducted on Glycom's 3-FL ingredient are also available. Characteristics of the 3-FL test articles used in these investigations are presented in Table 6.4-1 and the results of these studies are detailed in the following sections.

Table 6.4-1 Test Articles Used in Studies Conducted with 3-FL Preparations

Description	Glycom's 3-FL Produced by a Modified *Escherichia coli* K-12 Production Strain	DuPont's Highly Purified 3-FL Produced by a Modified *E. coli* K-12 Production Strain	Jennewein's HMO Mix
Manufacturing Overview	Microbial fermentation with a controlled DSP procedure	Microbial fermentation with a controlled DSP procedure	Individual microbial fermentation with a controlled DSP procedure, followed by blending with other HMOs
Production Organism	Derivative of *E. coli* K-12 MDO	*E. coli* K-12 MG1655	*E. coli* BL21 (DE3) JBT-3FL
Purity (3-FL Content)	Specified as ≥ 87%	94.6% (specified as > 90%)	10.4 to 16.0% (dry weight)

Table 6.4-1 Test Articles Used in Studies Conducted with 3-FL Preparations

Description	Glycom's 3-FL Produced by a Modified *Escherichia coli* K-12 Production Strain	DuPont's Highly Purified 3-FL Produced by a Modified *E. coli* K-12 Production Strain	Jennewein's HMO Mix
Other Specified Components	≤ 5.0% Lactose ≤ 1.0% Fucose ≤ 1.5% 3-Fucosyl-lactulose < 0.01% Protein ≤ 0.5% Ash	1.5% Lactose 1.2% Fucose 1.3% Glucose/galactose 1.4% Other carbohydrates ≤ 100 µg/g Protein ≤ 0.5% Ash 1.9% Moisture No detectable residual recombinant DNA	47.1 to 49.1% 2'-FL 23.7 to 19.9% LNT 3.5 to 4.1% 3'-SL 4.2 to 4.0% 6'-SL 5.1 to 12.9% Other carbohydrates
Toxicology/Safety Studies Conducted	• Bacterial reverse mutation test • *In vitro* micronucleus test • 14-day dose range-finding test • Subchronic oral toxicity in rats	• Bacterial reverse mutation test • *In vitro* micronucleus test • *In vitro c*hromosomal aberration test • *In vivo* micronucleus test • Acute oral toxicity in rats • Subchronic oral toxicity in rats	• Bacterial reverse mutation test • *In vitro* micronucleus test • 7-day pilot oral tolerance in rats • Subchronic oral toxicity in rats • 21-day oral toxicity study in piglets
References	Unpublished	Pitt *et al.* (2019)	Hanlon (2020); Parschat *et al.* (2020)

3-FL = 3-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; DNA = deoxyribonucleic acid; DSP = downstream processing; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose.

6.4.1 Oral Toxicology Studies

6.4.1.1 Acute Oral Toxicity Study in the Rat

3-FL (produced by *E. coli* K-12 MG1655) has been subject to an acute oral toxicity study in rats (Pitt *et al.*, 2019). Five female Crl:CD (SD) rats received a single bolus dose of 5,000 mg/kg body weight of 3-FL by gastric intubation at a dose volume of 20 mL/kg body weight. Observations for clinical signs of toxicity and body weights were recorded over a 14-day observation period.

No deaths or clinical signs of toxicity were observed, and all animals gained weight during the observation period. No macroscopic observations were noted at necropsy. The study authors concluded that 3-FL was non-toxic in this acute oral toxicity study.

6.4.1.2 Repeated Dose Oral Toxicity Study in the Rat

Subchronic Oral Toxicity: 3-FL (DuPont) – *Incorporated by Reference to GRN 951*

3-FL (produced by *E. coli* K-12 MG1655) was evaluated in a 90-day subchronic oral toxicity study in Crl:CD(SD) rats, conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 408 and U.S. FDA guidelines (OECD, 2018; Pitt *et al.*, 2019). Due to the nature of the use profile of the substance and information available in the published literature, the study investigators decided to test dietary concentrations of up to 10% (above the U.S. FDA guideline of 5%). To mitigate the potential for nutritional or adverse effects at this high dose, a low concentration test group comprising of rats fed 5% 3-FL in the diet was also included. At 7 weeks of age, four groups of 10 animals/sex were fed

either the basal diet or experimental diets containing 5% 3-FL, 10% 3-FL, or 10% fructo-oligosaccharide (FOS; reference control) *ad libitum* for at least 90 consecutive days.

Daily observations for general clinical conditions, detailed physical examination, and ophthalmological examinations were conducted. A cage-side clinical examination was performed daily, and a functional observational battery (FOB) and motor activity were evaluated prior to study initiation and near the end of the study. Animals were weighed periodically during the pre-test period, at the initiation of the experimental diet administration, and weekly thereafter. Food consumption was measured for each pair of animals and feed efficiency was calculated. The consumed 3-FL and FOS doses were calculated weekly. Blood and urine samples were collected during Week 12 for analysis of 3-FL concentration and kinetic evaluation. On the day of sacrifice, blood samples were collected. Hematology, coagulation parameters, serum chemistry, and urinalysis variables were analyzed. Gross necropsy was performed following euthanasia and absolute and relative organ weights were measured for brain, adrenals, heart, kidneys, liver, spleen, thymus, epididymides, testes, prostate seminal vesicles, ovaries, and uterus. Select tissues were preserved in fixatives, spliced, and stained with hematoxylin and eosin.

The attained doses of 3-FL among rats provided dietary concentrations of 5 and 10% were equivalent to 3,038 and 5,975 mg/kg body weight/day in males and 3,879 and 7,279 mg/kg body weight/day for females, respectively. In the reference control rats receiving 10% FOS, the mean attained dose was 6,224 and 7,412 mg/kg body weight/day for males and females, respectively.

All animals survived to scheduled sacrifice and no ophthalmological findings, clinical and detailed physical observations, or effects on neurobehavioral parameters were attributable to the consumption of 3-FL or FOS. No statistically significant or biologically-relevant differences in body weight, body weight gain, food consumption, or food efficiency were observed. No test article-related differences in hematology, coagulation parameters, clinical chemistry, or urinalysis endpoints were observed. Although a significantly higher mean cell volume and mean cell hemoglobin value in males receiving 5% 3-FL were detected, no dose-related response was observed and no accompanying changes in other hematology parameters were noted. A significantly lower red cell distribution width and cholesterol in females provided FOS was also reported; however, no correlative changes were observed in female rats and these observations were not seen in males.

No statistically significant, biologically- or toxicologically-relevant differences in organ weights were noted, and no macroscopic or microscopic observations attributed to the consumption of 3-FL or FOS were made by the study investigators. Microscopic observations in the study were consistent with normal background lesions in rats of this age and strain.

Overall, the study investigators deemed that there were no treatment-related effects of 3-FL on survival, ophthalmology, clinical observations, neurobehavioural parameters, body weight, body weight gain, food consumption, food efficiency, hematology, coagulation parameters, clinical chemistry, urinalysis, organ weights, and macroscopic or microscopic histopathological findings at dietary concentrations of 5 or 10%. The study authors considered the dietary level of 10% as the NOAEL (noted herein to be equivalent to 5,975 mg/kg body weight/day in males and 7,279 mg/kg body weight/day for females).

<u>Subchronic Oral Toxicity: 3-FL (Glycom's *E. coli* K-12 MDO derived production strain)</u>

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 3-FL (produced by Glycom's *E. coli* K-12 MDO derivative) when administered orally, by gavage, to neonatal rats from Day 7 of age (Unpublished). The study was conducted in compliance with the OECD principles of Good

Laboratory Practice (GLP) (OECD, 1998) and the most recent version of OECD TG 408 (OECD, 2018), but was adapted by using neonatal animals (as 3-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 paediatric 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).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (vehicle – water for irrigation), 1,000, 2,000, or 4,000 mg/kg body weight/day 3-FL, by oral gavage at a dose volume of 10 mL/kg body weight, once daily for 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 4,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose 3-FL group and identify any effects related to the general fiber-like characteristics of the reference material. Doses of 3-FL and the reference control were corrected to account for "other carbohydrates" within the test article batches (thus, the high dose corresponded to a total carbohydrate amount of 4,320 mg/kg body weight/day). A further 5 males and 5 females in the vehicle control, high-dose 3-FL, and reference control groups were also dosed once daily for at least 90 days and then kept undosed for 4 weeks, to assess the reversibility of any observed effects seen in the dosing period.

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

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

At the end of the dosing and recovery periods, all surviving animals were subjected to a gross macroscopic necropsy, where (for all animals after the dosing period, and for vehicle control, reference control, and high-dose 3-FL animals after the recovery period) selected organs (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, submandibular and sublingual salivary glands, seminal vesicles, spleen, testes, thymus, thyroid/parathyroid glands, and uterus/cervix) were weighed and fixed. At the end of the treatment period, a full list of tissues [adrenal glands, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, Harderian glands, head, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric and left axillary lymph nodes, esophagus, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerves, seminal vesicles, skeletal muscle, skin (with mammary glands), spinal cord, spleen, sternum, stomach, testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix), and vagina] for early decedents and animals in the vehicle control and high dose 3-FL groups were examined microscopically. Wet vaginal smears were collected by lavage from all females at necropsy to determine the stage of oestrous.

There were no deaths and no test item-related clinical signs, nor were there any ocular findings at the ophthalmic examinations in Week 13 of dosing. Body weight and food consumption were unaffected by 3-FL administration. There were no effects of 3-FL on the age or body weight at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation or vaginal opening for males and females, respectively) nor the age of attainment of the surface and air righting reflexes, performance in the pupil reflex and startle response tests, or mean ulna growth. Behavior of the animals during the in-hand and arena observations, as well as Morris maze performance, were similar across all groups. Oestrous cycles were unaffected by 3-FL administration, with most females in all groups showing an oestrus smear prior to termination.

No test item-related adverse effects on hematology, clinical biochemistry, or urinalysis parameters were observed. A statistically significant decrease in specific gravity was observed in all male 3-FL groups, in females given 2,000 or 4,000 mg/kg body weight/day, and in reference control males and females. In addition, males given 4,000 mg/kg body weight/day showed a slight, but statistically significant, increase in urinary pH, and a decrease in protein, creatinine, and glucose concentrations (both in total and concentration terms). However, reference control males also showed an increase in urine volume, and a decrease in urinary protein, glucose, and creatine concentration when compared to vehicle controls. No differences in these parameters were observed at the end of the 4-week recovery period.

No test article-related differences in organ weights were observed and there were no 3-FL-related macroscopic or histopathological abnormalities. The only findings observed were incidental and generally consistent with changes encountered in Sprague Dawley rats of this age kept under laboratory conditions.

The results to date demonstrate that once daily oral gavage administration of 3-FL to neonatal Crl:CD(SD) rats for 90 days (from Day 7 of age) at doses up to 4,000 mg/kg body weight/day (total carbohydrate amount of 4,320 mg/kg body weight/day) was well tolerated and not associated with any test article-related adverse effects.

HMO Mix Containing 16.0% 3-FL (produced by *E. coli* BL21 (DE3) JBT-3FL) - *Incorporated by Reference to GRN 925*

The oral toxicity of an HMO mixture containing 16.0% 3-FL was evaluated in a 7-day pilot oral tolerance study and a 13-week oral toxicity study in compliance with GLP and OECD TG 408 (OECD, 1998, 2018; Parschat *et al.*, 2020). In the 7-day pilot oral tolerance study, healthy female CD/Crl:CD rats (5/group) were allocated to receive either a standard certified commercial diet (ssniff-R/M-H V1530) or the same diet supplemented with 10% HMO Mix. This HMO mixture (called “HMO MIX I” in the study) was comprised of 16.0% 3-FL, 47.1% 2'-FL, 23.7% LNT, 4.1% 3'-SL, 4.0% 6'-SL, and 5.1% other carbohydrates on a dry weight basis. It is noted that no correction for the disparity in the nutrient profile of the control diet and the experimental diet was performed in this pilot study (nor in the subsequent main oral toxicity study described below). Animals were observed daily for viability, behavioral changes, and reactions to treatment or illness, and body weights were recorded at the time of group allocation and daily starting on the first day of treatment. Food consumption was also recorded daily and drinking water intake was monitored daily by visual inspection.

No premature mortalities, changes in behavior, appearance, and consistency of the feces, nor differences in body weight, body weight gain, or food consumption were observed between groups. As such, the concentration of 10% HMO Mix in the diet was selected as the high dose tested in the 13-week oral toxicity study.

In the 13-week study, two groups of 10 male and 10 female CD rats were provided either the standard commercial diet (control; ssniff-R/M-H V1530) or the same diet supplemented with 10% HMO mixture *ad libitum*[12]. The oligosaccharides were reportedly produced individually by fermentation and were subject to removal of the production strain, purification processes (not further disclosed), and concentration processes (not further disclosed). Drinking water was offered *ad libitum*. Each animal was kept in an individual Makrolon cage with a 12-hour light/dark cycle. The 10% HMO Mix diet was freshly prepared each week, with the components combined in an impact mill. Food residues were removed and weighed daily.

Animals were observed daily for clinical signs and detailed clinical observations were made on one occasion prior to the first exposure and on a weekly basis thereafter. In Test Week 13, all animals were tested for sensory reactivity to different stimuli (auditory, visual, and proprioceptive), grip strength, and locomotor activity. Observational screening included the righting reflex, body temperature, salivation, startle response, respiration, mouth breathing, urination, convulsions, pilo-erection, diarrhea, pupil size and response, lacrimation, impaired gait, stereotypic behavior, toe and tail pinch, wire maneuver, hind leg splay, positional passivity, tremors, positive geotropism, limb rotation, and auditory function. Mortality was checked every morning and afternoon. Body weight of each rat was recorded at the start of the adaptation period, at the time of group allocation, on the day exposure commenced, and weekly thereafter. Ophthalmological and auditory examinations were performed on all animals before (Day -1) and one week before the end of the treatment (Week 12). Blood and urine samples were taken from all animals fasted overnight at the end of Week 13 before necropsy on Day 92. On Day 92, animals were euthanized and dissected. Organ weights were determined, and histological analysis was carried out on specific organs.

One male in the control group was removed from analysis due to marked reductions in body weight and spontaneous and incidental changes in specific hematology and clinical chemistry parameters. In the remaining animals included in the analysis, no changes in food consumption between groups were observed. The mean intake of HMO Mix in the test group ranged from 5.01 to 6.88 g/kg body weight/day for males and 6.26 to 7.91 g/kg body weight/day in females. The resulting intake of 3-FL was equivalent to 0.80 to 1.1 g 3-FL/kg body weight/day in males and 1.0 to 1.3 g 3-FL/kg body weight/day in females.

No differences in body weight, body weight gain, or body weight on autopsy were observed between groups. No changes in behavior, external appearance, changes in the consistency of the feces, or other clinical observations were observed in any group. No changes in body posture, movement, or coordination capabilities were reported from baseline. Neurological screening at the end of the exposure period did not reveal any test item-related effects. Ophthalmological examination did not reveal any changes in the eyes and wider optic, or any impairment of auditory acuity between groups. Except for a statistically significant reduction in the absolute number of neutrophilic granulocytes in HMO Mix-treated females compared to control females, there were no significant changes between groups in any of the remaining hematological parameters. Although the absolute number of neutrophils in one treated female fell below the lower boundary of the historical control range, all neutrophil counts in the remaining males and females were within historical control ranges. As such, the study authors deemed the reduction in neutrophilic granulocytes to be not HMO Mix-related. Statistically significant changes in selected clinical chemistry parameters in rats receiving the HMO Mix were also noted; however, these were similarly deemed by the study authors not to be test article related. No changes in urinalysis parameters were observed with the exception of a statistically significant decrease in the specific gravity in female rats receiving the HMO Mix.

[12] Again, it is noted that no corrections for the nutrient disparities between the control and experimental diet was performed by the study investigators. The authors noted that the addition of the HMO Mix to the diet reduced the nutrient content of the diet by 10%.

However, this change was small in magnitude, within the historical control ranges, and deemed by study authors to be not HMO Mix-related.

Macroscopic inspection did not reveal any test item-related changes in the organs or tissues of animals receiving the HMO Mix. With respect to organ weights, the mean absolute body weights of brains in male rats receiving HMO Mix were statistically lower; the mean absolute weight of right kidneys were lower in female rats receiving HMO Mix; and the mean relative (to body) weights of the left and right kidneys were statistically lower in female rats receiving HMO Mix. Although there was a mild increase in the incidence and severity of hepatocellular lipid content in the periportal areas of the livers of males receiving HMO Mix, none of the organ weight changes were associated with histopathologic changes. Additionally, changes in kidney weights were not associated with adverse clinical chemistry effects or increases in the incidences and severities of histopathological changes. Furthermore, changes in brain and kidney weights were within the historical control ranges. As such, the study authors deemed the changes in absolute and relative organ weights as normal biological variation and not related to the HMO Mix.

In absence of any test item-related adverse effects, the NOAEL for the HMO Mix was concluded to be 5.67 g HMO Mix/kg body weight/day in males and 6.97 g HMO Mix/kg body weight/day in females, the highest doses tested. This was equivalent to 0.91 g 3-FL/kg body weight/day in males and 1.12 g 3-FL/kg body weight/day in females.

6.4.1.3 Twenty-One Day Feeding Study in Piglets with HMO Mix Containing 10% 3-FL (produced by E. coli BL21 (DE3) JBT-3FL) - Incorporated by Reference to GRN 925

The safety of a mixture of HiMOs was evaluated in a neonatal piglet model (Hanlon, 2020; U.S. FDA, 2021a). The study was conducted in accordance with GLP regulations following the U.S. FDA guidelines. Eighteen male and 18 female 2-day-old Domestic Yorkshire Crossbred Swine (sourced from a commercial farm) were received and provided an iron supplement and a broad-spectrum antibiotic prior to receipt at the laboratory, and at one week after receipt. Six male and 6 female piglets were non-randomly assigned to receive one of three diets: control (containing no HMOs), 5.75 g/L of HMO Mix, or 8.0 g/L of HMO Mix. On a dry-weight basis, this HMO Mix comprised of 10.4% 3-FL, 49.1% 2'-FL, 19.9% LNT, 3.5% 3'-SL, and 4.2% 6'-SL, with the remainder composed of other carbohydrates including lactose. The vehicle for all three diets was Land O'Lakes ProNurse Specialty Milk Replacer, reconstituted with deionized water. Diets were administered orally *via* a feeding bowl, six times a day for 21 days.

Mortality, clinical observations, body weight, food consumption, food efficiency, and compound consumption were monitored on a daily basis, and samples were obtained on Days 7 and 21 for hematology (including leukocyte count, erythrocyte count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, absolute reticulocytes, platelet count, red blood cell distribution width, and blood smear); coagulation parameters (including activated partial thromboplastin time, prothrombin time, and fibrinogen); and clinical chemistry (including alkaline phosphatase, total bilirubin, aspartate aminotransferase, alanine aminotransferase, *gamma*-glutamyl transferase, sorbitol dehydrogenase, urea nitrogen, creatinine, total protein, glutamate dehydrogenase, albumin, globulin, glucose, total cholesterol, triglycerides, low-density lipoprotein, sodium, potassium, chloride, calcium, and phosphorus). Samples for urinalysis (analyses including volume, color, appearance, protein, glucose, bilirubin, ketones, blood, urobilinogen, specific gravity, and pH) were collected on Study Days 7 and 22, and animals were subject to necropsy on Day 22.

Microscopic evaluation was conducted on the aorta, sternum, brain, epididymis, esophagus, eye, gallbladder, adrenal gland, Harderian gland, mammary gland, parathyroid gland, pituitary gland, prostate gland, seminal vesicle, thyroid gland, gut-associated lymphoid tissue, heart, kidney, large intestine, larynx, liver, lung, lymph node, skeletal muscle, nerves, ovary, pancreas, skin, small intestine, spinal cord, spleen, stomach, testis, thymus, tongue, trachea, urinary bladder, uterus/cervix, and vagina. Macroscopic inspection included the aforementioned organs, as well as nasal cavity, bone marrow smear, lumbar ganglion, salivary glands, tibial nerve, oviduct, and ureter.

With the exception of one male piglet, all animals survived to study termination. One male piglet receiving 8.0 g/L HMO Mix was euthanized on Day 7 due to poor clinical condition. Clinical and veterinary observations included yellow discolored feces, thin body condition, unkempt appearance, generalized muscle wasting, and lateral recumbency. Analyses of samples obtained from this piglet indicated that the decrease in physical condition was due to a bacterial infection that was likely obtained at the farm prior to enrollment into the study. The study investigator concluded that the effects in this animal were unrelated to the administration of HMO Mix.

No differences in body weight or food consumption were observed between controls and animals receiving the HMO Mix. Food efficiency was statistically lower in female piglets receiving 5.75 g/L HMOs compared to controls on Study Days 18 and 19; however, this was not observed on any other study days and was not seen in the higher dose group. Consumption of the HMO Mix was calculated to be equivalent to 2,556.2 and 3,576.4 mg/kg body weight/day in male animals receiving 5.75 and 8.0 g/L, respectively. In females, this was calculated as 2,603.9 and 3,659.8 mg/kg body weight/day in the 5.75 and 8.0 g/L groups, respectively. Clinical and veterinary observations were generally similar between the control and the test groups, seen infrequently, and/or considered common in neonatal piglets. A lower serum sodium and/or chloride was observed in animals on Study Day 7 and was considered by the study investigator as secondary to electrolyte loss associated with the watery feces that were also observed in these animals. No dose-dependent relationship was observed in this effect and the signs resolved by Study Day 21. No statistically significant differences were observed in hematology or urinalysis parameters. Though there were a few statistical differences in coagulation and clinical chemistry parameters, the study investigator deemed these differences were unrelated to treatment, as they were small in magnitude, were not dose-dependent, were not seen in both sexes, resolved between Study Day 7 and 21, and/or the changes were consistent with historical control data.

A statistically significant increase in the large intestinal weight relative to body weight was observed in male piglets provided the high dose of HMO Mix; this was not correlated with any microscopic findings and no other statistically significant differences in absolute or relative organ weights were noted. Macroscopic findings were incidental and/or consistent with those typically observed in piglets. Similarly, microscopic findings were low in incidence, lacked a dose response, occurred in control animals, or were otherwise consistent with observations in piglets of this age. Of note, the most common microscopic observation was polyarteritis (inflammation of small to medium-sized arteries) in a variety of tissues, observed in 6 animals receiving the control diet, 3 animals receiving 5.75 g/L of HMO Mix, and 5 animals receiving 8.0 g/L HMO Mix. The inflammation was mostly of minimal severity (72.2% of observations) and moderate severity (4.3%), with none scoring more than moderate severity.

Altogether, the study investigator concluded that administration of the HMO Mix to neonatal farm piglets at up to 8.0 g/L was not associated with any adverse outcomes.

6.4.2 Genotoxicity Studies

6.4.2.1 High Purity 3-FL

Bacterial Reverse Mutation Assay: 3-FL (DuPont) - *Incorporated by Reference to GRN 951*

The potential mutagenicity of 3-FL (produced by *E. coli* K-12 MG1655) was investigated in a bacterial reverse mutation assay, a mammalian cell micronucleus test, a chromosomal aberration test in human lymphocytes, and in an *in vitro* mammalian erythrocyte micronucleus test (Pitt *et al.*, 2019).

Briefly, a bacterial reverse mutation test was conducted in accordance with test guidelines established by the U.S. FDA, OECD, and European Commission (Pitt *et al.*, 2019). Tester strains *S.* Typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* strain WP2*uvr*A were tested in the absence and presence of exogenous metabolic activation (Aroclor-induced rat liver S9). 3-FL was tested at concentrations of 333, 667, 1,000, 3,333, and 5,000 µg/plate. Appropriate vehicle control and positive control groups were included for each tester strain and test condition.

No positive mutagenic response nor appreciable toxicity was observed in any test condition. Negative and positive controls exhibited the expected responses. The study authors concluded that 3-FL was not mutagenic in this test.

In Vitro Mammalian Cell Micronucleus Test: 3-FL (DuPont) - *Incorporated by Reference to GRN 951*

In the *in vitro* mammalian cell micronucleus test, Chinese Hamster Ovary (CHO-K1) cells were evaluated for the formation of micronuclei in the absence and presence of exogenous metabolic activation (Pitt *et al.*, 2019). Experiments were conducted in accordance with test guidelines established by the U.S. FDA, OECD, and European Commission. 3-FL (produced by *E. coli* K-12 MG1655) concentrations of 500, 1,000, 2,500, 3,500, and 5,000 ug/mL were tested; appropriate vehicle and positive control groups were included in each test condition. Cells were exposed to 3-FL for 4 hours in the presence and absence of exogenous metabolic activation; and for 24 hours in the absence of metabolic activation. Cells were harvested 24 hours after treatment initiation. Cells were analyzed by floc cytometry for micronuclei induction and toxicity.

No significant increases in the percentage of micronuclei (by Dunnett's test) were observed in any of the 3-FL experiments and all values were within the 95% confidence interval of the laboratory historical control ranges. However, a statistically significant trend (by William's Trend Test) was observed in the 4-hour S9-activated test condition at 3-FL concentrations of 2,500 µg/mL and higher. A confirmatory assay was conducted, which similarly indicated no significant differences in the percentage of micronuclei by Dunnett's test; however, a statistically significant concentration-related trend was again observed at 3-FL concentrations of 1,000 µg/mL and higher (by William's Trend Test). The cell cycle length during exposure was within acceptable limits and the positive controls exhibited the expected responses; as such, the study was considered valid. The study authors concluded that, based on the reproducible statistical trend test in the S9-activated test system, the study findings were equivocal and neither positive nor negative.

In Vitro Mammalian Cell Chromosomal Aberration Test: 3-FL (DuPont) - *Incorporated by Reference to GRN 951*

3-FL (produced by *E. coli* K-12 MG1655) was evaluated in an *in vitro* mammalian cell chromosomal aberration test in human lymphocytes in accordance with OECD TGs (OECD, 2016a; Pitt *et al.*, 2019). Concentrations of 1,250, 2,500, and 5,000 µg 3-FL/mL were tested for 4 and 24 hours in the absence of exogenous metabolic activation, and for 24 hours in the presence of exogenous metabolic activation (phenobarbital/β-naphthoflavone-induced rat liver S9). Appropriate vehicle and positive controls were included. Cells were incubated with demecolcine to induce mitotic arrest prior to expansion in 0.075 M hypotonic potassium chloride, fixation, and staining. The mitotic index was calculated by counting 1,000 lymphocyte nuclei per slide and expressed as a percentage of the vehicle control value.

No statistically significant differences in chromosome and chromatid breaks and exchanges, or chromosomal gaps were observed in metaphase cells of lymphocytes treated with 3-FL under any treatment conditions. Both positive and vehicle control responses were within the laboratory historical control ranges.

In Vivo Mammalian Micronucleus Test: 3-FL (DuPont) - *Incorporated by Reference to GRN 951*

An *in vivo* mammalian erythrocyte micronucleus test was conducted in Crl:CD1 (ICR) mice (Pitt *et al.*, 2019). The study was conducted in accordance with OECD TGs (OECD, 2016b). Groups of mice (at least 5/sex/group) received a single oral gavage dose of 3-FL (produced by *E. coli* K-12 MG1655) at 500, 1,000, or 2,000 mg/kg body weight. A negative control group received deionized water, and a positive control group received 30 mg/kg body weight of cyclophosphamide. Peripheral blood samples were collected at 48- and 72-hours post-dosing. A minimum of 20,000 reticulocytes were analyzed by flow cytometry. To confirm systemic exposure, blood samples were collected at 4 hours post-dosing from one vehicle control and four animals/sex were administered the low dose of 3-FL. 3-FL concentration was analyzed using ultra-high-performance liquid chromatography with tandem mass spectrometry detection.

No statistically significant or biologically relevant increases in the frequency of micronucleated reticulocytes in peripheral blood samples from mice administered 3-FL were observed. The analyzed 3-FL concentrations in plasma were 572 and 681 ng/mL in male and female mice, respectively, administered 500 mg/kg body weight. The positive and negative controls demonstrated the expected responses and were within the laboratory historical control data ranges.

Bacterial Reverse Mutation Test: 3-FL (Glycom)

The potential mutagenicity of 3-FL (produced by Glycom's *E. coli* K-12 MDO derivative) was evaluated in a bacterial reverse mutation test (Ames test), which was performed in compliance with the OECD principles of GLP (OECD, 1998) and according to OECD TG 471 (adopted 21 July 1997, corrected 26 June 2020 – OECD, 2020), Commission Regulation (EC) No 440/2008[13] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998), and U.S. FDA Redbook IV.C.1.a. (U.S. FDA, 2000) (Unpublished).

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

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were exposed to 3-FL at concentrations of up to 5,000 µg/plate (the OECD TG 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix). Water (purified by reverse osmosis) served as the vehicle for 3-FL and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence [2-aminoanthracene and benzo(a)pyrene in the presence of metabolic activation] of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity following exposure to 3-FL in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that 3-FL is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD TG 471 maximum recommended concentration).

In Vitro Mammalian Cell Micronucleus Test: 3-FL (Glycom)

The clastogenic and aneugenic potential of 3-FL (produced by Glycom's *E. coli* K-12 MDO derivative) was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes, in compliance with the OECD principles of GLP (OECD, 1998) and according to OECD Test Guideline 487 (OECD, 2016c) (Unpublished).

An initial preliminary cytotoxicity test was conducted using 3-FL at concentrations up to 2,000 µg/mL (the OECD TG 487 maximum recommended concentration) in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation. No precipitate was observed and there were no significant reductions in the cytokinesis-block proliferative index (CBPI) at any 3-FL concentration tested, compared with vehicle controls. In the main experiment for micronucleus analysis, human lymphocytes were exposed to concentrations of 3-FLat 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

There were no biologically relevant or statistically significant differences in the number of binucleate cells containing micronuclei for 3-FL exposed cultures compared with vehicle controls. Mean micronucleus frequencies for the vehicle control and test item-exposed cultures were all within the laboratory historical 95% confidence limits. The positive control compounds caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions (with all mean values falling within the laboratory's historical control ranges), demonstrating the efficacy of the S9 mix and the sensitivity of the test system. It was concluded, therefore, that 3-FL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD TG 487 maximum recommended concentration).

6.4.2.2 HMO Mix Containing 16.0% 3-FL

Bacterial Reverse Mutation Assay: HMO Mix (Jennewein) - *Incorporated by Reference to GRN 925*

The mutagenicity of the HMO Mix (containing 16.0% 3-FL) was evaluated in a bacterial reverse mutation assay conducted in compliance with OECD TG 471 (OECD, 2020; Parschat *et al.*, 2020). *S.* Typhimurium strains TA98, AT100, TA102, TA1535, and TA1537 were subject to a plate incorporation test and pre-incubation test in the presence and absence of exogenous metabolic activation (S9 mix). Concentrations of the HMO Mix tested were 5.0, 10.0, 31.6, 100, 316, and 600 mg HMO Mix/plate (equivalent to 3-FL concentrations of 0.8, 1.6, 5.1, 16, 51, and 96 mg 3-FL/plate). Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene, and mitomycin C) and presence [2-aminoanthracene and benzo(a)pyrene] of metabolic activation. A positive result for mutagenicity was defined as a reproducible and dose-significantly increased number of revertants compared to the solvent control (at least 2-fold for TA98, TA100, TA1535, and TA1537 and at least 1.5-fold for TA102) in two independent experiments.

No signs of cytotoxicity or mutagenicity were noted in any of the TA98, TA100, TA102, TA1535, and TA1537 test strains compared with vehicle control counts, neither in the plate incorporation test nor in the preincubation test. As such, no evidence of mutagenicity was observed at concentrations of up to 600 mg HMO Mix/plate (96 mg 3-FL/plate)

In Vitro Mammalian Cell Micronucleus Assay: HMO Mix (Jennewein) - *Incorporated by Reference to GRN 925*

The potential genotoxicity of the HMO Mix (containing 16.0% 3-FL) was evaluated in an *in vitro* mammalian cell micronucleus test conducted in compliance with OECD TG 487 (OECD, 2016c; Parschat *et al.*, 2020). Briefly, HMO Mix was tested at concentrations of up to the highest technically feasible concentration, 60 mg/mL, in human peripheral blood lymphocytes both in the presence (4 hours) and absence of metabolic activation (4 and 24 hours) with S9 mix. The vehicle ("highly purified water") was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. The cytokinesis-block technique was applied. At least 500 cells per replicate cell culture were scored and classified as mononucleates, binucleates, or multinucleates to estimate the proliferation index as a measure of toxicity. Micronucleus frequencies were analyzed in at least 2,000 binucleate cells per concentration

The results of these experiments revealed no indications of chromosomal damage. Therefore, HMO Mix was not clastogenic or aneugenic at concentrations of up to 60 mg HMO Mix/mL (9.6 mg 3-FL/mL).

6.4.3 Summary of Toxicological Data

Toxicological studies on 3-FL produced by biotechnology corroborate the safe consumption of 3-FL when included in foods at levels naturally occurring in human milk. A tabular summary of the published toxicological studies available on a highly purified 3-FL preparation (DuPont) and an HMO Mix containing 3-FL (Jennewein), and an unpublished study on Glycom's 3-FL, are provided below in Table 6.4.3-1 and 6.4.3-2.

Table 6.4.3-1 Summary of the Repeated-Dose Toxicology Studies Conducted with 3-FL

Test Article	Species	Route and Dose (mg/kg bw/day)	Duration	NOAEL	Reference
3-FL produced by microbial fermentation with *Escherichia coli* K-12 MG1655	Rats, Crl:CD(SD)	0, 5, or 10% in the diet; equivalent to 3,038 and 5,975 mg/kg bw/day for males and 3,870 and 7,270 mg/kg bw/day for females, respectively	90 days	10% in the diet (5,975 mg/kg bw/day for males; 7,270 mg/kg bw/day for females)	Pitt *et al.* (2019)
3-FL produced by microbial fermentation with Glycom's *E. coli* K-12 MDO derivative	Neonatal Crl:CD(SD) rats	0, 1,000, 2,000, or 4,000 mg/kg bw/day by gavage	90 days	4,000 mg/kg bw/day	Unpublished
HMO Mix, containing 16.0% 3-FL produced by microbial fermentation with *E. coli* BL21 (DE3) JBT-3FL	Rats, CD/Crl:CD	0 or 10% in the diet	7 days	Not applicable	Parschat *et al.* (2020)
	Rats, CD/Crl:CD	0 or 10% in the diet; equivalent to 5.67 g HMO Mix/kg bw/day in males and 6.97 g HMO Mix/kg bw/day in females	91 days	10% in the diet (5.67 g HMO Mix/kg bw/day in males and 6.97 g HMO Mix/kg bw/day in females)	
HMO Mix containing 10.4% 3-FL produced by microbial fermentation with *E. coli* BL21 (DE3) JBT-3FL	Piglets, Domestic Yorkshire Crossbred	0, 5.75 g/L, or 8.0 g/L of HMO Mix in milk replacer; equivalent to 0, 2,556.2, and 3,576.4 mg/kg bw/day in males and 0, 2,603.9, and 3,659.8 mg/kg bw/day in females	21 days	Not established by investigators	Hanlon (2020)

3-FL = 3-fucosyllactose; bw = body weight; HMO = human milk oligosaccharide; NOAEL = no-observed-adverse-effect level.

Table 6.4.3-2 Summary of the Genotoxicity Studies Conducted with 3-FL

Test Article	Assay	Concentrations or Doses Tested	Metabolic Activation	Result	Reference
3-FL produced by microbial fermentation with *Escherichia coli* K-12 MG1655	Bacterial Reverse Mutation Test	333, 667, 1,000, 3,333, and 5,000 µg/plate	± S9	Negative	Pitt *et al.* (2019)
	In Vitro Mammalian Cell Micronucleus Test	500, 1,000, 2,500, 3,500, and 5,000 µg/mL	± S9	Equivocal	
	In Vitro Mammalian Chromosomal Aberration Test	1,250, 2,500, and 5,000 µg/mL	± S9	Negative	
	In Vivo Mammalian Micronucleus Test	500, 1,000, or 2,000 mg/kg bw	NA	Negative	
3-FL produced by microbial fermentation with Glycom's *E. coli* K-12 MDO derivative	Bacterial Reverse Mutation Test	Up to 5,000 µg/plate	± S9	Negative	Unpublished
	In Vitro Mammalian Cell Micronucleus Test	Up to 2,000 µg/mL	± S9	Negative	
HMO Mix, containing 16.0% 3-FL produced by microbial fermentation with *E. coli* BL21 (DE3) JBT-3FL	Bacterial Reverse Mutation Test	5.0, 10.0, 31.6, 100, 316, and 600 mg HMO Mix/plate	± S9	Negative	Parschat *et al.* (2020)
	In Vitro Mammalian Cell Micronucleus Test	7.5, 15, 30, 60 mg HMO Mix/mL	± S9	Negative	

3-FL = 3-fucosyllactose; bw = body weight; HMO = human milk oligosaccharide; NA = not applicable.

6.5 Human Studies

One clinical infant study involving 3-FL was identified in the published literature. The growth, safety, and tolerability of an infant formula supplemented with a mixture of five HMOs (5HMO-Mix) during the first 4 months of life have been evaluated in a randomized, double-blind, multicenter[14], controlled, 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.

The basic infant formula providing the macro- and micro-nutrients required for infant nutrition was manufactured in compliance with regulations of the EU 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 5HMO-Mix was added at a concentration providing 5.75 g/L of the reconstituted infant formula. The concentration of individual HMOs from the 5HMO-Mix manufactured by Chr. Hansen HMO GmbH[15] (Rheinbreitbach, Germany) added to the Test formula is presented in Table 6.5-1 below. The

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

[15] Note Jennewein Biotechnologie GmbH was acquired by Chr. Hansen A/S on 22 Sept 2020

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

HMO	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; HMO = human 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 noninferior 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 World Health Organization (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)[16] and the per-protocol dataset (PPS)[17]. Growth parameters were evaluated in the FAS, while all other secondary outcomes were evaluated in the safety dataset (SS)[18].

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).

[16] 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 1 body weight value at baseline and after baseline.
[17] All subjects from the FAS without any major deviations.
[18] All subjects enrolled in the study who received at least one feeding and had any tolerability data available up to 4 months.

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[19].

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 two 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 3 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 effects 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 effects 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 HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) at concentrations similar to those naturally occurring in human milk supported normal infant growth and was safe and well-tolerated. The study results are supportive of the safe use of Glycom's 3-FL at 0.75 g/L in infant formula.

6.6 Allergenicity

3-FL is a high purity ingredient and is specified to contain ≤ 0.01% protein on a w/w basis.

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

Glycom has assessed the allergenic potential of the recombinant proteins introduced to the *E. coli* K-12 host using the search algorithms provided by the Allergen Online tool (ver. 21) of the University of Nebraska (FARRP, 2021). This database has been updated last on 14 February 2021 and contains sequences of 2,233 known and putative allergens. The online tool allows searches by three different search algorithms each with its own alert limit for potential allergenicity: (i) full sequence length (FASTA) comparison with an alert threshold of greater than 50% sequence similarity indicating potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert threshold of greater than 35% sequence identity; and (iii) 8 mer sequence segments (sliding window)[20]. No sequence alerts for potential cross-reactivity to known allergens were identified.

6.7 Other Considerations – Additive Dietary Intakes of 3-FL with Other HiMOs and Resistant Oligosaccharides

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, such as 2'-FL, 2'-FL/DFL, LNT, LNnT, 3'-SL, 6'-SL, and 3-FL will be used in combination to produce infant formula products that are as compositionally representative of human milk as possible, taking into account their natural variation. Glycom recognizes that there are known gastrointestinal tolerance issues that can develop if consumed levels of non-digestible carbohydrates, such as HiMOs or galacto-oligosaccharides (GOS)/FOS, are too high in sensitive populations including infants. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all geographies considering natural variation. While no adverse effects have been reported with the consumption of 3-FL (*i.e.,* the upper tolerable limit has not been identified for either 3-FL alone nor in combination with other non-digestible oligosaccharides), Glycom has selected a value that falls within the 95th percentile of mean values across all developmental stages, has been demonstrated to be well tolerated, and supports the growth and development of infants when formulated into infant formula (Parschat *et al*., 2021; Table 3.1.1.5-1). This approach ensures that the majority of infants will be provided with concentrations that fall within the population means and is well below the upper ranges of concentrations that have been reported for human milk samples, therefore ensuring that the intended uses levels are safe and well tolerated by all infants.

The maximum level of HiMOs used in combination (*i.e.*, an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in samples of mature human milk (Rudloff and Kunz, 2012; Bode, 2013; Xu *et al.,* 2017). In all cases where Glycom's HiMOs will be used in combination with other HiMOs, the total concentrations of HiMOs will fall within conservative means of the general population, thereby ensuring that levels provided will be of nutritional value and be safe and well tolerated. For example, the total quantities of HiMOs that could be theoretically added to infant formula based on existing and future GRAS Notifications for HiMOs manufactured by Glycom or others (*e.g*., 2'-FL, DFL, LNT, LNnT, 3'-SL, 6'-SL, and 3-FL) would be below 5.47 g/L at their maximum GRAS use levels[21]. This concentration is well below the concentrations of total HMOs naturally occurring in human milk samples which are in the region of 12 g/L for mature milk and as high as 25 g/L in colostrum (Rudloff and Kunz, 2012; Bode, 2013; Xu *et al.,* 2017). Therefore, considering that Glycom's HiMOs are identical to their natural

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

[21] Summation of maximum use levels for HiMO's: 2.4 g/L 2'-FL (GRN 650) + 0.6 g/L LNnT (GRN 659) + 0.32 g/L DFL (GRN 815) + 0.8 g/L LNT (GRN 833) (U.S. FDA, 2019) + 0.4 g/L 6'-SL (GRN 881) + 0.2 g/L 3'-SL (GRN 880) + 0.75 g/L 3-FL.

counterparts in human milk, the total amount of HMOs used singly and in combination in infant formula products are not a safety or tolerability concern in infants. In fact, the most sensitive consumer group, infants ages 1 to 4 days, is exposed to the highest concentrations of HMOs, as the early milk "colostrum" contains the highest levels of HMOs, and it is therefore apparent that infants have an inherent high tolerance for these compounds.

Glycom also recognized the possibility that the company's HiMOs may be used in combination with other non-digestible carbohydrate sources such as GOS and FOS, which have GRAS status for use in infant formula. Although Glycom is not a manufacturer of infant formula, and is therefore not in a position to comment on the levels of resistant oligosaccharides such as GOS or FOS that could be used with a HiMO, or even the likelihood that such combinations would be introduced to the market, Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the *Federal Food, Drug, and Cosmetic Act* [21 USC §350(a)]. Specifically, under Section 412(d)(1) of the *Federal Food, Drug, and Cosmetic Act*, a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula. Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination (*e.g.*, use of 3-FL with an indigestible oligosaccharide such as GOS) would be well tolerated as part of the Agency's 90-day notification procedure. Section 412 therefore ensures that any combination of HiMO, whether used singularly or on an additive basis with various HiMOs, will be the subject of corroborative safety and tolerance testing in infants.

The intended use level of 0.75 g/L of 3-FL represents a value that is conservative as it falls below the 95th percentile of mean values across the collective body of published studies covering all stages of lactation (*e.g.*, colostrum, transitional milk, mature and late milk). Additional reassurance of safety derives from the fact that the data analysis to arrive at the 95th percentile was applied on "pooled milk samples" (*i.e.*, across all milk phenotypes), while it is apparent from the published studies that 3-FL occurs at highest levels in the milk group 2. Milk group 2 represents a large proportion of mothers world-wide (approximately 20%) and there is no biological basis to assume that the milk from these mothers would not be considered generally safe for any infant. It is Glycom's view that it is not necessary to calculate a safe/tolerable intake levels of HiMOs for infants by utilizing mg/kg body weight calculations. Since dietary intakes of HiMOs by infants are exclusively provided by infant formula, reference levels for safe intakes should be conducted by comparing target concentrations in infant formula to concentrations in human milk. Accordingly, Glycom did not calculate a tolerable upper level as no concentration of 3-FL in human milk has been reported to be deleterious; however, it seems reasonable to conclude that the upper-range of values that have been reliably reported for human milk samples represents an observed safe level or highest observed intake (HOI) value that can be extrapolated to other population groups[22]. Glycom recognizes that 3-FL is proposed for addition to other foods that may substitute for infant formula as the infants age and that, therefore, there is the possibility that dietary intakes from infant formula and conventional foods will be at least partially additive on occasion. Glycom notes, however, that target concentrations of 3-FL in infant formula relative to background concentrations provide a significant margin of safety that would fall within the typical range

[22] In situations where no evidence of toxicity has been observed in the clinical dataset, derivation of an upper limit is not possible; use of the HOI has been suggested as an alternative. The HOI represents the "*highest intake with adequate data to show, with acceptable confidence, the absence of adverse effects up to that intake.*" [Hathcock and Kriengsinyos (2011)]

experienced by breast-fed infants, and therefore, such sporadic occurrences of added consumption from infant formula and other foods are not a safety or tolerance concern.

6.8 GRAS Panel Evaluation

Glycom has concluded that 3-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of 3-FL, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Professor Emeritus, Virginia Commonwealth University School of Medicine), Dr. George C. Fahey (Professor Emeritus, University of Illinois), and Dr. Ronald Kleinman (Professor, Harvard Medical School).

The GRAS Panel, convened by Glycom, independently and critically evaluated all data and information presented herein, and also concluded that 3-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and the evaluation of such data as it pertains to the proposed GRAS uses of 3-FL, is presented in Appendix B.

6.9 Conclusion

Based on the above data and information presented herein, Glycom has concluded that the intended uses of 3-FL in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, is GRAS based on scientific procedures. General recognition of Glycom's GRAS conclusion is supported by the unanimous consensus rendered by an independent GRAS Panel, qualified by experience and scientific training, to evaluate the use of 3-FL in infant formula and conventional food, who similarly concluded that the intended use of 3-FL in infant formula and conventional food as described herein is GRAS.

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

Part 7. List of Supporting Data and Information

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

Part	Section §	Last Amended	Section Title
101—Food labeling	101.12	4-1-20	Reference amounts customarily consumed per eating occasion
107—Infant formula	107.100	4-1-20	Nutrient specifications
170—Food additives	170.3	4-1-19	Definitions
	170.30	4-1-19	Eligibility for classification as generally recognized as safe (GRAS)

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

17 June 2022

Ellen Anderson
Regulatory Review Scientist
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 001037

Dear Ms. Anderson,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 17 May 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of 3-fucosyllactose (3-FL) filed by the Agency under GRN 001037.

FDA.1. *The intended uses of 3-FL described in the notice include use in non-exempt, infant formula for term infants. Please identify the protein source(s) included in the intended infant formula (e.g., cow milk-based, soy-based, etc.).*

Glycom does not intend to restrict the use of 3-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 3-FL to an infant formula product.

FDA.2. *We note that the intended uses listed in Table 1.3-1 of the notice do not include all previously notified uses of the 3-FL. For example, in GRN 000951, the intended uses of 3-FL included hot and ready-to-eat breakfast cereals, milk substitutes, non-dairy yogurts, and smoothies. Since the estimate of dietary exposure to a notified substance should include exposure from all sources in the diet, please discuss whether the dietary exposure assessment presented in GRN 001037 accounts for these additional uses.*

The exposure assessment in Glycom's GRAS notice (GRN 001037) was finalized in July of 2021, prior to the closure of GRN 000951 on Aug 12, 2021, and therefore did not account for the additional food uses. The dietary exposure assessment has been updated to include exposure from all proposed and previously notified uses of 3-FL. A summary of the individual food uses and use levels evaluated in the updated dietary exposure assessment is provided in Table 1, and results of the assessment are provided in Table 2.

Table 1 Summary of the Individual Proposed and Previously Notified as GRAS[a] Food Uses and Use Levels for 3-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021a)	Food Use*	Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk Meal Replacement and Nutritional Beverages[c]	2.00
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.25
Breakfast Cereals	**Hot Cereals**	**6.80**
	RTE Cereals	**8.80**
Dairy Product Analogs	**Milk Substitutes**	**0.26**
	Non-Dairy Yogurts	**2.64**
Infant and Toddler Foods	Term Infant Formulas	0.75
	Toddler Formulas[d]	2.00
	Other Baby Foods for Infants and Young Children	6.25
	Other Drinks for Young Children	2.00
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	25.00
	Cereal and Nutrition Bars	25.00
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	2.00
Milk Products	Buttermilk	2.00
	Flavored Milk	2.00
	Milk-Based Meal Replacement and Nutritional Beverages[c]	2.50
	Smoothies (Dairy and Non-Dairy)	**1.10**
	Yogurt Drinks, Probiotic Drinks	2.00
	Yogurt	12.50
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.25
	Fruit Juices and Nectars	**0.26**
Processed Vegetables and Vegetable Juices	**Vegetable Juice**	**0.26**
Foods for Special Dietary Use	**Special Dietary Purpose Ingredient in Oral and Enteral Tube Feeding (> 11 years)[e]**	**4.40**

3-FL = 3-fucosyllactose; CFR = Code of Federal Regulations; GRAS = Generally Recognized as Safe; RACC = Reference Amounts Customarily Consumed; RTE = Ready-to-Eat; U.S. = United States.

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

[a] Food uses that have been previously notified as GRAS to the U.S. FDA, and received a "no questions" letter from the Agency, are **bolded**.

[b] Use level expressed on a 3-FL basis in the final food, as consumed.

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

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

[e] Foods for special dietary use are assessed separately from the intended food uses of 3-FL in conventional foods, as they are intended for supplying a particular dietary need and/or supplementing the intake of a dietary component. Intake of 3-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources.

Table 2 summarizes the estimated total intake of 3-FL on an absolute basis (g/person/day) and on a per kilogram body weight basis (mg/kg body weight/day) from all proposed and previously notified food uses in the U.S. population group. The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 76.2% of the population groups consisted of consumers of food products in which 3-FL may be used. Infants aged 7 to <12 months had the greatest proportion of consumers at 100%. The consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of 3-FL were determined to be 1.29 and 2.57 g/person/day, respectively. Of the individual population groups, infants aged 7 to <12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of 3-FL on an absolute basis, at 2.05 and 3.74 g/person/day, respectively. On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of 3-FL were determined to be 22 and 49 mg/kg body weight/day, respectively. Among the individual population groups presented, infants aged 7 to <12 months were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 228 and 443 mg/kg body weight/day, respectively.

Table 2 Summary of the Estimated Daily Intake of 3-FL from Proposed and Previously Notified as GRAS Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	Consumer-Only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 6 m	76.2	139	0.91	1.72	135	250
Infants	7 to <12 m	100	124	2.05	3.74	228	443
Toddlers	1 to 2 y	99.9	306	1.54	2.52	125	215
Children	3 to 11 y	99.1	988	1.31	2.37	52	100
Female Teenagers	12 to 19 y	95.8	429	0.98	1.93	17	37
Male Teenagers	12 to 19 y	97.5	426	1.32	2.45	21	42
Female Adults of Childbearing Age	20 to 40 y	93.1	645	1.11	2.20	15	33
Female Adults	20 to 64 y	92.2	1,511	1.18	2.51	16	35
Male Adults	20 to 64 y	92.7	1,334	1.45	3.05	17	33
Elderly	65 y and older	94.8	991	1.29	2.51	17	35
Total Population	2 y and older	94.2	5,832	1.29	2.57	22	49

3-FL = 3-fucosyllactose; GRAS = Generally Recognized as Safe; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

FDA.3. *The notice includes data from the analysis of eight batches of 3-FL produced with and without the use of a crystallization step. Please clarify whether the batches tested are consecutive or non-consecutive.*

The four non-crystallized batches of 3-FL (GEMO2020_9_1, GEMO2020_13_1, GEMO2020_14_1, and 20193201) are from non-consecutive fermentations. Analytical results from these batches demonstrate the reproducibility of the manufacturing process of 3-FL without the optional crystallization step.

The crystallized 3-FL batches were produced from the same non-crystallized batch of 3-FL (Batch No. 20193201). As indicated above, this batch of non-crystallized 3-FL was demonstrated to be consistent to other batches of non-crystallized 3-FL produced from non-consecutive fermentations. Therefore, analytical results from batches of crystallized 3-FL (GEMO2020_17_1, GEMO2020_18_2, GEMO2020_19_4, and GEMO2020_20_4) demonstrate the reproducibility of the optional crystallization unit operation of the downstream processing.

FDA.4. *Please state whether all analytical methods used to analyze the batches for conformance with the stated specifications have been validated for that particular purpose.*

Glycom utilizes standard methods to evaluate specification parameters for 3-FL where applicable. As an exception, Glycom has developed methods for the analysis of carbohydrates (HPLC-402-4C4-001, HPAEC-HMO-021, HPLC-3FL-002, HPLC-3FL-003), water (KF-001), and residual protein by Bradford assay (UV-001). These methods have been internally validated for the evaluation of the conformance of each batch of 3-FL with specifications for 3-FL. Acetic acid is analyzed using the Megazyme acetic acid assay kit (K-ACETRM), which has been validated by the supplier for the analysis of acetic acid in foodstuffs, beverages and other materials[1].

Glycom noticed an error in the name of the method used for the analysis of acetic acid[2], and proposes to remove the version number of the method from the specification since a newer version number has become available from the supplier[3]. The revised method name proposed for the acetic acid specification for crystallized 3-FL is as follows:

Parameter	Specification	Method
Acetic acid[b]	≤ 1.0 w/w %	Megazyme K-ACETRM

[b] Relevant only for GlyCare™ 3FL 9000 crystallized with acetic acid.

[1] Validation report available at: https://www.megazyme.com/documents/Validation_Report/K-ACETRM_Validation_Report.pdf
[2] Correct spelling is Megazyme K-ACETRM, rather than Megazyme K-ACETTRM.
[3] Current version number is Megazyme K-ACETRM 04/20.

FDA.5. *We note that the results from batch analyses for iron presented in Table 2.3.2.3-1 of the notice include reported levels of <0.1 to <10, in addition to detected levels of 0.5 and 0.6. Please clarify if the results presented with a less-than value are due to the limits of the detection method used and discuss the reason for differences in the reported levels among the batches tested.*

The results of analysis for iron had been determined by two different accredited laboratories for the different batches of 3-FL, explaining the inconsistency of reported levels. The iron content from all batches has since been analyzed by the same laboratory, where the Limit of Quantification (LOQ) is defined as <0.5 mg/kg for iron. The updated results of analysis are provided in Table 3 below.

Table 3 Updated Batch Results for Iron Residues of 3-FL

Parameter	Manufacturing Batch No.							
	Non-Crystallized 3-FL				Crystallized 3-FL			
	GEMO2020_9_1	GEMO2020_13_1	GEMO2020_14_1	20193201	GEMO2020_17_1	GEMO2020_18_2	GEMO2020_19_4	GEMO2020_20_4
Iron (mg/kg)	0.5	< 0.5	< 0.5	< 0.5	0.6	1.0	< 0.5	< 0.5

3-FL = 3-fucosyllactose.

FDA.6. *We note that the description of the composition of the 3-FL test article listed in Table 6.4-1 for the unpublished study does not include the level of "other carbohydrates" as is included for the Dupont and Jennewein test articles. Please confirm the level of "other carbohydrates" for the test article of this study and whether the test article was produced using the optional crystallization step described in Section 2.2.2.*

The test article evaluated in the toxicological studies (Batch No. 20193201) was manufactured without the optional crystallization step. Product analyses (Section 2.3.2 of the notice), precautionary analyses (Section 2.3.3 of the notice), and bulk stability testing (Section 2.4.1 of the notice) were conducted on the same batch of non-crystallized 3-FL.

As indicated in Table 2.3.2-1 of the notice, the level of "other carbohydrates" in the batch of non-crystallized 3-FL evaluated in the toxicological studies (Batch No. 20193201) was 1.76 w/w%.

FDA.7. *On page 9 of the notice, Glycom states, "An overview of the modification events used for construction of strain MDO has been discussed previously and is hereby incorporated by reference to Section II.B.1.2 of GRAS Notice (GRN) 650" but does not summarize the information from Section II.B.1.2 of GRN 000650. As each GRAS notice stands on its own, for the administrative record, please briefly summarize the information incorporated by reference from Section II.B.1.2 of GRN 000650.*

The host strain *Escherichia coli* K-12 DH1 was optimized for general oligosaccharide expression features (used as a "platform host strain") by the introduction of 7 modification events related to the metabolism of various sugars, thereby improving the efficiency of the strain, which was then called MDO. The 7 modifications (deletions of *lacZ, nanKETA, lacA, melA, wcaJ, mdoH* genes and insertion of a Plac promoter upstream the *gmd* gene) that lead from DH1 to MDO are described below:

1. ***lacZ* knockout**. Deletion of the fragment of β-galactosidase gene *lacZ*, which encodes an enzyme that degrades lactose into galactose and glucose. Deletion of the *lacZ* gene prevents that the lactose that is used as a substrate for 3-FL production is hydrolysed to glucose and galactose.
2. **Insertion of Plac promoter.** Insertion of the Plac promoter upstream of the intrinsic GDP-mannose-4,6-dehydratase encoding gene *gmd*, enables an increase of GDP-fucose synthesis under isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. However, due to the further strain improvement (the replacement of the Plac promoter with the PglpF promoter), IPTG is not required (and not used) for 3-FL production.
3. ***nanKETA* knockout**. Deletion of the sialic acid metabolism-related gene cluster *nanKETA*. This modification is not relevant for 3-FL synthesis but makes the derived platform strain suitable for synthesis of sialylated oligosaccharides. The *nanKETA* cluster includes the genes *nanK*, *nanE*, *nanA*, and *nanT*, encoding the enzymes N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, N-acetylneuraminate lyase and the sialic acid transporter *nanT*, respectively.
4. ***lacA* knockout.** Deletion of the galactoside O-acetyltransferase gene *lacA*, which encodes an enzyme that acetylates the galactose residues of oligosaccharides and would thereby lead to increased carbohydrate-type impurities.
5. ***melA* knockout.** Deletion of the β-galactosidase gene *melA*. This modification is not relevant for 3-FL synthesis but makes the derived platform strain suitable for synthesis of α-linked oligosaccharides. As a result of this modification the strain is not able to grow on melibiose [Gal-α(1→6)-Glc].
6. ***wcaJ* knockout.** Deletion of the *wcaJ* gene that encodes the uridine diphosphate (UDP)-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, a lipid carrier transferase involved in colanic acid biosynthesis. Colanic acid is an extracellular polysaccharide containing fucose and its overproduction increases dramatically the viscosity of the culture medium. The *wcaJ* knock-out prevents high culture medium viscosity.
7. ***mdoH* knockout.** Deletion of the glucans biosynthesis glucosyltransferase H gene *mdoH*. The enzyme encoded by this gene is involved in the biosynthesis of periplasmic glucans, the presence of which would complicate the isolation and purification of other targeted oligosaccharides to be expressed by the strain.

The resulting strain was called *E. coli* K-12 DH1 MDO and constitutes a general HiMO "platform strain" for the generation of MDO-derived specific strains for fermentative synthesis of diverse oligosaccharides. The complete genotype of the HiMO production platform strain MDO is: F^-, λ^-, *endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17 lacZ-wcaF::Plac nanKETA lacA melA wcaJ mdo*. All HiMO production strains, including strain MAP1834, originate from the HiMO-platform strain MDO.

FDA.8. *In the notice, Glycom states, "Recent comparison of sequenced E. coli genomes shows that K-12 and its closely related "safety strains" possess 10 to 20% fewer genes than their pathogenic cousins … E. coli K-12-derived strains cannot colonize the human gastrointestinal system, and do not produce protein-type toxins" and "The safety of the production organism is based upon the long-history of safe use of E. coli K-12 in food production and, to date, eight HiMO ingredients produced by Glycom using the company's platform strain and similar downstream processing methods have been evaluated in toxicity studies in neonatal rats, and in in vitro genotoxicity assays without evidence of toxicity or genotoxic potential" (pages 8 and 44, respectively). For the administrative record, please state whether the production strain, Escherichia coli K-12 DH1 MDO strain "MAP1834", is non-pathogenic and non-toxigenic.*

The 3-FL production strain, *Escherichia coli* K-12 DH1 MDO MAP1834, is non-pathogenic and non-toxigenic.

FDA.9. *On page 9 of the notice, Glycom states, "The production strain is a genomically stable microorganism that provides high titers of 3-FL." For the administrative record, please briefly describe how stability of the production strain is ensured.*

The genomic stability of *Escherichia coli* K-12 DH1 MDO MAP1834 has been confirmed through whole genome sequencing and by the assessment of phenotypic stability in fermentation performance tests. Genetic modifications in the 3-FL production strain accumulated no mutations and were steadily inherited over more than 50 generations. In parallel, the fermentation performance tests on initial and aged production cell cultures showed that phenotypic titers and growth rates are stable over time.

FDA.10. *For the administrative record, please state whether E. coli K-12 DH1 MDO strain "MAP1834" is capable of DNA transfer to other organisms.*

The 3-FL production strain *Escherichia coli* K-12 DH1 MDO MAP1834 is derived from *E. coli* lineage K-12 DH1 which has F^-, λ^- genotype (Hanahan, 1983; Papadakis and Pedersen, 2021), thus, it is not capable of DNA transfer to other organisms via horizontal gene transfer. Lack of the conjugative F-plasmid prevents the spread of mobile genetic elements (plasmids and transposons) between bacteria *via* conjugation (Adelberg and Burns, 1960; Babic *et al.,* 2008). Secondly, the λ^- genotype indicates the absence of the temperate λ bacteriophage (Bachman, 1996), thus there is no risk of the DNA transfer *via* transduction.

FDA.11. *On page 9 of the notice, Glycom states, "The production strain … has been deposited in the DSMZ in Braunschweig, Germany." Please provide the deposit designation for E. coli K-12 DH1 MDO strain "MAP1834".*

The 3-FL production strain *Escherichia coli* K-12 DH1 MDO MAP1834 has been deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the deposition number DSM 33416.

FDA.12. *For the administrative record, please briefly specify how the purity of E. coli K-12 DH1 MDO strain "MAP1834" is ensured and state whether the fermentation process is conducted in a contained, sterile environment.*

Glycom applies a two-tiered cell banking system, in which a master cell bank (MCB) is produced from the initial cell clone (ICC) and used to generate working cell banks (WCB), which in turn are used to start any manufacturing batch. Such a cell banking system is generally accepted as the most practical approach to providing sufficient quantities of cells for continued manufacture of the product. The concept has been described by the ICH/FDA guidance "Q5D Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products" (EMEA, 1998; U.S. FDA, 1998). Performance qualification of the ICCs, MCBs and WCBs is carried out at Glycom R&D by running standardised head-to-head lab-scale fermentation processes against an appropriate control, confirming productivity, quality, purity, identity, and stability.

The production strain *Escherichia coli* K-12 DH1 MDO MAP1834 is used in compliance with Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. A compliance certificate is issued by the Danish Environmental Protection Agency (EPA) pursuant of Section 10(1) of the Danish EPA's Executive Order No. 225 of 19 March 2009.

FDA.13. *On page 14 of the notice, Glycom states, "Additional microbiological limits are established for 3-FL that is added during the dry blend stage and include the following (in addition to the parameters listed above): Cronobacter spp.: absent in 10 g; Listeria monocytogenes: absent in 25 g; Bacillus cereus: = 50 CFU/g." Please provide the complete citations for the analytical methods used to analyze for the presence of these three additional microbiological limits.*

3-FL is intended to be added to both liquid and powdered infant formulae. Updated specifications for 3-FL are provided below in Table 4 below, which include specifications for *Cronobacter spp.*, *Listeria monocytogenes*, and *Bacillus cereus*, as well as analytical methods for the analysis of the presence of these three additional microbiological parameters, for 3-FL intended for addition at the dry blending stage of infant formula manufacture.

Table 4 Specifications for 3-FL

Description		
GlyCare™ 3-FL 9000 and 9001 is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 87% of 3-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Color	White, white to off-white, off-white	ISO 6658
Identification by Retention Time	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-402-4C4-001
Assay (water-free) – Specified saccharides[a]	≥ 92.0 w/w %	Glycom method HPLC-402-4C4-001, HPAEC-HMO-021, HPLC-3FL-002 or HPLC-3FL-003

Table 4 Specifications for 3-FL

Description		
GlyCare™ 3-FL 9000 and 9001 is a purified carbohydrate powder or agglomerates obtained from microbial fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1 containing at least 87% of 3-fucosyllactose of dry matter		
Parameter	**Specification**	**Method**
Assay (water-free) – 3-Fucosyllactose	≥ 87.0 w/w %	Glycom method HPLC-402-4C4-001
L-Fucose	≤ 1.0 w/w %	Glycom method HPLC-3FL-002 or HPLC-3FL-003
D-Lactose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021
3-Fucosyl-lactulose	≤ 1.5 w/w %	Glycom method HPAEC-HMO-021
Sum of other carbohydrates	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021 + HPLC-3FL-003
pH in 5% solution (20°C)	3.2 – 6.0	Ph. Eur. 9.2 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-001
Ash, sulphated	≤ 0.5 w/w %	Ph. Eur. 9.2 2.4.14
Acetic acid[b]	≤ 1.0 w/w %	Megazyme K-ACETTRM 07/12
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Ph. Eur 2.6.14
Lead	≤ 0.1 mg/kg	EN 13805; EPA-6020A
Microbiological specifications		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	≤ 10 CFU/g	ISO 21528-2 or NMKL 144
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter (Enterobacter) sakazakii[c]	Absent in 10 g	ISO 22964
Listeria monocytogenes[c]	Absent in 25 g	ISO 11290-1
Bacillus cereus[c]	≤ 50 CFU/g	ISO 7932
Yeasts	≤ 100 CFU/g	ISO 21527-2
Molds	≤ 100 CFU/g	ISO 21527-2

3-FL = 3-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EPA = Environmental Protection Agency; E.U. = endotoxin units; HMO = human milk oligosaccharide HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; NMKL = Nordic Committee on Food Analysis; Ph. Eur. = European Pharmacopeia; RT = retention time; UV = ultraviolet.

[a] Specified saccharides include 3-fucosyllactose, D-lactose, L-fucose, and 3-fucosyl-lactulose.

[b] Relevant only for GlyCare™ 3FL 9000 crystallized with acetic acid.

[c] Applicable to 3-FL that is added during the dry-blending stage of infant formula manufacturing only.

FDA.14. *Please state whether any of the raw materials used in the fermentation are major allergens or are derived from major allergens. If any of the raw materials used are major allergens or are derived from major allergens, please discuss why these materials do not pose a safety concern.*

Glycom's 3-FL is produced using lactose derived from cow's milk. Therefore, the lactose substrate may contain traces of cow milk protein, which could theoretically be carried over in the final 3-FL ingredient.

However, any residual cow milk protein stemming from the lactose raw material is significantly diluted during the growth phase of the fermentation and most importantly all types of protein are removed at multiple steps during the manufacturing process of 3-FL including ultrafiltration, ion removal,

decolourization, and optional crystallization. The method used to measure residual protein in batches of 3-FL is the Bradford assay, which is a total protein method that doesn't allow any differentiation of the type of protein in regards to potential allergenicity. Although milk protein is not expected to be present in 3-FL at detectable levels, 3-FL produced from milk-derived lactose would be labelled as "contains milk" in accordance with requirements of the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA).

FDA.15. *On page 22 of the notice, Glycom states, "As a precautionary measure, production batches have been analyzed for secondary metabolites and cellular components that may potentially originate from the fermentation medium." For the administrative record, please clarify whether the production strain produces any secondary metabolites.*

Glycom interprets the term secondary metabolites as substances other than 3-FL that may be produced by the production strain during fermentation. Glycom has established internal quality control measures to monitor for secondary metabolites, and only those that have been confirmed to occur at detectable levels in the final 3-FL product are included in the specification for 3-FL. Most notably, these consist of carbohydrate-type products resulting from the biosynthesis of 3-FL by the production strain, including sialic acid, 3'-sialyl-lactulose, and other minor carbohydrates (specified as 'sum of other carbohydrates'). No significant detectable levels of other secondary metabolites potentially produced during fermentation (including biogenic amines and amino acids) have been identified in the finished 3-FL ingredient.

Moreover, Glycom accounts for Cytolysin A (HlyE) a protein-type cytotoxin, and lipopolysaccharide (LPS, endotoxin) an immunogenic pyrogen. Although these are not metabolites resulting from the biosynthesis of 3-FL, these compounds have been identified as bioactive secondary metabolites of *Escherichia coli* K-12 by EFSA. The concern for these secondary metabolites was highlighted in the publication: "Database on the taxonomical characterization and potential toxigenic capacities of microorganisms used for the industrial production of food enzymes and feed additives, which do not have a recommendation for Qualified Presumption of Safety" (EFSA, 2017). Out of the 474 bioactive secondary metabolites, 59 compounds were selected and examined for toxicology. Two of the 59 compounds are produced by *Escherichia coli* K-12, namely Cytolysin A (HlyE), and lipopolysaccharide (LPS, endotoxin). Cytolysin A is a pore-forming toxin known to cause lysis of mammalian cells. Under laboratory conditions, the Cytolysin A encoding gene, *hlyE*, appears to be silent (del Castillo *et al.,* 1997). To eliminate the risk of the activation of Cytolysin A production, Glycom has deleted the *hlyE* gene in the HiMO production strains. Lipopolysaccharides are a major component of the outer membrane of gram-negative bacteria. Consequently, Glycom allows no more than 10 E.U./mg residual endotoxin in the final 3-FL product.

FDA.16. *For completeness of the administrative record, please briefly describe the antibiotic resistance profile of E. coli K-12 DH1 MDO strain "MAP1834". Additionally, please briefly describe whether the production strain produces any antimicrobials and whether that impacts the safety conclusion.*

The genome of *E. coli* K-12 DH1 MDO strain MAP1834 has been assessed *in silico* for the presence of antimicrobial resistance genes using the Comprehensive Antibiotic Resistance Database (CARD; version 3.0.6), ResFinder (version 18-Feb-2020), and ARG-ANNOT (version 6), according to EFSA guidance (EFSA,

2018; EFSA NDA Panel, 2021). No acquired antimicrobial resistance genes resulting from the genetic modifications were identified.

Phenotypic antimicrobial susceptibility testing was also performed on the 3-FL production strain according to the requirements of the above EFSA guidance for phenotypic testing of Enterobacteriaceae bacteria. The minimum inhibitory concentration (MIC) results confirmed the susceptibility of *E. coli* K-12 DH1 MDO MAP1834 strain to all tested antimicrobials: ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, ciprofloxacin, colistin, fosfomycin, which are considered critically or highly important antimicrobials by WHO-AGISAR (2016).

As indicated in Section 2.2.1.1 of the GRAS notice, *E. coli* K-12 and its derivatives have been specifically developed and recognized as "safety strains", are among the preferred microorganisms for industrial biotechnology with wide application scope, and are used in the manufacture of several GRAS ingredients and food enzymes have been authorized in the U.S. A comprehensive safety assessment of *E. coli* K-12 and its derivatives has been conducted by the U.S. Environmental Protection Agency (EPA) and it was concluded that no negative effects to human health or environment have been documented (U.S. EPA, 1997). Although *E. coli* does not qualify for Qualified Presumption of Safety (QPS) status in the EU, Glycom's HiMO production strains derived from *E. coli* K-12 have not been subject to the characterization of antimicrobial activity according to EFSA (2018) under the presumption that they belong to a taxonomic unit known not to produce antimicrobials relevant to use in humans and animals. Furthermore, none of the genetic modifications applied to generate the production strain would result in antimicrobial production.

FDA.17. *On page 62 of the notice, Glycom states, "The intended use level of 0.75 g/L of 3-FL represents a value that is conservative as it falls below the 95th percentile of mean values across the collective body of published studies covering all stages of lactation (e.g., colostrum, transitional milk, mature, and late milk)." Please provide a further clarification of this statement by discussing the intended use level of 0.75 g/L within the context of the "body of published studies" as described in the statement. As part of this discussion, please provide the means and the upper limit of the 95% confidence interval for the means from these published studies. Please also note that it is unclear what aspect of "Appendix A: Comprehensive Report on the Quantitative Data for 3-FL in Human Milk" included in the GRAS notice would satisfy the standard of general recognition given that the method and data analysis have not been subject to peer review.*

In deriving the intended use level of 0.75 g/L for 3-FL, Glycom extracted and analyzed quantitative data relating to the distribution of 3-FL concentrations in human milk from a large number of individual published studies. In the GRAS notice, Glycom also took into account the level of 3-FL evaluated in the infant clinical trial by Parschat *et al*., 2021, where supplementation of up to 0.75 g/L of 3-FL from the 5HMO-Mix in infant formula during the first 4 months of life was reported to support normal infant growth and was safe and well-tolerated. The safe use of Glycom's 3-FL at 0.75 g/L in infant formula is further supported by a Scientific Opinion adopted by the European Food Safety Authority (EFSA) where it was concluded that the use of 3-FL in infant formula at a higher use level of 0.85 g/L is safe as it does not exceed the highest intake level of 3-FL in breastfed infants (EFSA, 2021). In fact, more recently, EFSA evaluated and concluded on the safety of 3-FL for addition at a maximum proposed use level of 0.9 g/L in infant formula (EFSA, 2022). Taken together, these data establish the general recognition of safety for the use 3-FL in infant formula at an intended use level of 0.75 g/L.

Specifically relating to Glycom's analysis of the distribution of 3-FL concentrations in human milk, the results of analysis for the mean of the 95 % confidence limit (CL)[4] by lactation stage across studies is reproduced in Table 5 below for ease of reference, whereby the intended use level of 3-FL in infant formula at 0.75 g/L is below the mean of the 95 % CL for each lactation stage. Glycom notes that there was slight error in reported means of the 95 % CL for mature milk (from Days 15 to 90 and Days 90 to 180), which have been corrected in Table 5 below, but result in the same conclusion.

Table 5 The 3-FL Concentrations in Human Milk over the Course of Lactation

Lactation Time	Mean of the 95 % CL (g/L)
Days 1 to 5 ("colostrum")	0.95
Day 6 to 14 ("transitional milk")	1.44
Days 15 to 90 ("mature milk")	1.56[a]
Days 90 to 180 ("mature milk")	2.05[a]
After 6 months ("late milk")	2.91

3-FL = 3-fucosyllactose; CL = confidence limit.

[a] Corrected value.

Means and upper limits of 95 % confidence intervals from individual published studies are provided by lactation stage in the tables that follow.

[4] 3-FL occurs in the milk of all mothers (all milk phenotypes). Many of the individual publications that evaluated the concentration of HMOs (including 3-FL) in human milk reported mean levels and standard deviations across samples from different mothers. Assuming a normal (Gauss) distribution of the levels of 3-FL in human milk, this allows to calculate for each such study the confidence interval into which the concentration of 3-FL from 95 % of samples fall, and to derive the mean of the upper limits of 95 % confidence intervals (95 % CL) across the different studies.

Days 1 to 5 ("Colostrum")

#	First author	Journal	Year	Number of sampled donors	Regions	Mean (or median*)	SD	95 % CL
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	0.34	0.06	0.46
7	Erney	JPGN	2000	381	Asia	0.78	n.a.	n.a.
10	Chaturvedi	Glycobiol.	2001	84	N. Amer.	0.30	n.a.	n.a.
13	Sumiyoshi	Br. J. Nutr.	2003	16	Asia	0.23	n.a.	n.a.
18	Sjögren	Pediatr. Allergy Immunol.	2007	20	Europe	0.11*	n.a.	n.a.
19	Asakuma	Eur. J. Clin. Nutr.	2008	12	Asia	0.25	0.18	0.60
22	Thurl	Br. J. Nutr.	2010	30	Europe	0.24	n.a.	n.a.
25	Gabrielli	Pediatr.	2011	63	Europe	1.17	0.63	2.43
33	Van Niekerk	J. Nutr.	2014	82	Africa	0.04	0.02	0.08
37	Spevacek	J. Nutr.	2015	25	N. Amer.	0.46	0.44	1.35
39	Aakko	Benef. Microbes	2017	11	Europe	0.05	0.03	0.12
46	Ma	Int. Diary J.	2018	46	Asia	0.43	0.42	1.27
49	Huang	J. Agric. Food Chem.	2019	33	Asia	0.35	0.31	0.96
53	Samuel	Scientific Rep.	2019	290	Europe	0.42	0.45	1.33
58	Ferreira	Nutrients	2020	147	LATAM	0.15*	n.a.	n.a.
63	Torres Roldan	Am. J. Clin. Nutr.	2020	55	LATAM	0.21*	n.a.	n.a.
65	Wu	Curr. Dev. Nutr.	2020	59	Asia	0.43	n.a.	n.a.
					MEAN	**0.35**	-	**0.95**
					MIN	**0.04**	-	**0.08**
					MAX	**1.17**	-	**2.43**
					COUNT	**17**	-	**9**

* = median; n.a. = not available; SD = standard deviation.

Days 6 to 14 ("Transitional Milk")

#	First author	Journal	Year	Number of sampled donors	Regions	Mean	SD	95 % CL
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	0.22	0.06	0.34
7	Erney	JPGN	2000	381	Asia	1.02	0.30	1.61
10	Chaturvedi	Glycobiol.	2001	84	N. Amer.	0.40	n.a.	n.a.
13	Sumiyoshi	Br. J. Nutr.	2003	16	Asia	0.28	n.a.	n.a.
22	Thurl	Br. J. Nutr.	2010	30	Europe	0.26	n.a.	n.a.
25	Gabrielli	Pediatr.	2011	63	Europe	1.40	0.94	3.27
37	Spevacek	J. Nutr.	2015	25	N. Amer.	0.59	0.45	1.48
38	Austin	Nutrients	2016	446	Asia	0.50	0.60	1.70
46	Ma	Int. Diary J.	2018	46	Asia	0.54	0.50	1.55
48	Austin	Nutrients	2019	500	Europe	0.36	0.26	0.88
49	Huang	J. Agric. Food Chem.	2019	33	Asia	0.48	0.40	1.27
51	McJarrow	Nutrients	2019	41	Asia	0.80	0.37	1.53
57	Borewicz	Scientific Rep.	2020	24	Europe	0.52	0.39	0.78
65	Wu	Curr. Dev. Nutr.	2020	59	Asia	0.42	n.a.	n.a.
					MEAN	**0.56**	**0.43**	**1.44**
					MIN	**0.22**	**0.06**	**0.34**
					MAX	**1.40**	**0.94**	**3.27**
					COUNT	**14**	**10**	**10**

n.a. = not available; SD = standard deviation.

Days 15 to 90 ("Mature Milk")

#	First author	Journal	Year	Number of sampled donors	Regions	Mean (or median*)	SD	95 % CL
3	Chaturvedi	Anal. Biochem.	1997	50	LATAM	0.36	n.a.	n.a.
4	Coppa	Acta Paediatr. Suppl.	1999	18	Europe	0.51	0.16	0.83
5	Kunz	Br. J. Nutr.	1999	10	Europe	0.07	0.08	0.23
7	Erney	JPGN	2000	381	Asia	1.04	0.50	2.04
10	Chaturvedi	Glycobiol.	2001	84	N. Amer.	0.63	n.a.	n.a.
13	Sumiyoshi	Br. J. Nutr.	2003	16	Asia	0.43	n.a.	n.a.
22	Thurl	Br. J. Nutr.	2010	30	Europe	0.49	0.12	0.73
24	Coppa	JPGN	2011	39	Europe	0.38	0.11	0.59
25	Gabrielli	Pediatr.	2011	63	Europe	1.22	0.77	2.76
28	Smilowitz	J. Nutr.	2013	52	N. Amer.	1.23	0.52	2.26
33	Van Niekerk	J. Nutr.	2014	82	Africa	0.04	0.02	0.08
34	Alderete	Am. J. Clin. Nutr.	2015	25	N. Amer.	0.16*	n.a.	n.a.
37	Spevacek	J. Nutr.	2015	25	N. Amer.	0.87	0.67	2.20
38	Austin	Nutrients	2016	446	Asia	0.65	0.52	1.68
46	Ma	Int. Diary J.	2018	46	Asia	1.04	0.78	2.61
48	Austin	Nutrients	2019	500	Europe	0.64	0.35	1.34
49	Huang	J. Agric. Food Chem.	2019	33	Asia	0.73	0.55	1.82
53	Samuel	Scientific Rep.	2019	290	Europe	0.85	0.66	2.17
55	Tonon	Nutrients	2019	77	LATAM	0.82	0.44	1.71
57	Borewicz	Scientific Rep.	2020	24	Europe	0.89	0.53	1.94
58	Ferreira	Nutrients	2020	147	LATAM	0.18*	n.a.	n.a.
60	Lefebvre	Front. Nutr.	2020	156	Europe	1.77	n.a.	n.a.
61	Lagström	Am. J. Clin. Nutr.	2020	802	Europe	0.14*	n.a.	n.a.
62	Saben	Nutrients	2020	136	N. Amer.	1.87*	n.a.	n.a.
63	Torres Roldan	Am. J. Clin. Nutr.	2020	55	LATAM	0.20*	n.a.	n.a.
65	Wu	Curr. Dev. Nutr.	2020	59	Asia	0.76	n.a.	n.a.
					MEAN	**0.69**	-	**1.56**
					MIN	**0.04**	-	**0.08**
					MAX	**1.87**	-	**2.76**
					COUNT	**26**	-	**17**

* = median; n.a. = not available; SD = standard deviation.

Days 90 to 180 ("Mature Milk")

#	First author	Journal	Year	Number of sampled donors	Regions	Mean (or median*)	SD	95 % CL
10	**Chaturvedi**	**Glycobiol.**	**2001**	**84**	**N. Amer.**	1.18	n.a.	n.a.
13	**Sumiyoshi**	**Br. J. Nutr.**	**2003**	**16**	**Asia**	0.45	n.a.	n.a.
34	**Alderete**	**Am. J. Clin. Nutr.**	**2015**	**25**	**N. Amer.**	0.52*	n.a.	n.a.
43	**Williams**	**J. Human Lact.**	**2017**	**16**	**N. Amer.**	0.10	n.a.	n.a.
44	**Azad**	**J. Nutr.**	**2018**	**427**	**N. Amer.**	0.24	0.15	0.55
46	**Ma**	**Int. Diary J.**	**2018**	**46**	**Asia**	1.35	0.85	3.05
48	**Austin**	**Nutrients**	**2019**	**500**	**Europe**	1.02	0.55	2.11
50	**Larsson**	**Front Pediatr.**	**2019**	**22**	**Europe**	0.23*	n.a.	n.a.
51	**McJarrow**	**Nutrients**	**2019**	**41**	**Asia**	1.47	0.21	1.88
53	**Samuel**	**Scientific Rep.**	**2019**	**290**	**Europe**	1.21	0.72	2.64
58	**Ferreira**	**Nutrients**	**2020**	**147**	**LATAM**	1.01*	n.a.	n.a.
60	**Lefebvre**	**Front. Nutr.**	**2020**	**156**	**Europe**	2.26	n.a.	n.a.
					MEAN	**0.92**	**-**	**2.05**
					MIN	**0.10**	**-**	**0.55**
					MAX	**2.26**	**-**	**3.05**
					COUNT	**12**	**-**	**7**

* = median; n.a. = not available; SD = standard deviation.

After 6 Months ("Late Milk")

#	First author	Journal	Year	Number of sampled donors	Regions	Mean (or median*)	SD	95 % CL
10	**Chaturvedi**	**Glycobiol.**	**2001**	**84**	**N. Amer.**	1.20	n.a.	n.a.
46	**Ma**	**Int. Diary J.**	**2018**	**46**	**Asia**	1.36	0.77	2.91
50	**Larsson**	**Front Pediatr.**	**2019**	**22**	**Europe**	0.33*	n.a.	n.a.
60	**Lefebvre**	**Front. Nutr.**	**2020**	**156**	**Europe**	2.40	n.a.	n.a.
					MEAN	**1.32**	**0.77**	**2.91**
					MIN	**0.33**	**0.77**	**2.91**
					MAX	**2.40**	**0.77**	**2.91**
					COUNT	**4**	**1**	**1**

* = median; n.a. = not available; SD = standard deviation.

FDA.18. *On page 62 of the notice, Glycom states, "It is Glycom's view that it is not necessary to calculate a safe/tolerable intake levels [sic] of HiMOs for infants by utilizing mg/kg body weight calculations. Since dietary intakes of HiMOs by infants are exclusively provided by infant formula, reference levels for safe intakes should be conducted by comparing target concentrations in infant formula to concentrations in human milk. Accordingly, Glycom did not calculate a tolerable upper level as no concentration of 3-FL in human milk has been reported to be deleterious; however, it seems reasonable to conclude that the upper-range of values that have been reliably reported for human milk samples represents an observed safe level or highest observed intake (HOI) value that can be extrapolated to other population groups.[22]"*

A) *We note that the concentration of 3-FL in human milk increases throughout the course of lactation (Thurl et al., 2017; Soyyilmaz et al., 2021)[1] whereas consumption of infant formula provides a constant concentration of 3-FL throughout infancy. If reference levels for safe intakes of 3-FL should be derived from concentrations in human milk as Glycom states, please provide a discussion that explains why a constant level of 3-FL at 0.75 g/L is safe and tolerable for all infants (0-12 months) given that younger infants consume a lower concentration of 3-FL in human milk than do older infants. We note that the intended use level of 0.75 g/L is above the upper limit of the 95% confidence interval of mean values for lactation days 0-30 as reported by Thurl et al., 2017 (Table S2-A) and above the upper limit of the 95% confidence interval of mean values in colostrum, transitional, and mature milk reported by Soyyilmaz et al., 2021 (Table S2).[2]*

B) *Table 1.6.3-1 (Highest Intakes of 3-FL from Breast Milk) in Appendix A (page 28) of the GRAS notice appears inconsistent with Glycom's assertion that a tolerable upper level of 3-FL intake on a body weight basis was not calculated. Please provide a statement that reconciles the purpose of Table 1.6.3-1 with Glycom's statements above. If Glycom does intend to calculate a safe/tolerable intake level for 3-FL on a body weight basis, please discuss why Glycom utilizes values from EFSA for intakes of breast milk (i.e., 800 mL and 1200 mL) and infant body weight (6.7 kg), but selects reference values for 3-FL in human milk which are much higher than what is utilized by EFSA to calculate daily infant intakes of 3-FL (EFSA, 2021).[3]*

C) *Please provide a more detailed discussion regarding the use of the Highest Observed Intake (HOI)/Observed Safe Level (OSL) as a generally recognized methodology to assess the safety of 3-FL in infant formula. As part of this discussion, please address the following points:*

   i. *Please indicate if the HOI/OSL methodology has been utilized by other regulatory or scientific bodies as an approach to assess the safe intake of 3-FL by infants consuming formula as sole source nutrition.*

   ii. *Hathcock and Kriengsinyos 2011 note, "The HOI is not literally the highest observed intake, but is the highest with adequate data to support safety." Given that 3-FL concentrations in human milk increase throughout lactation, please explain how HOI/OSL can be used to appropriately extrapolate a safe level and dietary exposure to all infants expected to consume infant formula.*

   iii. *Please provide more precise definitions for the following terms: "upper range of values", "reliably reported", and "other population groups".*

Part A)

Indeed, both Thurl *et al*. (2017) and Soyyilmaz *et al*. (2021) reported that the concentration of 3-FL in human milk increases throughout the course of lactation, where the concentration of 3-FL was observed to increase by approximately 2-fold from colostrum to 3-4 months of lactation. Nevertheless, when considering the full distribution of 3-FL concentrations in human milk by lactation stage, the intended use level of 0.75 g/L in infant formula is within the range of 3-FL concentrations reported to occur in early milk. For example, based on the results of Glycom's analysis (see Table 1.6.4-1 of Appendix A of the GRAS notice), the intended use level of 3-FL of 0.75 g/L in infant formula is within the range of mean concentrations of 3-FL that have been reported for each lactation stage, and below the mean of the 95 % CL and upper ranges. Values are reproduced in Table 6 below for ease of reference.

Table 6 Distribution of 3-FL Concentrations in Human Milk over the Course of Lactation

Parameter	3-FL Concentration (g/L)				
	Colostrum (<5 days)	Transitional (6-14 days)	Mature (15-90 days)	Mature (90-180 days)	Late (> 6 months)
Range of means	0.04-1.17	0.22-1.40	0.04-1.87	0.1-2.26	0.33-2.40
Mean of 95 % CL	0.95	1.44	1.56[a]	2.05[a]	2.91
Mean of Upper Ranges	1.22	1.58	3.00	3.14	3.80
Max of Upper Range	2.47	2.80	5.72	6.03	4.32

3-FL = 3-fucosyllactose; CL = confidence limit.

[a] Corrected value.

The long history of wet nursing has also established that milk of different mothers, and from different lactation stages, is safe between infants.

Summary statistics reported by Glycom are generally higher than those reported by Thurl *et al*. (2017) and Soyyilmaz *et al*. (2021) for two reasons:

- **Type of summary statistic:** Glycom's analysis considered the variability of 3-FL concentrations in milk samples from individual publications, whereas Thurl *et al*. (2017) and Soyyilmaz *et al*. (2021) focused solely on mean concentrations of 3-FL from individual publications. More specifically, Thurl *et al*. (2017) reported the 95 % CL of the mean across publications, whereas Soyyilmaz *et al*. (2021) reported the mean of means across publications. Notably, the objective of the review by Soyyilmaz *et al*. (2021) was to determine a ranking of individual HMOs by global means across different milk phenotypes, regardless of individual variations.

- **Types of studies reporting on 3-FL levels in human milk included in the analysis:** Glycom's analysis included all milk phenotypes, whereas Thurl *et al*. (2017) reported 3-FL concentrations from Secretor milk only and Soyyilmaz *et al*. (2021) reported 3-FL concentrations from pooled milk only. As indicated in the GRAS notice, 3-FL occurs at highest concentration in the milk phenotype 2 of mothers that are FUT2-negative (non-Secretor phenotype) but FUT3-positive (Lewis phenotype). Furthermore, Glycom's analysis considered both term and preterm studies. Although Thurl *et al*. (2017) considered term and preterm studies for summary statistics reported throughout all lactation periods (see Table 3 of the publication), only term studies were considered for results reported by individual lactation period (see Table S2-A of the publication). Soyyilmaz *et al*. (2021) did not include preterm milk in their approach. As preterm

infants are more vulnerable than term infants, Glycom considers concentrations of 3-FL from preterm human milk to also be safe for term infants.

Summary statistics on concentrations of 3-FL in human milk as well as methodological differences between Glycom's analysis and reviews conducted by Thurl *et al*. (2017) and Soyyilmaz *et al*. (2021) are summarized in Table 7 below.

Table 7 Comparison of Summary Statistics on Concentrations of 3-FL in Human Milk

Parameter	Method	Milk Phenotype and Length of Gestation	3-FL Concentration (g/L)				
			Colostrum (<5 days)	Transitional (6-14 days)	Mature (15-90 days)	Mature (90-180 days)	Late (> 6 months)
GRAS Notice (Appendix A)	Mean of the 95 % CL	All milk phenotypes; Mothers delivering term and preterm infants	0.95	1.44	1.60	1.87	2.91
Soyyilmaz *et al*. (2021)	Mean of means	Pooled milk; Mothers delivering term infants	0.38	0.61	0.74	0.95	
Thurl *et al*. (2017)	CL of the mean	Secretor milk; Mothers delivering term infants	0.34	~0.38-0.43	~0.43-0.78	~0.78-1.44	

3-FL = 3-fucosyllactose; CL = confidence limit; GRAS = Generally Recognized as Safe.

Part B)

The purpose of Table 1.6.3-1 in Appendix A of the GRAS notice was to provide a reference frame of the highest anticipated daily intakes of 3-FL by breastfed infants as an initial comparison to estimated daily intakes of 3-FL from the proposed conditions of use for all population groups (Table 3.2.2-1 of the GRAS notice). It was never Glycom's intention to calculate a tolerable upper intake level (UL) of 3-FL, as the potential risk of adverse effects above levels of exposure in breastfed infants remains largely unknown. Rather, exposure to 3-FL by breastfed infants, a vulnerable population group, serves as the safe reference range of 3-FL intake from the proposed conditions of use for all population groups.

Part C)

As indicated in response to Question 17, general recognition of safety of the intended use of Glycom's 3-FL at a level of 0.75 g/L in infant formula is based on the distribution of 3-FL concentrations in human milk from a large number of individual published studies, and by levels of 3-FL determined to be safe in a recent clinical trial (0.75 g/L from 5HMO-Mix in infant formula – Parschat *et al*., 2021) and by EFSA for approval in the EU (0.85 g/L in infant formula – EFSA, 2021; EU, 2021).

Specifically relating to Glycom's analysis of the distribution of 3-FL concentrations in human milk, the proposed use level of 3-FL in infant formula (0.75 g/L) is within the range of mean concentrations of 3-FL in human milk reported from individual published studies and below the mean of the 95 % CL calculated for each lactation stage (see response to Part a). Moreover, the proposed use level of 0.75 g/L in infant formula is well-below upper ranges of 3-FL that has been reported to naturally occur in human milk for any of the lactation stages (see Table 1.6.4-1 of Appendix A of the GRAS notice).

Responses to specific points are provided below.

i) In their Scientific Opinions for 3-FL, EFSA evaluated the anticipated daily intake of 3-FL from the consumptions of infant formula only against the highest intake level of 3-FL in breastfed infants on a body weight basis (EFSA, 2021, 2022). They also considered the anticipated daily intake of 3-FL from all proposed conditions of use in population groups from EU dietary surveys against the highest intake level of 3-FL in breastfed infants on a body weight basis, stating that the intake in breastfed infants is expected to be safe also for other population groups. In order to derive the highest intake level of 3-FL in breastfed infants, the Panel considered in their 2021 assessment the highest 95 % CL of the mean from any lactation stage reported for 3-FL by Thurl et al. (2017), while the maximum mean concentration of 3-FL reported by Buket *et al*. (2021) was considered in the Panel's 2022 assessment. As a result, 3-FL was determined to be safe under the proposed conditions of use in both scientific opinions [maximum use level in infant formula of 0.85 g/L (EFSA, 2021) and 0.9 g/L (EFSA, 2022)].

ii) Based on the definition of the HOI by Hathcock and Kriengsinyos (2011), Glycom agrees that it is not appropriate to designate the highest reported occurrence levels of 3-FL in human milk as the HOI. The intention was to indicate that there are currently no data suggesting that exposure to 3-FL at concentrations above the mean range reported in human milk is unsafe in breastfed infants.

iii) Definitions are as follows:

- **Upper range of values:** the range of maximum concentrations of 3-FL that have been reported for individual human milk samples from studies included in Glycom's analysis.

- **Reliably reported:** summary statistics obtained from data reported in published, peer-reviewed, journals.

- **Other population groups:** non-breastfeeding population groups.

FDA.19. *Please provide a narrative that discusses the safety of 3-FL in toddler formula given that the intended use level (2.0 g/L) is much higher than the intended use level in infant formula (0.75 g/L). As part of the discussion, please include relevant information on the physiological differences between infants (0-12 months) and toddlers (1-2 years) that may impact the absorption, distribution, metabolism, and/or excretion of 3-FL at the intended use level of 2.0 g/L.*

As discussed above, the concentration of 3-FL in human milk increases throughout the course of lactation. For this reason, a higher use level is proposed for the use of 3-FL in toddler formula (2.0 g/L) than infant formula (0.75 g/L). The intended use of 3-FL in toddler formula at a maximum use level of 2.0 g/L does not exceed the maximum mean concentration of 3-FL in late milk reported in the review by Soyyilmaz *et al*. (2021) (2.57 g/L after 90 days of lactation; see Table 6 of the publication) nor in Glycom's analysis (2.40 g/L after 6 months of lactation; see Table 1.6.4-1 of Appendix A of the GRAS notice).

As noted in Appendix A, 3-FL occurs at highest concentration in Milk Group 2, representing mothers that are FUT2-negative (non-Secretor phenotype) but FUT3-positive (Lewis phenotype). The high amount of

3-FL in Milk Group 2 is partially explained by an increased activity in the Lewis enzyme (Thurl *et al*., 2010). Similarly, the increasing concentration of 3-FL over the course of lactation can also be explained by changes in the activity of the Lewis enzyme and fucosyltransferases. In fact, an inverse relationship between the concentration 3-FL and 2'-FL as lactation progresses has been recognized (*i.e.*, increasing 3-FL and decreasing 2'-FL), and is hypothesized to result from the co-regulation of fucosyltransferases responsible for the synthesis of these HMOs or from competition for a limited supply of substrate required by the fucosylating enzymes for the synthesis of these HMOs (Thum *et al*., 2021).

The infant gut microbiota is dominated by *Bifidobacteria* until the introduction of complementary foods at around 4 to 6 months of age, altering the microbiota towards a more diverse flora (Moore and Townsend, 2019). Increased consumption of dietary fiber through complementary feeding increases the diversity and proportion of fiber-degrading bacteria, while increased protein consumption provides an additional energy source (*i.e*., partially digested proteins/peptides) to gut microbiota (Laursen, 2021). The cessation of breastfeeding is attributed to shifting the microbiota to an adult-like state (Moore and Townsend, 2019). This demonstrates the diet's influence on the composition of the gut microbiota. It is expected that toddlers exposed to infant formula or other complementary foods containing 3-FL will maintain bacteria capable of digesting this HiMO in their microbiota. As 3-FL produced by Glycom has been demonstrated to be analytically and structurally identical to its naturally occurring counterpart in human milk, the absorption, distribution, metabolism, and excretion of 3-FL at the intended use level of 2.0 g/L in toddler formula is expected to be similar to infants receiving late milk who are also consuming complementary foods (*i.e.*, > 6 months of lactation – maximum mean concentration of 2.40 g 3-FL/L).

FDA.20. *On page 61 of the notice, we note that footnote 21 refers to the summation of maximum use levels of HMOs concluded to be GRAS that are manufactured by Glycom or others. However, there is an error regarding 3'-SL. The maximum intended use level concluded to be GRAS is 0.28 g/L, not 0.20 g/L. Please provide a statement that corrects footnote 21.*

We apologize for this oversight of GRAS notices for 3'-SL submitted by other manufacturers. Indeed, the maximum intended use level of 3'-SL concluded to be GRAS is 0.28 g/L (GRN 921).

Therefore, the total quantities of HiMOs that could be theoretically added to infant formula based on existing and future GRAS Notifications for HiMOs manufactured by Glycom or others (*e.g*., 2'-FL, DFL, LNT, LNnT, 3'-SL, 6'-SL, and 3-FL) would be below 5.55 g/L at their maximum GRAS use levels[5].

FDA.21. *During our search of the literature, we identified a recent toxicity study (i.e., online ahead of print) that utilizes a 3-FL test article manufactured by Glycom (Phipps KR, Lozon D, Stannard DR, et al. Neonatal subchronic toxicity and in vitro genotoxicity studies of the human-identical milk oligosaccharide 3-fucosyllactose [published online ahead of print, 2022 May 5]. J Appl Toxicol. 2022;10.1002/jat.4335. doi:10.1002/jat.4335). Please comment on whether this forthcoming publication is the same toxicity study as cited in the GRAS notice as "unpublished."*

The toxicity study cited above (Phipps *et al*., 2022), is the recent publication of the same 90-day repeat dose toxicity study and genotoxicity studies (bacterial reverse mutation test and *in vitro* mammalian cell

[5] Summation of maximum use levels for HiMOs: 2.4 g/L 2'-FL (GRN 650) + 0.6 g/L LNnT (GRN 659) + 0.32 g/L DFL (GRN 815) + 0.8 g/L LNT (GRN 833) + 0.4 g/L 6'-SL (GRN 881) + 0.28 g/L 3'-SL (GRN 921) + 0.75 g/L 3-FL.

micronucleus test) of 3-FL produced by microbial fermentation with Glycom's *E. coli* K-12 MDO derivative cited as 'unpublished' in the GRAS notice. The manuscript was accepted for publication and first published (05 May 2022) only after the submission of the GRAS notice (04 November 2021).

FDA.22. *In the notice, Glycom includes a reference to "bioburden" (page 21). We note, that "bioburden" is a pharmaceutical term, and is not appropriate for ingredients added to conventional foods. For the administrative record, please make a statement that corrects this reference.*

We thank the FDA for bringing this to our attention and are in agreement with the inappropriate use of the term for ingredients added to conventional foods. Please see the revised statement below.

The microbiological purity of 3-FL production batches has been assessed for non-pathogenic microorganisms (bacteria, yeasts, and molds) as general hygiene indicators, and for selected food-borne pathogens (*Salmonella* spp., *Cronobacter* spp., *Listeria monocytogenes*, and *Bacillus cereus*).

Aerobic mesophilic total plate count, yeasts and molds levels, and the count of *Enterobacteriaceae* give an indication of the microbiological quality and the absence of the production strain in 3-FL. 3-FL was also tested for the absence of potentially pathogenic bacteria, namely *Salmonella* spp., *Cronobacter* spp., and *Listeria monocytogenes*. Spore-forming bacteria *Bacillus cereus*, which is a frequent contaminator of heat-treated or spray-dried foods, was also measured to control the number of surviving spores in the final product. The results of these analyses consistently indicate low microbial contamination in the finished 3-FL product.

FDA.23. *In the GRAS Final Rule published in the Federal Register on August 17, 2016, we stated that convening an external GRAS panel is not required for a conclusion of GRAS status (81 FR 54960 at 55026). Regarding Glycom's GRAS Panel Evaluation that was included as Appendix B in Glycom's GRAS submission, we wish to note the following for the administrative record:*

A) On page 3 of Appendix B, footnote 2 states that "Infant formula products to which 3-FL would be added are most likely to be formula containing partially hydrolyzed cow's milk protein as a protein base." We note that Glycom's GRAS notice does not mention this specific protein base within the context of the intended use of 3-FL in infant formula.

B) On page A-1 of Appendix B, Table A-1 lists the proposed use level of 3-FL in non-exempt infant formula for term infants as 2.0 g/L, whereas Glycom's GRAS notice states that the proposed use level in non-exempt infant formula for term infants is 0.75 g/L. Please confirm that the proposed use level in non-exempt infant formula for term infants is 0.75 g/L.

C) Related to the inconsistency in the stated proposed use levels in Appendix B and Glycom's GRAS notice, we note that the estimated exposures to 3-FL from proposed food uses listed in Table 1 (page 4) of Appendix B are different for the infant population subgroups compared to the estimated exposures listed in Table 3.2.2-2 (page 40) of Glycom's GRAS notice. Please confirm that the values listed in Table 3.2.2-2 of the GRAS notice are the correct exposure estimates.

D) On page 63 of the GRAS notice, Glycom states, "The GRAS Panel, convened by Glycom, independently and critically evaluated all data and information presented herein…" Given that the Parschat et al., 2021 clinical study was published in August 2021 and the statement of the GRAS Panel is dated May 2021, please clarify if the GRAS Panel evaluated the Parschat et al., 2021 study. If the GRAS Panel did not evaluate the Parschat et al., 2021 study and its findings, please amend the statement on page 63 so that it is correct.

Part A)

As indicated in response to Question 1, Glycom does not intend to restrict the use of 3-FL according to the protein source included in the infant formula. However, Glycom's other HiMOs that have been notified as GRAS and received a "no questions" letter from the FDA have been historically predominantly added to infant formula containing partially hydrolyzed cow's milk protein as a protein base.

Part B)

Glycom confirms that the proposed use level of 3-FL in non-exempt infant formula for term infants is 0.75 g/L, as specified in the GRAS notice.

The GRAS Panel evaluated and concluded on the safety of the addition of 3-FL at a higher use level to non-exempt infant formula for term infants (*i.e.*, 2.0 g/L), as specified in the GRAS Panel Consensus Statement (Appendix B of the notice).

Part C)

Glycom confirms that the values listed in Table 3.2.2-2 of the GRAS notice are the correct exposure estimates. These exposure estimates reflect the intended conditions of use of 3-FL from Table 1.3-1 of the GRAS notice, where the proposed use level of 3-FL in non-exempt infant formula for term infants is 0.75 g/L.

The exposure estimates presented in Table 1 of Appendix B are based on the proposed conditions of use of 3-FL evaluated by the GRAS Panel (Table A-1 of Appendix B). As indicated in response to Part B) of this question, the GRAS Panel evaluated and concluded on the safety of the addition of 3-FL at a higher use level to non-exempt infant formula for term infants (*i.e.*, 2.0 g/L), which resulted in higher daily intakes in infant population groups.

Part D)

We thank the FDA for catching this error. Indeed, the GRAS Panel did not evaluate the Parschat *et al*. (2021) infant clinical study and its findings as the study was published after the GRAS Panel completed its evaluation and signed the consensus statement. The statement on page 63 of the GRAS notice has been amended below:

The GRAS Panel, convened by Glycom, independently and critically evaluated all data and information presented herein, except for the Parschat *et al*. (2021) infant clinical study that was published after the GRAS Panel completed its evaluation of 3-FL under the intended conditions of use. Nevertheless, even in the absence of the Parschat *et al*. (2021) study that established the safety of infant formula supplemented with a mixture of 5 HMOs (including 3-FL) at levels similar to those in human milk, the GRAS Panel concluded that 3-FL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and the evaluation of such data as it pertains to the proposed GRAS uses of 3-FL, is presented in Appendix B.

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We hope this information adequately addresses the Agency's questions on GRN 001037, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
Date: 2022.06.17 14:25:23 -04'00'

Maryse Darch
Sr. Regulatory Affairs Specialist
Glycom A/S

ADDITIONAL REFERENCES NOT CITED IN THE NOTICE

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

04 October 2022

Ellen Anderson
Regulatory Review Scientist
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 001037

Dear Ms. Anderson,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s email correspondence dated 08 September 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of 3-fucosyllactose (3-FL) filed by the Agency under GRN 001037.

FDA.1. *For the administrative record, please state whether the production strain, E. coli K-12 DH1 MDO strain DSM 33416, contains any plasmids or episomal vectors.*

The 3-FL production strain *E. coli* K-12 DH1 MDO MAP1834, deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany under the deposition number DSM 33416, is a fully genomic strain, which does not contain any plasmids or episomal vectors.

FDA.2. *On pages 9 and 10 of the notice, Glycom states, "To enable the MDO strain to biosynthesize 3-FL, the strain has been modified by replacement of a promoter element, insertion of four gene cassettes encoding enzymes for biosynthesis and export of 3-FL and resulting in the deletion of four genes at each targeted insertion site, and subsequently the deletion of four additional genes to optimize 3-FL production" and "In addition, the lacI gene, which encodes a transcriptional repressor of the Plac promoter has been deleted. An additional three gene deletions have been implemented to inhibit synthesis of unwanted mixed-acid metabolites and to improve the safety of the final product". For the administrative record, please describe these eight gene deletions.*

Four expression cassettes were integrated into the strain *E. coli* K-12 DH1 MDO genome in four different loci – genes encoding enzymes involved in carbohydrate metabolism. Due to these genetic modifications, the 3-FL production strain *E. coli* K-12 DH1 MDO MAP1834 is unable to utilize four carbohydrates.

Additionally, the following four genes have been deleted:

1. The gene encoding the repressor, LacI, of the lac operon.
2. The gene encoding D-lactate dehydrogenase.
3. The operon encoding a formate channel and a pyruvate formate-lysate enzyme.
4. The gene encoding Cytolysin A, a pore-forming protein toxin, which may cause lysis of the cell membrane. Even though, the Cytolysin A was not expressed neither in the laboratory nor under production conditions, it was deleted to eliminate any potential risk.

FDA.3. *On pages 14 and 22 of the notice, Glycom lists a specification for Cronobacter spp. In the June amendment, Glycom lists a specification for C. sakazakii (Table 4), and states that the method used is ISO 22964. The current version of this method is ISO 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp." For the administrative record, please clarify whether Glycom tests for the presence of Cronobacter spp. or C. sakazakii, specifically. If it is the former, please state whether presumptive positives are further analyzed to determine if the isolate is C. sakazakii.*

Glycom tests for the presence of *Cronobacter* spp. for 3-FL that is added during the dry blend stage. Indeed, the specification parameter was misreported in Table 4 of the June amendment.

The presence of *Cronobacter spp.* is tested according to the current ISO 22964:2017 version of the *"Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp."* method. As per 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 3-FL that is added during the dry-blending stage of infant formula manufacture require for *Cronobacter* spp. to be absent in 10 g.

FDA.4. *C. sakazakii has been isolated from foods intended for very young children and can cause infection in infant and young children. Because Glycom lists intended use of 3-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, there remains a potential risk to these vulnerable populations if C. sakazakii is not controlled for during the production of 3-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:*

- *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.*
- *Forsythe, S. J. (2015). New insights into the emergent bacterial pathogen Cronobacter. In Food Safety (pp. 265-308). Academic Press.*

On page 22 of the notice, the notifier states, "Accordingly, suitable specifications have been established for the ingredient when intended for inclusion at the wet blending stage of infant formula manufacture (i.e., prior to retort) and also for conventional food products … More restrictive release specifications (including additional limits for Cronobacter spp., L. monocytogenes, and B. cereus) are established for 3-FL intended for addition at the dry blending stage of infant formula manufacture, where subsequent heat-treatment is not applied". Please elaborate on this statement as it relates to the intended use of 3-FL in formula and drinks intended for young children (>12 months of age) and in foods intended for infants and young children and describe why a C. sakazakii specification is not provided for these products.

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, 2021). Hence, as part of the system of process controls to prevent adulteration of powdered infant formula from microorganisms, the specifications for 3-FL added during the dry blending stage of infant formula manufacture include this specification limit for *Cronobacter* spp.

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 3-FL added during the wet-blending stage of the manufacturing process are subject to heat-treatment[1] 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).

[1] *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.

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 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 3-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 3-FL ingredient. Furthermore, the specifications for 3-FL under all intended conditions of use include a specification limit for the *Enterobacteriaceae* family, which encompasses the *Cronobacter* genus.

FDA.5. *In response to question 19 in the June amendment, Glycom notes that since 3-FL increases throughout lactation, 3-FL concentrations in late milk may be an appropriate reference for use in formula for young children (i.e., toddler formula). However, it is unclear from the response why Glycom proposes a use level in infant formula that falls within the 95% CI of mean values for 3-FL in human milk, but a use level for formula for young children that falls outside of the 95% CI for mean values of 3-FL in human milk (i.e., 95% CI: 0.90-0.95 g/L in late milk from Soyyilmaz et al., 2021). In other words, different statistical parameters (i.e., 95% CI vs. a maximum mean concentration) are being used to justify the safety of a particular use level.*

Glycom further states that the introduction of complementary foods alters the microbiota toward a more diverse flora, but we note that the "complementary feeding period coincides with a phase of drastic changes in the gut microbiota . . ., including a rapid decline in HMO-degrading Bifidobacterium species." Please provide additional scientific rationale, based on generally available and generally accepted data and information, that justifies the safe and tolerable use of 3-FL in formula for young children at the proposed use level of 2.0 g/L.

Soyyilmaz *et al*. (2021) reported the 95% confidence interval of the mean concentration of 3-FL in late (pooled) milk (mean of means: 0.92; 95% CI of the mean: 0.90-0.95 g/L). However, Glycom's proposed approach for the determination of the maximum safe level of 3-FL in formula for young children considers the distribution of 3-FL concentrations in mature and late human milk, where the upper limit of the 95% confidence interval (calculated as the sum of the mean plus 2 times its standard deviation) is averaged across publications.

Nevertheless, Glycom acknowledges that general recognition of safety of 3-FL and other human-identical milk oligosaccharides (HiMO) in healthy term infants has historically been supported at the mean (or mean of means) levels reported for human milk. As such, Glycom has decided to lower the proposed use level of 3-FL in toddler formulas and other drinks for young children to 0.90 g/L, similar to the mean of means concentration of 3-FL reported in late milk by Soyyilmaz *et al*. (2021).

The revised food use table (Table 1.3-1 of the GRAS notice) is presented below.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 3-FL in the U.S. [REVISED]†

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk Meal Replacement and Nutritional Beverages[c]	240	0.48	2.00
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	360	0.45	1.25
Infant and Toddler Foods	Term Infant Formulas	100[d]	0.075	0.75
	Toddler Formulas[e]	100[d]	0.09	0.90
	Other Baby Foods for Infants and Young Children	7 to 170	0.04 to 1.06	6.25
	Other Drinks for Young Children	120	0.11	0.90
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40	1.00	25.00
	Cereal and Nutrition Bars	40	1.00	25.00
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk*	240	0.48	2.00
Milk Products	Buttermilk*	240	0.48	2.00
	Flavored Milk	240	0.48	2.00
	Milk-Based Meal Replacement and Nutritional Beverages[c]	240	0.60	2.50
	Yogurt Drinks, Probiotic Drinks	80 to 207[f]	0.16 to 0.41	2.00
	Yogurt*	170	2.13	12.50
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	240	0.30	1.25

3-FL = 3-fucosyllactose; CFR = *Code of Federal Regulations*; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

† Revised values are indicated in green.

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

[b] Use level expressed on a 3-FL basis in the final food, as consumed.

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

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

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

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

Furthermore, cumulative estimated daily intakes of 3-FL were calculated considering Glycom's proposed conditions of use (revised Table 1.3-1 above) and those previously notified as GRAS to the U.S. FDA that received a no questions letter from the Agency (GRNs 925 and 951). Food uses and maximum use levels evaluated in the cumulative dietary exposure assessment of 3-FL are presented in Table 1 below.

Table 1 Summary of the Individual Proposed and Previously Notified as GRAS[a] Food Uses and Use Levels for 3-FL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021a)	Proposed Food Use[b]	Proposed Maximum Use Level[c] (g/kg or g/L)
Beverages and Beverage Bases	Non-Milk Meal Replacement and Nutritional Beverages[d]	2.00
	Sports, Isotonic, and Energy Drinks, Soft Drinks, Enhanced or Fortified Waters	1.25
Breakfast Cereals	**Hot Cereals**	**6.80**
	RTE Cereals	**8.80**
Dairy Product Analogs	**Milk Substitutes**	**0.26**
	Non-Dairy Yogurts	**2.64**
Infant and Toddler Foods	Term Infant Formulas	0.75
	Toddler Formulas[e]	0.90
	Other Baby Foods for Infants and Young Children	6.25
	Other Drinks for Young Children	0.90
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	25.00
	Cereal and Nutrition Bars	25.00
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk	2.00
Milk Products	Buttermilk	2.00
	Flavored Milk	2.00
	Milk-Based Meal Replacement and Nutritional Beverages[d]	2.50
	Smoothies (Dairy and Non-Dairy)	**1.10**
	Yogurt Drinks, Probiotic Drinks	2.00
	Yogurt	12.50
Processed Fruits and Fruit Juices	Fruit Drinks and Ades	1.25
	Fruit Juices and Nectars	**0.26**
Processed Vegetables and Vegetable Juices	**Vegetable Juice**	**0.26**
Foods for Special Dietary Use	**Special Dietary Purpose Ingredient in Oral and Enteral Tube Feeding (> 11 years)[f]**	**0.88 g/serving**

3-FL = 3-fucosyllactose; CFR = Code of Federal Regulations; GRAS = Generally Recognized as Safe; RACC = Reference Amounts Customarily Consumed; RTE = Ready-to-Eat; U.S. = United States.

[a] Food uses that have been previously notified as GRAS to the U.S. FDA, and received a "no questions" letter, are **bolded**.

[b] 3-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169, do not permit its addition in standardized products.

[c] Use level expressed on a 3-FL basis in the final food, as consumed.

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

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

[f] Foods for special dietary use are assessed separately from the intended food uses of 3-FL in conventional foods, as they are intended for supplying a particular dietary need and/or supplementing the intake of a dietary component. Intake of 3-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources and was not included in the cumulative dietary exposure assessment.

Estimated daily intakes from the cumulative dietary exposure assessment of 3-FL, considering the lower proposed use level of 3-FL in toddler formulas and other drinks for young children (*i.e.*, 0.9 g/L), are presented in the revised Tables 3.2.2-1 and 3.2.2-2 of the GRAS notice below. Intake estimates of 3-FL from the cumulative dietary exposure assessment are generally similar to those originally provided in the GRAS notice (*i.e.*, from proposed conditions of use only), with slight increases in certain population groups as a result of the additional conditions of use of 3-FL previously notified as GRAS.

Table 3.2.2-1 Summary of the Cumulative Estimated Daily Intake of 3-FL in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	0.66	1.47	76.2	139	0.87	1.72
Infants	7 to < 12 m	1.99	3.51	100	124	1.99	3.51
Toddlers	1 to 2 y	1.52	2.46	99.9	306	1.52	2.46
Children	3 to 11 y	1.30	2.36	99.1	988	1.31	2.37
Female Teenagers	12 to 19 y	0.94	1.93	95.8	429	0.98	1.93
Male Teenagers	12 to 19 y	1.29	2.41	97.5	426	1.32	2.42
Female Adults, CBA	20 to 40 y	1.03	2.17	93.1	645	1.11	2.20
Female Adults	20 to 64 y	1.08	2.41	92.2	1,511	1.18	2.51
Male Adults	20 to 64 y	1.34	2.90	92.7	1,334	1.45	3.05
Elderly	65 y and older	1.22	2.43	94.8	991	1.29	2.51
Total Population	2 y and older	1.21	2.54	94.2	5,832	1.29	2.57

3-FL = 3-fucosyllactose; CBA = childbearing age; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

Table 3.2.2-2 Summary of the Cumulative Estimated Daily Per Kilogram Body Weight Intake of 3-FL in the U.S. by Population Group (2017-2018 NHANES Data) [REVISED]

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	99	226	76.2	139	130	247
Infants	7 to < 12 m	221	413	100	124	221	413
Toddlers	1 to 2 y	123	211	99.9	297	124	211
Children	3 to 11 y	51	100	99.1	985	52	100
Female Teenagers	12 to 19 y	16	34	95.7	422	17	37
Male Teenagers	12 to 19 y	20	42	97.4	423	21	42
Female Adults, CBA	20 to 40 y	14	32	93.1	644	15	33
Female Adults	20 to 64 y	15	35	92.2	1,504	16	35
Male Adults	20 to 64 y	15	33	92.7	1,326	17	33
Elderly	65 y and older	16	34	95.0	974	17	35
Total Population	2 y and older	21	47	94.2	5,780	22	49

3-FL = 3-fucosyllactose; bw = body weight; CBA = childbearing age; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

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We hope this information adequately addresses the Agency's questions on GRN 001037, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
Date: 2022.10.04 10:03:42 -04'00'

Maryse Darch
Regulatory & Scientific Affairs Manager
Glycom A/S

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