Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

#815

20 September, 2018

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SEP 24 2018
OFFICE OF
FOOD ADDITIVE SAFETY

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 2'-fucosyllactose / difucosyllactose (2'-FL / DFL)

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 2'-fucosyllactose / difucosyllactose (2'-FL / DFL) produced by microbial fermentation with an *E. coli* K12 DH1-derived strain, is GRAS on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products across multiple categories; these food uses of 2'-FL/DFL 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.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

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.
Senior Scientist
Head of Regulatory & Scientific Affairs
Glycom A/S

GRAS NOTICE FOR 2'-FUCOSYLLACTOSE/ DIFUCOSYLLACTOSE (2'-FL/DFL)

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:
20 September 2018

GRAS Notice For 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)

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GRAS Notice for 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)

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 (Glycom) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that 2'-fucosyllactose/difucosyllactose (2'-FL/DFL), 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 2'-FL/DFL as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

[redacted]

20 Sept 2018

Christoph H. Röhrig, Ph.D.
Senior Scientist
Head of Regulatory & Scientific Affairs
Glycom A/S
Christoph.Roehrig@glycom.com

Date

1.1 Name and Address of Notifier

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark
Tel: +45 2826 3724
Fax: +45 4593 3968

1.2 Common Name of Notified Substance

2'-Fucosyllactose/Difucosyllactose; 2'-FL/DFL

1.3 Conditions of Use

2'-FL/DFL is intended to be added as a food ingredient to foods targeted to infants and young children, including non-exempt term infant formula, as well as uses in specific conventional food products used by the general population (Table 1.3-1). Food uses of 2'-FL/DFL in infant formula (*i.e.*, infants up to 12 months) will provide 2'-FL/DFL at a use-level of 1.6 g/L, follow-on formula at a use-level of 1.2 g/L, infant-specific foods and foods for young children at a use-level of 1.2 g/L in ready-to-drink and reconstituted products,

and up to 10 g/kg for products other than beverages (*e.g.*, baby foods). The maximum use-levels are proposed on the basis of providing similar levels of 2'-FL and DFL on a body weight basis as those consumed by breast-fed infants (see Section 3.1.3).

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use-Levels for 2'-FL/DFL in the U.S.

Food Category	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use-Level (g/RACC)	Proposed Maximum Use-Level (g/kg or g/L)[d]
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.96	4.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.72	2.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.16	1.6
	Toddler Formulas	100 mL[c]	0.12	1.2
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.07 to 1.70	10
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	1.6	40
	Cereal and Granola Bars	40 g	0.8	20
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.48	2.0
Milk Products	Buttermilk*	240 mL	0.48	2.0
	Flavored Milk	240 mL	0.48	2.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.96	4.0
	Yogurt*	170 g	3.4	20

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose; CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2018a). 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] Includes ready-to-drink and powder forms.

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

[d] Use level defined g of dry powder per serving on a weight/weight basis.

*2'-FL/DFL is intended for use in unstandardized products when standards of identity do not permit its addition.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2018b), Glycom has concluded that the intended uses of 2'-FL/DFL as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the 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. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2'-FL/DFL comprises 2 structurally and biologically related HiMOs produced as a mixture by fermentation using a modified strain of *Escherichia coli* (*E. coli*) K-12. The ingredient is described as a white to off-white amorphous powder or agglomerate. Amorphous powders do not possess defined melting points. 2'-FL/DFL is readily soluble in aqueous solutions (max. 500 mg/mL, 25°C), with poor solubility in any organic solvents. 2'-FL consists of a lactose molecule carrying 1 fucose unit, and DFL consists of a 2'-FL molecule carrying a second fucose unit (Table 2.1-1).

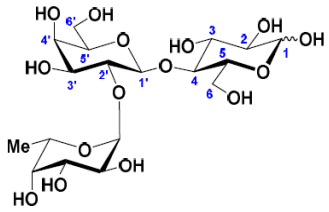
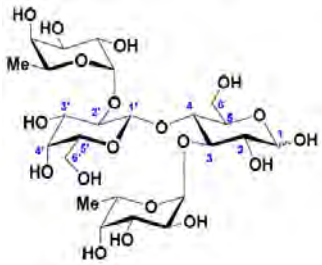
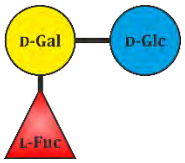
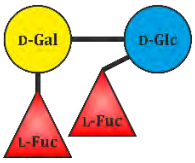
The main constituent, 2'-FL, is a neutral trisaccharide that is secreted naturally in large amounts into human milk and is also found in minor amounts in other mammalian milks. 2'-FL is the most abundant human milk oligosaccharide (HMO) in human milk (*ca.* 2.5 g/L). 2'-FL is comprised of L-fucose, D-galactose, and D-glucose. Alternatively, the molecular constitution can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an α(1→2) bond to form the trisaccharide. 2'-FL is a chemically defined trisaccharide that occurs only as one specific constitutional isomer.

The second component, DFL, is a derivative of 2'-FL where a second fucose sugar has been added to the 3-glucose position of 2'-FL. It is a neutral tetrasaccharide that is present in human milk at lesser amounts than 2'-FL (*ca.* 0.5 g/L). Additional information on HMO composition and the "Secretor" phenotype of human milk is presented and described in Section 3.1.3. Besides its prominent role in human milk, DFL has been reported as a characteristic milk oligosaccharide in the milk of the lemur aye-aye (Taufik *et al.*, 2012), and is a principal carbohydrate in the milk of monotremes such as the platypus and the echidna (Messer and Kerry, 1973). DFL is composed of two units of L-fucose, one unit of D-galactose, and one unit of D-glucose.

Table 2.1-1 Description of Identity of the 2'-FL/DFL Product

Product Name	2'-Fucosyllactose/Difucosyllactose	
Abbreviations	2'-FL/DFL	
	2'-Fucosyllactose (2'-FL)	Difucosyllactose (DFL)
IUPAC Name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucopyranose
IUPAC Abbreviation (extended)	α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-D-Glc	α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-[α-L-Fuc*p*-(1-2)]-D-Glc*p*
IUPAC Abbreviation	Fuc-(α1-2)-Gal-(β1-4)-Glc	Fuc-(α1-2)-Gal-(β1-4)-[Fuc-(α1-3)]-Glc

Table 2.1-1 Description of Identity of the 2'-FL/DFL Product

(condensed)		
Molecular Structure		
Symbol Nomenclature		
Molecular Formula	$C_{18}H_{32}O_{15}$	$C_{24}H_{42}O_{19}$
Molecular Mass (weight)	488.44	634.58
CAS Number	41263-94-9	20768-11-0
CAS Name	D-Glucose, *O*-6-deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)	D-Glucose, O-6-deoxy-α-L-galactopyranosyl-(1→3)-*O*-[6-deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)]
Synonyms	2'-*O*-Fucosyllactose	Lactodifucotetraose (LDFT)

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

2'-FL and DFL obtained from microbial fermentation are chemically and structurally identical to 2'-FL and DFL that are secreted into human breast milk, as confirmed by 1H- and 2D-nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

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 (*λ⁻ gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*), which has been used as a host strain for further development of the 2'-FL/DFL production strain, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (deposited under

DSM No. 4235)[1]. *E. coli* K-12 and its derivatives[2] 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% less genes than their pathogenic cousins (Lukjancenko *et al.*, 2010). *E. coli* K-12-derived strains cannot colonize in the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). *E. coli* K-12 derivatives are currently among the preferred microorganisms for industrial biotechnology with wide application scope (Chen *et al.*, 2013; Theisen and Liao, 2017) and several GRAS ingredients and food enzymes have been authorized in the U.S. that were manufactured from *E. coli* K-12 derivatives [*e.g.*, 2'-FL (U.S. FDA, 2015, 2016a), lacto-N-neotetraose (LNnT) (U.S. FDA, 2016b), alpha cyclodextrin (U.S. FDA, 2004), chymosin (U.S. FDA, 2015c), L-leucine (U.S. FDA, 2010), and β-galactosidase (U.S. FDA, 2014)].

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

Characteristics of *Escherichia Coli* K-12 DH1	
Genotype	*F⁻, λ-, gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44.*
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli* (*E. 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

The host strain *E. coli* K-12 DH1 (DSMZ, 2015) was then optimized for general oligosaccharide expression features (used as a "platform host strain") by introduction of 7 modifications related to carbohydrate metabolism, thereby improving the efficiency of the strain. This strain was given the designation "MDO". An overview of the modification events used for construction of strain MDO has been discussed previously and is hereby incorporated by reference to Section II.B.1.2 of GRAS Notice (GRN) 650. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing (at the level of MDO strain, Steps 1 to 7) and colony polymerase chain reaction (PCR) targeted sequencing methods.

The HiMO platform strain MDO was then transformed with the aid of (i) a helper plasmid, which is responsible for expression of genes required for double strand DNA recombineering, and (ii) a donor plasmid containing DNA fragments surrounding the site of insertion, a promoter fragment, the gene(s) of interest, and a transcriptional terminator sequence. Defined DNA sequences from donor microorganisms (*i.e.*, the colanic acid operon from *E. coli*, and a fucosyltransferase-encoding gene from *Helicobacter pylori*) 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 synthesized

[1] www.dsmz.de

[2] Note: In the scientific literature, the term *E. coli* 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).

by DNA synthesis. Such synthesized DNA sequences were integrated into the chromosomal DNA of the parental strain *E. coli* K-12 DH1 to construct the 2'-FL/DFL production strain *E. coli* (K-12 DH1 MDO) MAP1001. As the donor genes were not isolated (or directly amplified) from the actual donor strains (or their genomic DNA) but were derived from *de novo* DNA synthesis (based on sequence information identified from bioinformatics databases), there is no risk of introducing extraneous or unknown DNA from the donor organisms. Three donor plasmids containing the integrated genes of interest (genetic cassettes), were used for construction of the modified strain. Plasmids were incorporated into the host genome by homologous recombination at 3 site-specific loci involved in sugar metabolism. During the construction of the strain, both donor and helper plasmids were removed with standardized molecular biology procedures, leaving no antibiotic resistant markers behind in the strain. In consequence, the MAP1001 strain does not contain any plasmids nor vectors as well as any antibiotic marker genes. Following the construction, the MAP1001 strain has been tested to be sensitive to the antibiotics, which were used as antibiotic resistance markers during the strain development process, which confirmed the absence of the markers. The enzyme functions of the donor genes are described below in Table 2.2.1.1-1. The enzyme identities are well characterized and based on their enzymatic functions it was concluded that the introduced genes would not confer toxicogenic/pathogenic properties to the host organism. The genetic modifications made to the production strains result in the expression of proteins that are involved in the normal carbohydrate processing within their donor microbial (bacterial) sources. These proteins are "carbohydrate-active enzymes" ("CAZymes"), a panel of enzymes that can degrade, modify, or create glycosidic bonds, and accordingly are involved in the metabolism of complex carbohydrates[3]. When expressed together in the recipient strains, these proteins work in concert to convert the starting carbohydrates (lactose and glucose[4]) into oligosaccharides that are identical to those in human breastmilk. 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; Popoff, 2018; Wilson *et al.*, 2002). Indeed, bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the *E. coli* K-12 (DH1) MAP1001 strain by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens. Table 2.2.1.2-1 provides a summary of the recombinant enzymes and their functions incorporated into the production strain MAP1001. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods.

Table 2.2.1.1-1 Donor Plasmids used for Construction of the 2'-FL/DFL Production Strain *E. coli* MAP1001

Plasmid Name	Expression Product	Copy Number	Function	Marker Gene
pMAP379	*colanic acid operon enzymes*	*1*	*de novo GDP-fucose synthesis*	*bla*
pMAP395	*fucosyltransferase*	*2*	*fucosylation*	*kanR*
pMAP398	*fucosyltransferase*	*2*	*fucosylation*	*kanR*

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; *bla* = beta-lactamase (ampicillin); *E. coli* = *Escherichia coli*; *kanR* = kanamycin resistance.

Figure 2.2.1.2-1 shows the biochemical pathway by which the production strain generates 2'-FL (and in turn DFL from 2'-FL) using lactose as a substrate and glucose as a carbon source[4]. Notably, the primary substrate for the fucosyltransferase is lactose, generating 2'-FL by adding a fucose moiety in the 2-galactose position of lactose (with concurrent release of guanosine 5'-diphosphate). However, 2'-FL itself is also accepted as a

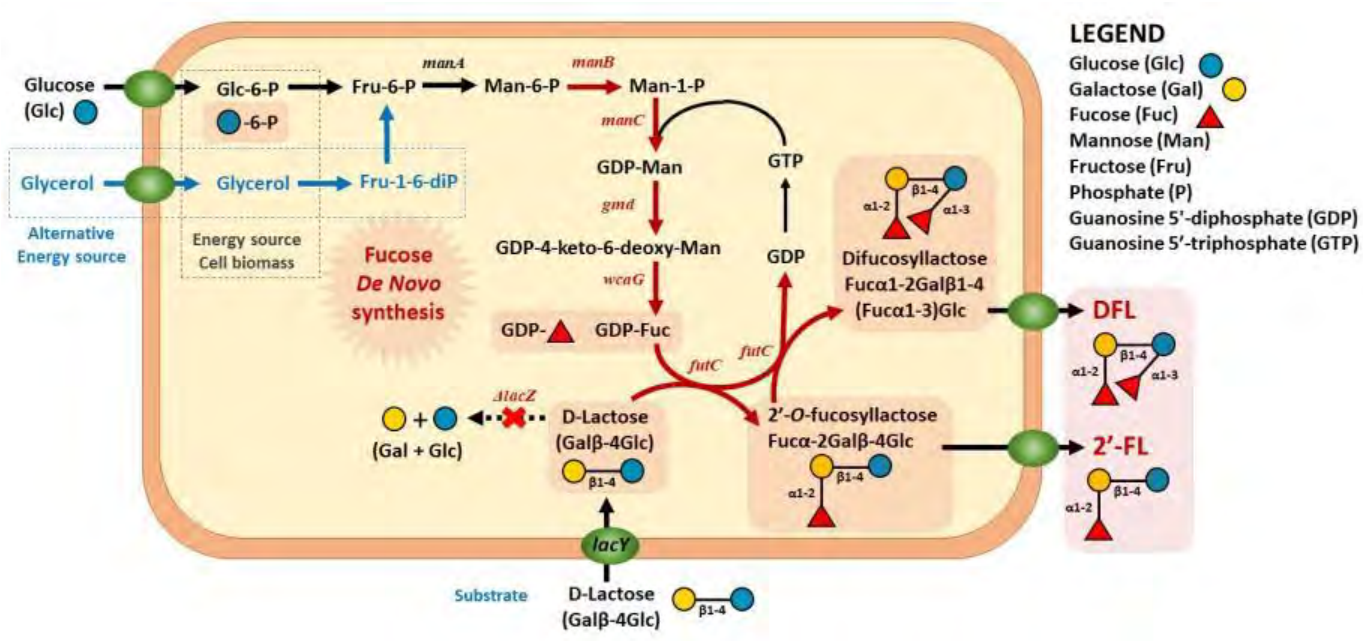
[3] Database of Carbohydrate-Active EnZYmes: http://www.cazy.org

[4] Glycerol or sucrose are alternative carbon sources to glucose.

substrate by the same fucosyltransferase, which as a side activity possesses the ability to add another fucose moiety at the 3-glucose position of 2'-FL, thus converting it into DFL. However, the equilibrium favors the formation of 2'-FL over DFL and the result is a mixture of 2'-FL and DFL at a similar ratio as is found in human milk.

During manufacture, the production strain remains intact, secretes the 2'-FL and DFL 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 Engineered Pathway for 2'-Fucosyllactose and Difucosyllactose (2'-FL and DFL) Biosynthesis in the Production Strain *Escherichia coli* MAP1001



2.2.2 Description of the Production Process

Glycom's 2'-FL/DFL is manufactured in compliance with cGMP and the principles of Hazard Analysis Critical Control Point (HACCP) and is largely comparable to the production processes previously evaluated for other HiMOs with GRAS status (*i.e.*, 2'-FL and LNnT; GRN 650 and GRN 659, respectively) (U.S. FDA, 2016a,b). All additives, processing-aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously concluded to be GRAS for their respective uses, or have been the subject of an effective food contact notification. The manufacturing process can be broadly divided into 2 stages.

In Stage 1 [upstream processing (USP)], D-lactose is converted by subsequent fucosylation to 2'-FL and DFL by the adapted cellular metabolism of the 2'-FL/DFL production microorganism, which uses D-glucose (or optionally D-glycerol or D-sucrose) as an exclusive energy and carbon source and D-lactose as a substrate for 2'-FL and DFL 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 2'-FL/DFL.

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

Table 2.2.2-1 Overview of the Manufacturing Process for the 2'-FL/DFL product

STAGE 1		**Upstream Processing (USP)**
STEPS	**1**	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase
	4B	Feeding (Fed-Batch) Phase
	5	Removal of Microorganism
STAGE 2		
STEPS	**6**	Purification/Concentration 1
	7	Ion Removal
	8	Decolorization
	9	Purification/Concentration 2
	10	Drying (*e.g.*, spray drying, freeze-drying)
	11	Sampling and Packaging
	12	Quality Control and Batch Release

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose.

2.2.3 Quality Control

The manufacture of 2'-FL/DFL by microbial fermentation is conducted in accordance with cGMP and HACCP principles.

Since all principal raw materials and the final products are chemically well-characterized compounds, the entire 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 Tables 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 comprises a number of in-process controls to minimize the amount of impurities to the level technically possible. The Glycom production process (including all processing aids, raw materials, unit operations, and filter aids) and the food safety management system comply with the following standards and certifications: Food Safety Systems Certification 22000 (FSSC 22000), ISO 9001, Kosher, Halal, Bisphenol-A free, Phthalate-free, Latex-free, and Allergen-free (except for milk-derived allergens).

The production microorganism is efficiently removed by the ultrafiltration step, which is applied directly after the fermentation. There are several subsequent purification steps involved in the down-stream processing stage to obtain a highly purified mixture of 2'-FL and DFL, free of the bacterial cells and residual fermentation by-products. The absence of the microorganisms is demonstrated by measuring

Enterobacteriaceae in the final product according to internationally recognized methods (ISO 21528-1:2004, MSZ ISO 21528-2:2007). The ISO 21528-1:2004 method explicitly includes the pre-enrichment step, which is specifically applicable for microorganisms sought to be expected to need resuscitation before enrichment. The specification for Enterobacteriaceae is set at "absent in 10 g" of test article, which ensures absence of the microorganism as *E. coli* that belongs to Enterobacteriaceae family.

E. coli K-12 is a gram-negative bacterium and these bacteria possess complex glycolipids of high molecular weight, called lipopolysaccharides (LPS), in their cell membrane. LPS elicits an immune response when it is recognized by immune cells after entering the blood stream. Therefore, LPS can cause serious effects in infusion therapy and parenteral nutrition and is also called endotoxin (not to be confused with protein-type toxins). Lipopolysaccharides are comparably harmless when ingested, most likely due to a combination of deactivation by stomach acid and a low absorption due to the high molecular weight. To control potential residual endotoxin levels, a strict specification for endotoxins has been set for the ingredient and all batch results confirm high purity in terms of endotoxins.

After fermentation with strain MAP1001 and purification of 2'-FL/DFL, the absence of traces of residual DNA in the product is confirmed by 3 different validated quantitative PCR (qPCR) methods which target short subsequences of the introduced genes as well as a short subsequence of the multicopy DNA sequence encoding the 23S ribosomal subunit of *E. coli*. These methods have been validated to detect traces of DNA down to 4 µg/kg(4 ppb) and the negative batch results ensures that no amount of any significance of DNA stemming from the production microorganism is present in the final ingredient. All the qPCR tests were conducted with the5 batches and the results were below the limit of quantification (LOQ) in all tested batches (see Table 2.3.3.3-1).

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Specifications for 2'-FL/DFL are presented in Table 2.3.1-1. As a simple carbohydrate mixture, the composition is specified by a combination of parameters but foremost by the sum of 2'-FL and DFL which makes up at least 85.0% of the dry powder. On an individual level, 2'-FL is specified by assay with at least 75.0% (water-free), and DFL with no more than 20.0% (water-free), respectively, based on high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). The main advantages of the PAD detector used is high sensitivity and selectivity, wide dynamic detection levels, reliable performance with excellent precision and more consistent response over wide range of chemical structures (*e.g.*, no chromophores are required for detection).

The specification limits for 2'-FL/DFL reflect the naturally occurring ratio between 2'-FL and DFL (see Section 3.1.3). Since lactose and fucose are further components of the product that are also naturally present in human milk, an additional quality parameter relevant to the infant nutrition uses has been introduced with 92.0% as the sum of HiMS to ensure a consistent product quality in context of that particular proposed use. Another specified carbohydrate is 2'-fucosyllactulose, which is a common isomerization product of glucose-containing carbohydrates such as glucose (forming minor levels of fructose), lactose (forming minor levels of lactulose) and 2'-FL (forming minor levels of 2'-fucosyllactulose). In all cases, it is the glucose at the reducing end that isomerizes to a fructose unit.

All methods of analysis are either internationally-recognized or developed and validated internally by Glycom and confirmed by independent accredited external laboratories [International Laboratory Accreditation Cooperation (ILAC)-accredited laboratory WESSLING Hungary Kft., and Eurofins Medigenomix

GmbH (Germany) accredited against ISO/IEC 17025:2005 by Die Deutsche Akkreditierungsstelle GmbH (DakkS)].

Table 2.3.1-1 Specifications for 2'-FL/DFL

Definition					
2'-Fucosyllactose/difucosyllactose (2'-FL/DFL) is a purified white to off-white powder that is produced by a microbial process.					
Source					
A modified strain of *Escherichia coli* K-12 DH1.					
Parameter	**Specification**	**AVE**	**±**	**SD**	**Method**
Appearance	Powder or agglomerates	Complies			ISO 6658:2007
Color	White to off white	Complies			ISO 6658:2007
Identification (2'-FL/DFL)	RT of standard ± 3%	Complies			Glycom method HPAEC-HMO-011
Assay (water free) – Sum of HiMS[a]	Not less than 92.0 w/w %	94	±	0.4	Glycom method HPAEC-HMO-011,012
Assay (water free) – Sum of 2'-FL and DFL	Not less than 85.0 w/w %	92	±	1	Glycom method HPAEC-HMO-012
Assay (water free) – 2'-FL	Not less than 75.0 w/w %	81	±	2	Glycom method HPAEC-HMO-012
Assay (water free) – DFL	Not more than 20.0 w/w %	11	±	1	Glycom method HPAEC-HMO-012
D-Lactose	Not more than 10.0 w/w %	1.9	±	1.3	Glycom method HPAEC-HMO-011
L-Fucose	Not more than 1.0 w/w %	0.1	±	0.0	Glycom method HPAEC-HMO-011
2'-Fucosyl-D-lactulose	Not more than 2.0 w/w %	0.9	±	0.1	Glycom method HPAEC-HMO-011
Sum of other carbohydrates	Not more than 6.0 w/w %	2.2	±	0.1	Glycom method HPAEC-HMO-011
pH (20°C, 5% solution)	3.5 to 5.4	4.5	±	0.3	Ph. Eur. 9.2 2.2.3 (07/2016:20203)
Water	Not more than 6.0 w/w %	0.4	±	0.0	Glycom method KF-001
Ash, sulphated	Not more than 0.8 w/w %	0.04	±	0.03	Ph. Eur. 9.2 2.4.14 (04/2010:20414)
Residual protein by Bradford assay	Not more than 0.01 w/w %	< 0.0017			Glycom method UV-001
Microbiological Parameters					
Aerobic mesophilic total plate count	Not more than 500 CFU/g	< 10			ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	Complies			ISO 21528-1:2004, ISO 21528-2:2007
Salmonella spp.	Absent in 25 g	Complies			ISO 6579:2006
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies			ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	Complies			ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	< 10			ISO 7932:2005
Yeasts	Not more than 10 CFU/g	< 10			ISO 7954:1999
Molds	Not more than 10 CFU/g	< 10			ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	0.02	±	0.03	Eur. Ph. 2.6.14

2'-FL = 2'-fucosyllactose; AVE = average; CFU = colony-forming units; DFL = difucosyllactose; EPA = Environmental Protection Agency; EU = endotoxin units; Eur. Ph. = European Pharmacopoeia; HiMS = Human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; MPN = most probable number; RT= retention time.

[a] HiMS = Sum of 2'-FL, 3-fucosyllactose, DFL, lactose and fucose.

2.3.2 Product Analysis

2.3.2.1 Main Products and Other Carbohydrates

The analytical results of 5 independent production batches of 2'-FL/DFL together with averaged values (AVE) and standard deviations (SD) are provided in the Table 2.3.2-1 below. Results of the HPAEC-PAD analyses

demonstrate that the 2 major HiMO constituents, 2'-FL and DFL, constitute on average *ca.* 81 and *ca.* 11% by weight of the final composition, respectively (as shown in Table 2.3.2-1). 2'-FL and DFL are present in an approximate weight ratio of on average 7:1 (range from 6:1 to 8:1, which is similar to the range of ratios naturally present in human milk; see Section 3.1.3). Together with other related saccharides, that are also natural constituents of breast milk (*e.g.*, lactose, fucose, 3-fucosyllactose), the sum of HiMS comprises on average 94% of the total batch weight. The small remaining portion of the product consists mainly of other carbohydrate-type compounds structurally related to 2'-FL and DFL [*e.g.*, 2'-fucosyl-D-lactulose, 3-fucosyllactose (3-FL), 2'-fucosyl-galactose, lactitol, 2'-fucosyllactitol, difucosyllactitol, glucose, isomaltose, sorbitol and fucosyl-fucosyllactose (FFL)]. The water-free total specified carbohydrates fraction of 2'-FL/DFL adds up to 97% of the final batch weight.

Table 2.3.2-1 Batch Results for Fermentation Main Products and Other Carbohydrates for 2'-FL/DFL

Parameters	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Assay (water free) – Sum of HiMS[a] [%]	93.6	93.2	93.9	94.0	94.4	**94**	±	**0.4**
Assay (water free) – Sum of 2'-FL and DFL	90.6	92.1	92.6	90.3	93.7	**92**	±	**1**
Assay (water free) – 2'-FL [%]	79.1	82.5	81.7	78.8	81.7	**81**	±	**2**
Assay (water free) DFL [%]	11.5	9.6	10.9	11.5	12.0	**11**	±	**1**
Ratio 2'-FL:DFL	6.9	8.6	7.5	6.9	6.8	**7**	±	**1**
D-Lactose [%]	3.0	1.0	1.3	3.6	0.6	**1.9**	±	**1.3**
L-Fucose [%]	0.1	0.1	0.04	0.1	0.1	**0.1**	±	**0.0**
2'-Fucosyl-D-lactulose [%]	1.1	0.9	0.8	0.8	0.9	**0.9**	±	**0.1**
Sum of other carbohydrates [%]	2.1	2.4	2.1	2.0	2.2	**2.2**	±	**0.1**
Total specified carbohydrates (water free) [%]	96.9	96.5	96.8	96.8	97.5	**97**	±	**0.4**

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; HiMS = human-identical milk saccharides; SD = standard deviation.

[a] Sum of HiMS = Sum of 2'-FL, 3-fucosyllactose, DFL, lactose, and fucose.

2'-Fucosyl-D-lactulose is an isomer of 2'-FL, arising from the isomerization of the terminal glucose moiety of 2'-FL to fructose. This type of isomerization is pH and temperature dependent and has been commonly reported for the closely related conversion of 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 2'-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 2'-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 2.0%) in 2'-FL/DFL batches are negligible and not biologically/nutritionally relevant.

2.3.2.2 Non-carbohydrate Residues

Analytical testing for non-carbohydrate residues included several substances that could potentially be present based on the properties and metabolism of the production microorganism. Such precautionary parameters included amino acids, amino acid metabolites, biogenic amines, microbial endotoxins, residual proteins, as well as anions, trace elements, and heavy metals (described in Section 2.3.3). None of these substances were detected in the purified product and are therefore not considered to contribute to the composition of 2'-FL/DFL. Certificates of Analysis demonstrating the absence of these parameters can be provided upon request.

All independent batches were tested for residual water and sulphated ash, which are minor contributors in the composition of 2'-FL/DFL (see Table 2.3.2.2-1).

Table 2.3.2.2-1 Batch Results for Water and Sulphated Ash Content of 2'-FL/DFL

Parameter	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Water [%]	0.38	0.44	0.42	0.44	0.47	**0.43**	±	**0.03**
Ash, sulphated [%]	0.02	0.06	0.03	0.01	0.07	**0.04**	±	**0.03**

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; SD = standard deviation.

2.3.2.3 Microbiological Contaminants

The microbiological purity of 2'-FL/DFL production batches has been assessed for non-pathogenic microorganisms (bacteria, yeasts, and molds) as general hygiene indicators, and for selected food-borne pathogens (Table 2.3.2.3-1).

Aerobic mesophilic total plate count, yeasts and molds levels and the presence of Enterobacteriaceae give an indication of a level of total contamination (bioburden) and the absence of the production strain in the 2'-FL/DFL preparation, respectively. 2'-FL/DFL was also tested for the presence of pathogenic bacteria, *Salmonella* spp., *Cronobacter sakazakii* and *Listeria monocytogenes*. Spore-forming bacteria *Bacillus cereus*, a frequent contaminator of heat-treated or spray-dried foods, was also evaluated to control the number of surviving spores in the final product.

Table 2.3.2.3-1 Batch Results for Microbiological Analysis of 2'-FL/DFL

Microbiological Parameters	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**		
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	**Absent in 10 g**		
Salmonella spp.	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	**Absent in 25 g**		
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	**Absent in 10 g**		
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	**Absent in 25 g**		
Bacillus cereus [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**		

Table 2.3.2.3-1 Batch Results for Microbiological Analysis of 2'-FL/DFL

Microbiological Parameters	Manufacturing Batch Numbers:					
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE ± SD
Yeasts [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**
Molds [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; CFU = colony-forming units; SD = standard deviation.

2.3.3 Manufacturing By-Products, Impurities and Contaminants

The final 2'-FL/DFL preparation contains the 2 major HiMO ingredients, 2'-FL and DFL, and significant amounts of lactose but only minor quantities of other compounds predominantly carbohydrate-type metabolites structurally related to 2'-FL and DFL which are described in detail in Section 2.3.2.1.

Besides the carbohydrate-type by-products which are detectable, specified impurities, the Quality control measures included the precautionary confirmation of absence of a range of potential residual compounds and trace elements.. These include amino acids and biogenic amines, microbial endotoxins and residual proteins, trace elements, and the presence/absence of genes characteristic for the production microorganism. Those that have been confirmed as absent are not proposed for addition to the product specifications. .

2.3.3.1 Amino Acids and Biogenic Amines

2'-FL and DFL are secreted into the fermentation broth and no disruption of the production microorganism is required during manufacture. 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 (*e.g.*, histamine, tyramine, spermidine, cadaverine, and putrescine), and amino acids and their metabolites (*e.g.*, 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 the 2'-FL/DFL final product.

2.3.3.2 Microbial Endotoxins and Residual Proteins

The parental strain, *E. coli* K-12, is a gram-negative bacterium which possess complex glycolipids of high molecular weight, called either endotoxins or LPS, in their cell walls. Internal specifications for LPS have been established (max. 10 EU/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 of 2'-FL/DFL produced by fermentation, was assayed using the *Limulus* amoebocyte lysate kinetic chromogenic assay.

Similarly, a sensitive residual protein test (based on the Bradford assay) has been applied. Because batch analyses of 2'-FL/DFL demonstrated extremely low endotoxin and residual protein concentrations, they were not considered as compositional or safety-related data of the 2'-FL/DFL final product (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 2.3.1-1).

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

Parameters	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Residual endotoxins [EU/mg]	0.003	0.008	0.016	0.008	0.071	**0.021**	±	**0.028**
Residual protein by Bradford assay [%]	< LOR[a]	< LOR	< LOR	< LOR	< LOR	**< 0.0017**		

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; EU = endotoxin units; LOR = limit of reporting; SD = standard deviation.
[a] LOR = 0.0017% (w/w).

2.3.3.3 Absence of Production Organism and its DNA

The production microorganism is efficiently removed by the ultrafiltration (Step 5) during USP, which is applied directly after fermentation. Various sequential filtration and purification processes are applied during DSP (see Table 2.3.3.3-1) to ensure the final purity of 2'-FL/DFL. The absence of the production microorganisms in the bulk product is demonstrated by testing of final batches for bacteria from the *Enterobacteriaceae* family according to internationally-recognized methods (ISO 21528-1:2004, MSZ ISO 21528-2:2007). The ISO 21528-1:2004 method includes a pre-enrichment step to allow for resuscitation of the microorganism before enrichment and enumeration.

To increase the level of reassurance, an additional and *E. coli*-specific method (ISO 7251:2005) has been applied for all regulatory batches and the success criteria has been set and confirmed to be "absent in 10 g" of test article.

The absence of the production organism in the finished ingredient is also supported by analyses for residual DNA in final production batches. As demonstrated in Table 2.3.3.3-1, the absence of residual DNA from the production organism is confirmed by 3 different validated qPCR methods. These qPCR methods target short subsequences of the inserted fucosyltransferase and colonic acid genes as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of 5 independent batches of 2'-FL/DFL product demonstrate no detectable levels of residual DNA (limit of quantification of 4 µg/kg or 4 ppb) present in the final ingredient.

Table 2.3.3.3-1 Levels of Residual DNA in 5 Batches of 2'-FL/DFL Produced by Fermentation

Parameter	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Residual DNA by qPCR (fucosyltransferase gene)	< LOQ[a]	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		
Residual DNA by qPCR (colonic acid synthesis gene)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		
Residual DNA by qPCR (23S assay)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; DNA = deoxyribonucleic acid; LOQ = limit of quantitation; qPCR = quantitative polymerase chain reaction; SD = standard deviation.
[a] LOQ = 4 µg/kg (parts per billion).

2.3.3.4 Residual Anions, Trace Elements, and Heavy Metals

Due to the nature of the fermentation process, the 2'-FL/DFL preparation would theoretically have a potential to contain trace elements and minerals (as carry-over from the fermentation medium). However, the use of nanofiltration and ion-exchange is sufficient to reduce any appreciable carry-over of minerals from fermentation into the final ingredient. The results of trace element analyses are presented in Table 2.3.3.4-1. The averaged sum of sulphated ash does not exceed 0.05%; therefore, trace elements were not considered as compositional data of 2'-FL/DFL. In addition, the trace element measurements included the confirmation that toxic heavy metals (such as lead) are not present.

Table 2.3.3.4-1 Levels of Anions, Trace Elements, and Heavy Metals in 5 Batches of 2'-FL/DFL Produced by Fermentation

Parameter	Manufacturing Batch Numbers:							
	CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	±	SD
Orthophosphate by UV [%]	0.0178	0.0009	< 0.0006	< 0.0008	< 0.0007	**0.0042**	±	**0.0076**
Sulphate by IC [%]	0.07	< 0.01	< 0.01	< 0.01	< 0.01	**0.02**	±	**0.03**
Chloride by IC [%]	< 0.002	< 0.002	0.010	0.013	< 0.002	**0.006**	±	**0.005**
Ammonium by UV [%]	< 0.0002	< 0.0002	< 0.0002	< 0.0003	< 0.0002	**0.0002**	±	**0.0000**
Sodium (Na) [mg/kg]	160	150	130	230	50	**144**	±	**65**
Potassium (K) [mg/kg]	< 50	< 50	< 50	< 50	< 50	**< 50**		
Magnesium (Mg) [mg/kg]	50	40	70	50	70	**56**	±	**13**
Iron (Fe) [mg/kg]	< 10	< 10	< 10	< 10	< 10	**< 10**		
Copper (Cu) [mg/kg]	< 0.1	0.2	< 0.1	< 0.1	< 0.1	**0.1**	±	**0.0**
Manganese (Mn) [mg/kg]	< 0.1	< 0.1	< 0.1	0.2	< 0.1	**0.1**	±	**0.0**
Zinc (Zn) [mg/kg]	0.3	0.3	0.3	0.2	0.5	**0.3**	±	**0.1**
Lead (Pb) [mg/kg]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	**< 0.1**		
Ash, sulphated [%]	0.02	0.06	0.03	0.01	0.07	**0.04**	±	**0.03**

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; IC = ion chromatography; SD = standard deviation; UV = ultraviolet.

2.4 Stability

Storage (real-time and accelerated) and stressed (forced) stability studies on the pure ("bulk") powdered 2'-FL/DFL were conducted by accredited external laboratories in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Guidelines (*Stability Testing of New Drug Substances and Products*) (ICH, 2003), in order to:

i) Test the 2'-FL/DFL stability during storage;
ii) Investigate degradation pathways when exposed to selected stress factors; and
iii) Define the optimal storage conditions and corresponding re-test dates or shelf-lives.

For the bulk 2'-FL/DFL product, experiments were performed in solid state (in form of amorphous powder) and in liquid form (as aqueous solutions). An accelerated stability study for the bulk 2'-FL product in solid state was conducted.

Stability studies in processed foods included new studies in powdered infant formula and previous studies with 2'-FL in powdered infant formula and other foods.

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of the powdered 2'-FL/DFL produced from microbial fermentation, as described herein, was investigated, under both real-time conditions [25°C, 60% relative humidity (RH)] and accelerated conditions (40°C, 75% RH). The chemical, physical, microbiological, and sensory testing was performed in an ongoing 5-year storage study (25°C, 60% RH) on 2 representative batches (No. CPN6317 1000517 FD and CPN6317 1000917 FD), with interim results available up to 9 months at the time of filing (see Table 2.4.1.1-1). The results further confirm that the ingredient is stable when stored at ambient room temperature for at least 9 months.

2.4.1.2 Accelerated Stability

The 2-year accelerated stability evaluation was performed for powdered 2'-FL/DFL under controlled storage conditions (40°C, 75% RH), with interim results available to 9 months at the time of filing (see Table 2.4.1.2-1). Chemical, physical, microbiological, and sensory testing was conducted. As with the real-time stability testing, no appreciable changes in organoleptic properties nor degradation of the ingredient or alterations in impurity profiles were reported.

2'-FL/DFL was stable throughout the 6-month storage period with no measurable loss of 2'-FL/DFL, other carbohydrates or change in impurities content. As with the real-time stability testing, no appreciable degradation of the ingredient or alterations in impurity profiles were reported. Based on the results of the accelerated stability study and using the Arrhenius equation (Peleg *et al.*, 2012), the stability of the ingredient was calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity. A 2-year accelerated stability study and a real-time 5-year stability study on representative batches of 2'-FL/DFL are currently ongoing.

Table 2.4.1.1-1 Representative Interim Results of the 5-Year Real-Time Stability Study on 2'-FL/DFL (25°C, 60% Relative Humidity, RH) for 2 Representative Batches (No. CPN6317 1000517 FD and CPN6317 1000917 FD)

Parameter	Sample Time (Months)							
	0		3		6		9	
	Manufacturing Batch Numbers:							
	CPN6317 1000517	CPN6317 1000917	CPN6317 1000517	CPN6317 1000917	CPN6317 1000517	CPN6317 1000917	CPN6317 1000517	CPN6317 1000917
Physical Properties								
Color	White	White	White	White	White	White	White	NT
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Powder	Powder	Uniform sized, non-agglutinated powder	NT
Purity								
Water content [%]	0.44	1.24	0.60	1.09	0.40	1.03	0.51	NT
Assay (water free) 2'-FL [%]	82.5	79.2	82.8	79.0	83.0	78.03	82.9	NT
Assay (water free) DFL [%]	9.64	11.43	9.65	11.69	9.85	11.48	9.90	NT
D-Lactose [%]	0.96	3.38	0.98	3.44	0.89	3.41	1.01	NT
L-Fucose [%]	0.06	0.10	0.06	0.09	0.08	0.09	0.08	NT
2'-Fucosyl-D-lactulose [%]	0.94	0.81	0.95	0.87	0.72	0.56	0.61	NT
Glucose [%]	0.00	0.03	0.03	0.03	0.02	0.02	0.02	NT
2-Fucosyl-galactose [%]	0.38	0.53	0.45	0.52	0.49	0.44	0.47	NT
3-FL [%]	0.00	NT	0.00	NT	0.00	NT	0.00	NT
Assay (water free) HiMS [%]	93.2	94.3	93.5	94.3	93.8	93.1	93.9	NT
Microbiological Quality								
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	NT	NT	NT	NT	NT	NT
Salmonella spp.	Absent in 25 g	Absent in 25 g	NT	NT	NT	NT	NT	NT
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent in 10 g	NT	NT	NT	NT	NT	NT
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	NT	NT	NT	NT	NT	NT
Bacillus cereus	< 10	< 10	NT	NT	NT	NT	NT	NT
Yeasts [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT
Molds [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; 3-FL = 3-Fucosyllactose; CFU = colony-forming units; HiMS = human-identical milk saccharides = Sum of 2'-FL, 3-FL, DFL, lactose and fucose; NT = not tested.

Table 2.4.1.2-1 Representative Interim Results of the 2-Year Accelerated Stability Study on 2'-FL/DFL (40°C, 70% Relative Humidity, RH) for 2 Representative Batches (No. CPN6317 1000517 FD and No. CPN6317 1000917 FD)

Parameter	Sample Time (Months)											
	0		1		2		3		6		9	
	Manufacturing Batch Number:											
	CPN6317 1000517 FD	CPN6317 1000917 FD	CPN6317 1000517 FD	CPN6317 1000917 FD	CPN6317 1000517 FD	CPN6317 1000917 FD	CPN6317 1000517 FD	CPN6317 1000917 FD	CPN6317 1000517 FD	CPN6317 1000917 FD	CPN6317 1000517 FD	CPN6317 1000917 FD
Physical Properties												
Color	White	White	White	White	White	White	White	White	White	White	White	NT
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Non-agglutinated uniformly sized powder	Fine powder	Non-agglutinated uniformly sized powder	NT
Purity												
Water content [%]	0.44	1.24	0.62	0.87	0.52	0.82	0.66	0.75	0.40	0.55	0.40	NT
Assay (water free) 2'-FL [%]	82.5	79.2	84.3	78.1	82.9	78.3	83.0	77.9	82.4	78.88	82.4	NT
Assay (water free) DFL [%]	9.64	11.43	9.77	11.37	9.65	11.51	9.63	11.61	9.75	11.57	9.83	NT
D-Lactose [%]	0.96	3.38	0.99	3.37	0.98	3.54	0.99	3.42	0.94	3.35	1.02	NT
L-Fucose [%]	0.06	0.10	0.06	0.11	0.06	0.09	0.06	0.10	0.08	0.09	0.08	NT
2'-Fucosyl-D-lactulose [%]	0.94	0.72	0.76	0.78	0.75	0.73	0.96	0.63	0.73	0.57	0.62	NT
Glucose [%]	0.00	0.03	0.01	0.02	0.01	0.03	0.03	0.03	0.03	0.02	0.03	NT
2-Fucosyl-galactose [%]	0.38	0.53	0.42	0.47	0.39	0.53	0.44	0.52	0.49	0.45	0.47	NT
3-FL [%]	0.00	NT	0.00	n.d.	0.00	NT	0.00	NT	0.00	NT	0.00	NT
Assay (water free) HiMS [%]	93.2	94.3	95.2	93.1	93.6	93.5	93.7	93.1	93.2	94	93.3	NT
Microbiological Quality												
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT	< 10	< 10	NT	NT

Table 2.4.1.2-1 Representative Interim Results of the 2-Year Accelerated Stability Study on 2'-FL/DFL (40°C, 70% Relative Humidity, RH) for 2 Representative Batches (No. CPN6317 1000517 FD and No. CPN6317 1000917 FD)

Enterobacteriaceae	Absent in 10 g	Absent in 10 g	NT	NT	NT	NT	NT	NT	Absent in 10 g	Absent in 10 g	NT	NT
Salmonella spp.	Absent in 25 g	Absent in 25 g	NT	NT	NT	NT	NT	NT	Absent in 25 g	Absent in 25 g	NT	NT
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent in 10 g	NT	NT	NT	NT	NT	NT	Absent in 10 g	Absent in 10 g	NT	NT
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	NT	NT	NT	NT	NT	NT	Absent in 25 g	Absent in 25 g	NT	NT
Bacillus cereus	< 10	< 10	NT	NT	NT	NT	NT	NT	< 10	< 10	NT	NT
Yeasts [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT	< 10	< 10	NT	NT
Molds [CFU/g]	< 10	< 10	NT	NT	NT	NT	NT	NT	< 10	< 10	NT	NT

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; 3-FL = 3-fucosyllactose; CFU = colony-forming units; HiMS = human-identical milk saccharides = Sum of 2'-FL, 3FL, DFL, lactose and fucose; NT = not tested

2.4.1.3 Stress/Forced Stability

The stress (forced) stability studies described herein, were performed according to the International Conference on Harmonisation Guidelines (*Stability Testing of New Drug Substances and Products*) and aimed to identify the likely degradation products under harsh, stress conditions.

Forced stability tests of the bulk 2'-FL/DFL in aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively:

- Amorphous 2'-FL/DFL powder at acidic pH (4.1 and 4.5)
- Crystalline 2'-FL powder at slightly acidic pH (5.4) as reference
- Amorphous 2'-FL/DFL powder at neutral pH (6.2 and 7.0)

The results of these studies showed the action of 2 potential pH-dependent chemical degradation pathways in aqueous solutions of 2'-FL/DFL, namely hydrolysis at pH <5.0 and isomerization at pH >6.0. At neutral pH, 2'-FL and DFL underwent minor isomerization to fucosyl-lactulose and difucosyl-lactulose, correspondingly. At low pH, 2'-FL hydrolyzed mainly to fucose and lactose, and slightly to 2-fucosyl-galactose and glucose; the hydrolyzation of DFL to 3-fucosyllactose and fucose was not reported. The latter reaction has been documented in an additional forced stability test conducted on 2'-FL/DFL syrup, thermally treated (80°C for 1 hour) and stored at 60°C for 4 weeks.

2.4.2 Stability Under the Intended Conditions of Use

2.4.2.1 Stability in Powdered Infant Formula

The stability of 2'-FL/DFL in powdered infant formula was investigated using a high-performance liquid chromatography (HPLC) with fluorescent detection following long-term storage at temperatures of 4, 20, 30 and 37°C.

The infant formula powder tested was a whey-based commercially available starter formula supplemented with 2'-FL/DFL. The infant formula also contained long chain polyunsaturated fatty acids, and vitamins and minerals at concentrations intended for full nutritional support of infants from birth to 6 months of age. The interim results available up to 12 months are presented in Table 2.4.2.1-1. No losses were reported for 2'-FL and DFL between T0 and the 3, 6, 9 and 12-months, which demonstrates good stability of these compounds after 12 months storage under the test conditions.

Table 2.4.2.1-1 Interim Results of Stability of 2'-FL/DFL in a Commercially Representative Infant Formula Following Storage for up to 12 months at Various Temperatures

Temperature	Target*	T0	Sample Time (Months)			
	g/100g IF		3	6	9	12
2'-FL						
4°C	1.77	1.84	1.88	1.84	1.98	2.00
20°C			1.94	1.90	1.92	1.95
30°C			1.82	1.88	1.99	1.98
37°C			1.86	1.86	1.88	1.94
DFL						
4°C	0.25	0.24	0.24	0.24	0.25	0.25

20°C	0.25	0.25	0.25	0.25
30°C	0.23	0.24	0.26	0.25
37°C	0.24	0.24	0.24	0.25

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; IF = infant formula.
* Targeted concentration of human identical milk oligosaccharide per 100 g of IF.

2.4.2.2 Stability in Other Food Matrices

The stability of chemically-synthesized 2'-FL preparations formulated into various food matrices (including yogurts, ready-to-drink flavored milk, and citrus fruit beverages) has been previously discussed in Section II.D.2.2 of GRN 546 (U.S. FDA, 2015a). Stability studies were conducted using formulations representative of commercial food products on the market and under typical processing (*i.e.*, pasteurization and/or UHT heating) and typical storage conditions (*e.g.*, temperature and shelf-life) for such products. The analytical data demonstrated no loss of 2'-FL in yoghurt, a citrus fruit drink, or ready-to drink chocolate-flavored milk at each time point tested when compared to the initial 2'-FL concentration. Testing in a citrus fruit drink and ready-to drink chocolate-flavored milk also demonstrated no loss of 2'-FL between pre-processing and the start of the stability study; pasteurization and/or UHT heating did not affect the stability of 2'-FL in these foods. The analytical data demonstrated that 2'-FL was stable when added to yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk following typical processing conditions and when stored at 4°C for the duration of the shelf-life of these foods. Liquid matrices, moderate changes of pH and diverse heat challenges did not impact the stability of 2'-FL to a significant degree.

Part 3. Dietary Exposure

3.1 History of Use of the GRAS Substance and/or of its Source

3.1.1 2'-FL/DFL

To the best of our knowledge, 2'-FL/DFL as described herein, has not been previously concluded to be GRAS.

2'-FL, the major constituent of 2'-FL/DFL, produced by chemical synthesis or by fermentation, has GRAS status for use in infant formula and other conventional food products in the U.S. (GRN 546, 571, 650, 735, 749). A number of 2'-FL preparations manufactured by Glycom and other manufacturers have been authorized in the European Union (EU), Israel, and Singapore as novel food ingredients. Market authorizations are pending worldwide. The results of the evaluations that have been completed to date are summarized in the following sections.

All authorized approaches to manufacture 2'-FL to date lead to some levels of DFL being co-isolated, and consequently, some 2'-FL sources have been authorized as "2'-FL alone" with up to 5.0% of DFL.

3.1.2 2'-FL Alone or in Combination with LNnT

3.1.2.1 United States

2'-FL Produced by Fermentation

Glycom's 2'-FL product obtained by fermentation from a modified strain of *E. coli* K-12 has been concluded to be GRAS for use in term infant formula at a maximum level of 2.4 g 2'-FL per liter, and for use in various

other foods [including other baby foods and drinks for infants and young children such as toddler formula, beverages and beverage bases, dairy product analogues, grain products and pastas, milk (whole and skim), milk products, processed fruits and juices, and processed vegetables and juices] at maximum levels ranging from 0.084 to 2.4 g of 2'-FL per serving. The GRAS status was notified to the U.S. Food and Drug Administration (FDA) in 2016 and filed as GRN 650 without objection (U.S. FDA, 2016a). The DFL content of this 2'-FL preparation (GRN 650) was specified as less than 1.0%.

DuPont Nutrition and Health (DuPont)'s 2'-FL product obtained from fermentation of lactose and sucrose using a modified strain of *E. coli* K-12 (MG1655 INB3051) was concluded to be GRAS for use in term infant formula, infant and toddler foods, and in toddler drinks up to 2.4 g/L, 12 g/kg, and 1.2g/L, respectively. The GRAS status was notified to the FDA in 2017 and filed without objection by the agency under GRN 749 (U.S. FDA, 2018c). DuPont describes its 2'-FL (GRN 749) product as containing a minimum of 82% 2'-FL, and with a DFL content less than 7%.

Glycosyn, LLC (Glycosyn) and FrieslandCampina Domo B.V. (FrieslandCampina)'s 2'-FL products obtained from fermentation of lactose and sucrose *E. coli* K-12 (GI724 referred to as E997) also was concluded to be GRAS for use in milk and soy-based term infant formula (up to 2.4 g/L), infant and toddler foods (at 0.24 to 1.2 g/serving), and in various other foods (0.28 to 1.2 g/serving; beverages and beverage bases, breakfast cereals, dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; jams and jellies; milk and milk products; processed fruits and fruit juices; and sweet sauces, toppings, and syrups). The GRAS status was notified to the FDA in 2017 and filed by the agency without objection under GRN 735 (U.S. FDA, 201d8). Glycosyn and FrieslandCampina describes its 2'-FL (GRN 735) ingredient as containing a minimum of 90% 2'-FL (DFL is not included in the specifications provided in the FDA response letter).

Jennewein Biotechnologie GmbH, Germany, another manufacturer of 2'-FL, uses a different fermentation method and concluded the use of their ingredient to be GRAS when used in term infant formula and toddler formula at levels of up to 2.0 g 2'-FL per liter (GRN 571), to which the FDA has issued a "no questions" response (U.S. FDA, 2015b). This ingredient is prepared from the fermentation of *E. coli* BL21 (DE3) derived strain. The DFL content of this 2'-FL preparation (GRN 571) has been specified as less than 5.0%.

2'-FL Produced by Chemical Synthesis

2'-FL produced by Glycom using chemical synthesis has been concluded to be GRAS for use as an ingredient in term infant formula at a maximum level of 2.4 g 2'-FL per liter, and in various foods [including baked goods and baking mixes, beverages and beverage bases, coffee and tea, dairy product analogues, infant and toddler foods, grain products and pastas, milk (whole and skim), processed fruits and juices, processed vegetables and juices, and sugar substitutes] at maximum levels ranging from 0.084 to 2.4 g of 2'-FL per serving. Glycom's GRAS conclusion was notified to the U.S. FDA on October 10th, 2014, and filed without objection by the Agency under GRN 546 (U.S. FDA, 2015a). The DFL content of 2'-FL produced by chemical synthesis is specified as less than 1.0%.

3.1.2.2 EU

2'-FL from both synthetic and microbial processes has been approved for use in a number of food applications (See Table 3.1.2.2-1) under Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods[5].

Table 3.1.2.2-1 Permitted Uses of 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470]

Authorised Novel Food	Conditions under which the Novel Food May Be Used	Additional Specific Labelling Requirements	her Requirements
2'-Fucosyllactose	***Specified Food Category***	***Maximum Levels***	1. The designation of the novel food on the labelling of the foodstuffs containing it shall be '2'-fucosyllactose'. 2. The labelling of food supplements containing 2'-fucosyllactose shall bear a statement that the supplements should not be used if other foods with added 2'-fucosyllactose are consumed the same day. 3. The labelling of food supplements containing 2'-fucosyllactose intended for young children shall bear a statement that the supplements should not be used if breast milk or other foods with added 2'-fucosyllactose are consumed the same day
	Unflavoured pasteurised and sterilised (including UHT) milk-based products	1,2 g/L	
	Unflavoured fermented milk-based products	1,2 g/L beverages	
		19,2 g/kg products other than beverages	
	Flavoured fermented milk-based products including heat-treated products	1,2 g/L beverages	
		19,2 g/kg products other than beverages	
	Dairy analogues, including beverage whiteners	1,2 g/L beverages	
		12 g/kg for products other than beverages	
		400 g/kg for whitener	
	Cereal bars	12 g/kg	
	Table-top sweeteners	200 g/kg	
	Infant formula as defined in Regulation (EU) No 609/2013[a]	1,2 g/L alone or in combination with up to 0,6 g/L of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Follow-on formula as defined in Regulation (EU) No 609/2013	1,2 g/L alone or in combination with up to 0,6 g/L of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Processed cereal-based food and baby food for infants and young children as defined in Regulation (EU) No 609/2013	12 g/kg for products other than beverages	
		1,2 g/L for liquid food ready for use, marketed as such or reconstituted as instructed by the manufacturer	

[5] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, p. 72–201.

Table 3.1.2.2-1 Permitted Uses of 2'-FL [Reproduced from Table 1 of Commission Implementing Regulation (EU) 2017/2470]

Authorised Novel Food	Conditions under which the Novel Food May Be Used	Additional Specific Labelling Requirements	Other Requirements
	Milk-based drinks and similar products intended for young children	1,2 g/L for milk-based drinks and similar products added alone or in combination with up to 0,6 g/l lacto-*N*-neotetraose, at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	
	Foods for special medical purposes as defined in Regulation (EU) No 609/2013	In accordance with the particular nutritional requirements of the persons for whom the products are intended	
	Total diet replacement for weight control as defined in Regulation (EU) No 609/2013	4,8 g/L for drinks	
		40 g/kg for bars	
	Bread and pasta products bearing statements on the absence or reduced presence of gluten in accordance with the requirements of Commission Implementing Regulation (EU) No 828/2014[b]	60 g/kg	
	Flavoured drinks	1,2 g/L	
	Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; tea, herbal and fruit infusions, and chicory extracts; tea, plant, fruit, and cereal preparations for infusions, as well as mixes and instant mixes of these products	9,6 g/L – the maximum level refers to the products ready to use	
	Food supplements as defined in Directive 2002/46/EC[c], excluding food supplements for infants	3,0 g/day for general population	
		1,2 g/day for young children	

2'-FL = 2'-fucosyllactose; EU = European Union; UHT = ultra-high temperature.

[a] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181, 29.6.2013, p. 35–56.

[b] Commission Implementing Regulation (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. OJ L 228, 31.7.2014, p. 5–8.

[c] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183, 12.7.2002, p. 51–57.

The abovementioned Union list of novel foods covers:

1. The previous EU formal approval obtained by Glycom under Regulation (EC) No 258/97, Commission Implementing Decision (EU) 2016/376[6] of 11 March 2016 authorizing the placing on the market of 2'-*O*-fucosyllactose as a novel food ingredient, which is based upon the European Food Safety Authority (EFSA) Scientific Opinion on the safety of 2'-O-fucosyllactose as a novel food ingredient and the EFSA Statement on the safety of LNnT and 2'-O-fucosyllactose as novel food ingredients in food supplements for children (EFSA, 2015a,b). The DFL content of chemical synthetic 2'-FL has been specified as less than 1.0%.

2. The Substantial Equivalence Notification of Glycom for the same uses for 2'-*O*-fucosyllactose from the microbial source "Genetically modified strain of *Escherichia coli* K-12", which was based on the Safety Assessment from the Food Safety Authority of Ireland (FSAI, 2016). The DFL content of microbial 2'-FL from *E. coli* K-12 has been specified as less than 1.0%.

3. Commission Implementing Decision (EU) 2017/2201 of 27 November 2017[7] authorizing the placing on the market of 2'-fucosyllactose produced with a genetically modified strain of *Escherichia coli* BL21 as a novel food ingredient, which is based on the opinion of the expert Committee of the Netherlands plus Member States comments and applicant responses (CBG-MEB, 2016). There is no EFSA opinion in this case. The DFL content of microbial 2'-FL from *E. coli* BL21 has been specified as less than 5.0%.

4. The Substantial Equivalence Notification of DuPont Nutrition & Biosciences ApS for 2'-fucosyllactose from the microbial source "Genetically modified strain of *Escherichia coli* K-12", which was based on the Safety Assessment from the Novel Food Unit of the Health Council of the Netherlands (CBG-MEB, 2017a). The DFL content of this 2'-FL source has been specified as less than 2.0%.

5. The Substantial Equivalence Notification of FrieslandCampina Nederland BV for 2'-fucosyllactose from the microbial source "Genetically modified strain of *Escherichia coli* K-12", which was based on the Safety Assessment from the Novel Food Unit of the Health Council of the Netherlands (CBG-MEB, 2017b). The DFL content of this 2'-FL source has not been disclosed.

3.1.2.3 Israel

2'-FL obtained by fermentation as produced by Glycom and as produced by Jennewein has been authorized as a novel food in Israel for use in milk-based infant formulas (ages 0 to 6 months) and follow-on formulas (ages 6 to 12 months), at levels of up to 2.0 g 2'-FL per liter of the ready-to-feed product (Israel MOH, 2017, 2018). The DFL content of the Jennewein preparation has not been disclosed in the publicly available part of the authorization but is presumed to be less than 5.0% as it is likely the same product as described in GRN 571.

[6] Commission Implementing Decision (EU) 2016/376 of 11 March 2016 authorizing the placing on the market of 2'-O-fucosyllactose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2016) 1423). OJ L 70, 16.3.2016, p. 27–31.

[7] Commission Implementing Decision (EU) 2017/2201 of 27 November 2017 authorising the placing on the market of 2'-fucosyllactose produced with *Escherichia coli* strain BL21 as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2017) 7662). OJ L 313, 29.11.2017, p. 5–9.

3.1.2.4 Singapore

In 2017, the Agri-Food & Veterinary Authority of Singapore (AVA) concluded that 2'-FL obtained by fermentation, as produced by Glycom, is permitted as an ingredient in infant formula (including follow-on formula) for infants 0 to 12 months old and growing up milk (for children aged 12 to 36 months), up to a level of 1.2 g 2'-FL per liter of reconstituted formula. The AVA has indicated that growing up milks containing the 2'-FL ingredient at the proposed level of use is now permitted for sale in Singapore, and the Food Regulations has been amended accordingly[8]. The DFL content of this 2'-FL preparation has been specified as less than 1.0%.

3.1.3 History of Consumption in Breast Milk

3.1.3.1 Human biology background relevant to 2'-FL and DFL

2'-FL and DFL are 2 important and significant components 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 (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). These are known as HMOs because they were first discovered in human breast milk (Malpress and Hytten, 1958) at much higher concentrations than in any other mammalian milk (Urashima *et al.*, 2001). More than 140 members of this family have been fully described on a structural basis (Urashima *et al.*, 2011; Chen, 2015), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al*., 1999; Wu *et al*., 2010, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012) although high variations are reported on individual level and in dependency of the lactation period and the genotype of the mother. Concentrations of oligosaccharides in bovine milk are approximately 20-fold lower than levels in human milk and consists of a less complex oligosaccharide mixture (Tao *et al.*, 2009; Aldredge *et al*., 2013; Urashima *et al*., 2013) that is largely absent fucosylated oligosaccharides (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Urashima *et al*., 2012).

2'-FL and DFL belong to the "fucosylated" sub-fraction of HMOs, oligosaccharides that contain the sugar fucose and which is reported to constitute on average around 70% of the total HMO fraction. The fucosylated sub-fraction of HMOs is biosynthesized from lactose or the "core" HMOs (HMOs that are not decorated by either fucose or sialic acid) by specific enzymes called the fucosyltransferases. The fucosyltransferase-encoding genes are better known as Lewis or Secretor genes and are intrinsically connected to the human blood groups. Fucosylated HMOs played an important role in the discovery of the human blood and Lewis groups. Ground-breaking work performed by Victor Ginsburg (Kobata *et al*., 2004) and Akira Kobata (Endo, 2010) in the 1960s has revealed the enzymatic basis for the human blood groups, which are carbohydrate-based cell-surface antigens, and their close structural and biosynthetic relationship to the freely occurring milk oligosaccharides (Kobata *et al*., 1968; Shen *et al*., 1968; Grollman *et al*., 1969). In this context, it was initially recognized in 1967 that not all mothers excrete 2'-FL (nor DFL) into their milk, because they do not express a specific enzyme that is needed for 2'-FL (or DFL) biosynthesis (Grollman and Ginsburg, 1967). This observation is not limited to the freely occurring 2'-FL in milk but includes other cell-surface bound "2'-FL" epitopes[9] that are typically secreted into other bodily fluids like blood and saliva. Therefore, the term "non-Secretor" was coined for this phenotype. The majority of mothers (roughly 80%

[8] Available at: https://www.ava.gov.sg/docs/default-source/legislation/sale-of-food-act/food-(amendment)-regulations-2018.pdf?sfvrsn=2 (Singapore Agri-Food & Veterinary Authority, 2018).

[9] The structure of 2'-FL represents a simple structural mimic of the "H-epitope" of human blood group epitopes, where the N-acetylglucosamine carbohydrate of the H-antigen has been replaced by a glucose carbohydrate in 2'-FL.

of the global population) express 2'-FL and DFL in their milk, and are termed "Secretors", as described in further detail below. Based on this initial finding, it was established that human milk can be categorized into 4 different "milk groups" (*i.e.*, phenotypes) based on the presence (or absence) of distinct structural features in their oligosaccharide fraction and that these different phenotypes contain a significantly different amount of fucosylated HMOs in general and 2'-FL (or DFL) in particular.

The categorization of milk types is related and comparable to "blood groups", but with the important difference that all mothers are "universal donors" of milk, as can be concluded from the long and safe tradition of wet nursing that was commonplace before the invention of infant formulas or the use of donor milk by milk banks (Stevens *et al.*, 2009; Bertino *et al*., 2013; Mason *et al.*, 2013). It means that all milk groups are fundamentally safe for an infant; however, there are indeed differences in the nutritional effects of each milk group for the infant which were reported in observational clinical trials where mother-child pairs were stratified according to milk phenotypes. The scientific basis and structural consequences of the 4 different milk groups are summarized below, since these are fundamental to understanding the biological roles of 2'-FL and DFL. The milk groups are categorized according to the presence (or absence) of the specific oligosaccharide products of 2 distinct fucosyltransferase enzymes, namely α1,2- and α1,4 fucosyltransferases, encoded by the genes FUT2 and FUT3, respectively (see Table 3.1.3.1-1).

Table 3.1.3.1-1 Milk Groups by Maternal Secretor and Lewis Phenotypes

Milk Group	**1**	**2**	**3**	**4**
Milk Phenotype	Se^{+} / $Le^{(a-b+)}$	Se^{-} / $Le^{(a+b-)}$	Se^{+} / $Le^{(a-b-)}$	Se^{-} / $Le^{(a-b-)}$
α-1,2-fucosylated HMOs produced by α-1,2-FT enzyme gene FUT2	**+**	**–**	**+**	**–**
α-1,3-fucosylated HMOs produced by α-1,3-FT enzyme genes FUT3, FUT5 and FUT6[a]	**+**	**+**	**+**	**+**
A-1,4-fucosylated HMOs produced by α-1,4-FT enzyme gene FUT3[b]	**+**	**+**	**–**	**–**
Typically reported frequency[c]	~ 70%	~ 20%	~ 9%	~ 1%

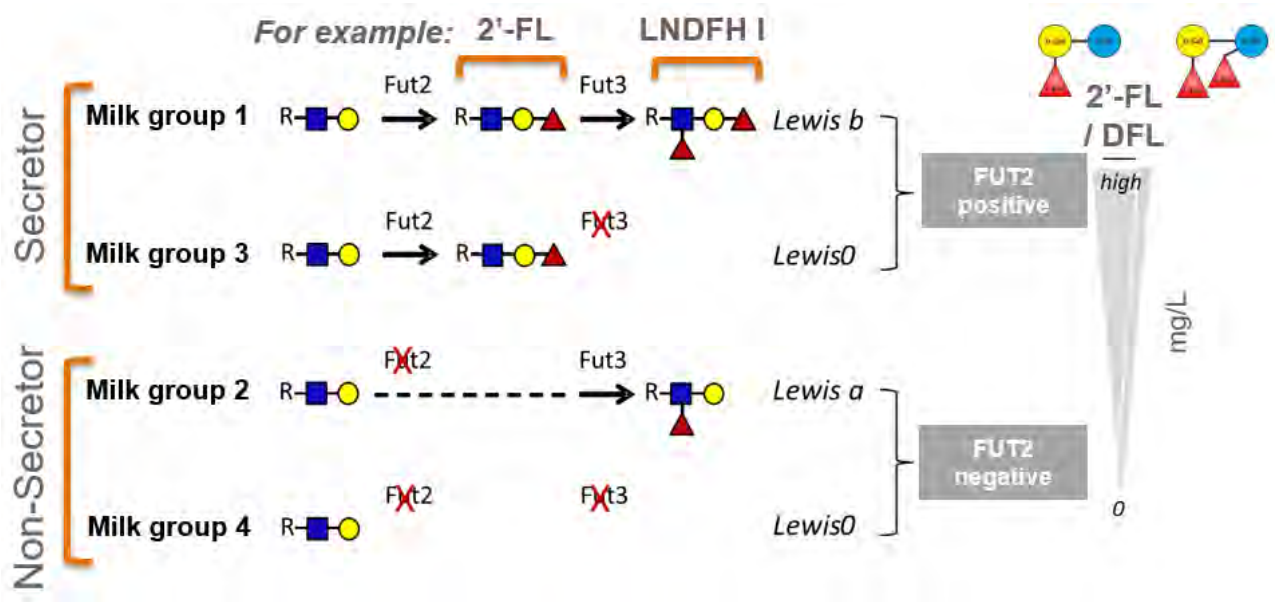
HMOs = human milk oligosaccharides; FT = fucosyltransferase; Se^{+} = Secretor; Se^{-} = non-Secretor; Le = Lewis blood group.
[a] α-1,3-fucosylated structures are synthesized by different FT enzymes.
[b] The FUT3 gene possesses α-1,3 and α-1,4 FT activity.
[c] As reported in the literature (Thurl *et al*., 2010; Castanys-Muñoz *et al*., 2013; Austin *et al*., 2016).

Expressed in words:

- Group 1. Lewis-positive Secretor (Se^{+}) mothers with Lewis blood group (Le^{a-b+}) express both FUT2 and FUT3 and thus can synthesize both α-1,2-fucosylated HMO and α-1,4-fucosylated HMOs.
- Group 2. Lewis-positive non-Secretor (Se^{-}) mothers with Lewis blood group (Le^{a+b-}) express FUT3 but not FUT2 and thus produce α-1,4-fucosylated HMOs but not α-1,2-fucosylated HMOs.
- Group 3. Lewis-negative Secretor (Se^{+}) mothers with Lewis blood group (Le^{a-b-}) express FUT2 but not FUT3 and therefore synthesize α-1,2-fucosylated HMOs but not α-1,4-fucosylated HMOs.
- Group 4. Lewis-negative non-Secretor (Se^{-}) mothers with Lewis blood group (Le^{a-b-}) express neither FUT2 nor FUT3 and cannot produce either α-1,2-fucosylated HMOs nor α-1,4-fucosylated HMOs.

The concentration of fucosylated HMO in mothers' milk, and particularly that of 2'-FL and DFL, is in ascending order from milk group 4 < 2 < 3 < 1 as is shown in the Figure 3.1.3.1-1. Simplified[10], it can be said that 2'-FL and DFL occur only in the milk of mothers with the Secretor phenotype (milk groups 1 and 3), and that they always occur concurrently.

Figure 3.1.3.1-1 Concentration of 2'-Fucosyllactose and Difucosyllactose (2'-FL and DFL) Increases from Milk Group 4 < 2 < 3 < 1



The prevalence of these 4 milk groups varies to some extent globally according to ethnicity or geographical location but is typically in most ethnicities including the Caucasian and Chinese population; around 70% Group 1 (express FUT2 and FUT3), 19% Group 2 (express FUT3 but not FUT2), 10% Group 3 (express FUT2 but not FUT3), and 1% Group 4 (no expression of either FUT2 or FUT3) (Thurl *et al*., 2010; Castanys-Muñoz *et al.*, 2013; Austin *et al*., 2016). it can be stated that roughly 80% of the global population are Secretors and express 2'-FL and DFL into their milk. These phenotypes (*i.e.*, Secretor and Lewis) can be best understood in the context of general human polymorphisms for the carbohydrate histo-blood group and Lewis antigens (refer to excellent overview articles for more detail) (Oriol *et al*., 1986; Ferrer-Admetlla *et al*., 2009; Hod *et al*., 2009).

3.1.3.2 Quantity of 2'-FL in Breast Milk

The concentration of 2'-FL in human milk has been measured and reported to date in at least 28 independent publications [reviewed recently by Thurl *et al*. (2017)]. The following table summarizes the levels of 2'-FL that have been reported in breast milk across these various studies independently (Table 3.1.3.2-1). The data demonstrate that 2'-FL is the most abundant HMO of pooled human milk, though approximately 20% of women (termed "non-Secretors") do not express the α-1,2-fucosyltransferase enzyme in their mammary glands and thus their milk does not contain 2'-FL (Castanys-Muñoz *et al.*, 2013; Austin *et al.*, 2016). This enzyme is responsible for the fucosylation of oligosaccharides and lactose at the 2-position of galactose, resulting in the production of 2'-FL among others. In this context it is noted that the

[10] "Simplified" because the relationship of genotype to phenotype is rather complex when looking at the polymorphism in a population as a whole, due to a high number of different mutations in the affected genes that may lead to full or partial knock-out of a phenotype [*e.g.*, see Ferrer-Admetlla *et al.* (2009)].

diversity ("polymorphism") of the female population in regard to the Secretor genotype appears to have been maintained over evolutionary times due to a "parent-offspring conflict" (Springer and Gagneux, 2013). While the non-Secretor phenotype (*i.e.*, the resulting absence of characteristic cell-surface glycans) appears to provide a net benefit to the mother to escape some infectious agents (Marionneau *et al.*, 2005; Lindén *et al.,* 2008; Carlsson *et al.*, 2009), there is a growing body of evidence that the breastfed infant actually benefits from the inverse situation: the Secretor phenotype as expressed into milk (*i.e.*, presence of the same characteristic glycans as free oligosaccharides in milk) (Morrow *et al*., 2004; Newburg *et al*., 2004; Lewis *et al*., 2015; Smith-Brown *et al*., 2016).

Table 3.1.3.2-1 The 2'-FL Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Pooled Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 1.0 to 8.4 g/L** Average: 3.2 g/L	Erney *et al.* (2000); Morrow *et al.* (2004); Asakuma *et al.* (2008); Spevacek *et al.* (2015)
Days 5 to 14 ("transitional milk")	**Reported Range: 2.1 to 2.8 g/L** Average: 2.5 g/L	Erney *et al.* (2000); Spevacek *et al.* (2015); Austin *et al.* (2016)
Days 10 to 60 ("mature milk")	**Reported Range: 0.7 to 3.9 g/L** Average: 2.2 g/L	Chaturvedi *et al.* (1997, 2001a); Erney *et al.* (2000, 2001); Musumeci *et al.* (2006); Spevacek *et al.* (2015); Austin *et al.* (2016); McGuire *et al.* (2017)
After 2 months ("mature milk")	**Reported Range: 0.7 to 3.4 g/L** Average: 1.9 g/L	Erney *et al.* (2000); Asakuma *et al.* (2011); Smilowitz *et al.* (2013); Austin *et al.* (2016); McGuire *et al.* (2017)
Secretor Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 3.9 to 4.1 g/L** Average: 4.0 g/L	Coppa *et al.* (1999); Leo *et al.* (2009); Kunz *et al.* (2017)
Days 5 to 14 ("transitional milk")	**Reported Range: 3.0 to 3.6 g/L** Average: 3.3 g/L	Coppa *et al.* (1999); Kunz *et al.* (2017)
Days 10 to 60 ("mature milk")	**Reported Range: 1.0 to 7.8 g/L** Average: 3.0 g/L	Coppa *et al.* (1999, 2011); Leo *et al.* (2009); Galeotti *et al.* (2012, 2014); Bao *et al.* (2013); Hong *et al.* (2014); Olivares *et al.* (2015); Kunz *et al.* (2017); Sprenger *et al.* (2017); McGuire *et al.* (2017)
After 2 months ("mature milk")	**Reported Range: 1.0 to 3.6 g/L** Average: 2.4 g/L	Thurl *et al.* (1996); Coppa *et al.* (1999); Kunz *et al.* (2017); Sprenger *et al.* (2017); McGuire *et al*. (2017)

2'-FL = 2'-fucosyllactose.

The average levels of 2'-FL in pooled milk are highest in colostrum (3.2 g/L), followed by transitional milk (2.5 g/L) and continue to decline slowly in mature milk (2.2 g/L) and mature milk from a lactation stage later than 2 months (1.9 g/L). In the context of relative abundance, 2'-FL ranks first with approximately 15 to 20 w/w % (corresponding to 24 to 30 mol %) of the total HMO biomass (Castanys-Muñoz *et al*., 2013). In milk from Secretor mothers, the corresponding levels are significantly higher, with average levels reported at 4.0 g/L in colostrum, 3.3 g/L in transitional milk, 3.0 g/L in mature milk and 2.4 g/L in mature milk from a lactation stage later than 2 months. 2'-FL concentrations between different mothers is highly variable, with reported levels reaching beyond 5 g/L (8.4 g/L as the highest level reported).

Several studies reported the regional dependency of the 2'-FL concentration of milk and reveal that the correlation to the Secretor frequency within a population is predictive. There are negligible differences of average 2'-FL concentrations (2.2 to 2.4 g/L) between Asia, China, Europe, and the U.S., regions which all possess Secretor frequencies between 70 and 80% (see table below). In Mexico, Peru, and the Hispanic populations of the U.S., where the Secretor frequency is reported to reach nearly 100%, the average concentration of 2'-FL is highest with 3.2 to 3.4 g/L.

Using the range of average levels of 2'-FL reported for breast milk over different times of lactation, of 1.9 to 4.0 g/L (see Table 3.1.3.2-1), combined with the estimated formula intakes for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of 2'-FL from breastfeeding can be estimated at between 494 to 1,040 mg/kg body weight/day.

3.1.3.3 Quantity of DFL in Breast Milk

DFL can be generated in the mammary gland from 2'-FL by action of any of the known fucosyltransferases with α-1,3-fucosylation activity, namely FUT3, FUT5 or FUT6 (compare to Table 3.1.3-1). 2'-FL is never detected without concurrent presence of DFL, although the 2'-FL levels are typically 3 to 12 times as high as the DFL levels (on average 7 times as high but reported variation of the 2'-FL/DFL ratio is high).

The average content of DFL in pooled milk is highest in colostrum (0.5 g/L), followed by transitional milk (0.4 g/L) and continues to decline slowly in mature milk (0.3 g/L) as presented in Table 3.1.3.2-1. However, the reported ranges of DFL in human milks widely vary from 0.1 to 1.0 g per liter of milk. In milk from Secretor mothers, with the functional FUT2 gene, 2'-FL was the major milk oligosaccharide, followed by lacto-N-fucopentaose I (LNFP I) and DFL according to the results from an open observatory, longitudinal cohort study with quantitative human milk collection (Sprenger *et al.,* 2017). Moreover, a recently published systematic review by Thurl *et al.* (2017) presenting a meta-analysis of the concentrations of oligosaccharides in human milk reported mean values of 2'-FL and DFL in milk from Secretor mothers to be 2.74 and 0.42 g/L, respectively, with corresponding 95% CL of 2.43 to 3.04 and 0.32 to 0.51 g/L.

Table 3.1.3.2-1 The DFL Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Pooled Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 0.2 to 1.0 g/L** Average: 0.5 g/L	Erney *et al.* (2000); Asakuma *et al.* (2008); Spevacek *et al.* (2015)
Days 5 to 14 ("transitional milk")	**Reported Range: 0.1 to 0.7 g/L** Average: 0.4 g/L	Erney *et al.* (2000); Spevacek *et al.* (2015)
Days 10 to 60 ("mature milk")	**Reported Range: 0.1 to 0.6 g/L** Average: 0.3 g/L	Chaturvedi *et al.* (1997); Nakhla *et al.* (1999); Erney *et al.* (2000, 2001); Spevacek *et al.* (2015); McGuire *et al.* (2017)
After 2 months ("mature milk")	**Reported Value: 0.3 g/L** Average: not applicable	Smilowitz *et al.* (2013)
Secretor Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 0.4 to 0.5 g/L** Average: 0.4 g/L	Thurl *et al.* (2010); Kunz *et al.* (2017)
Days 5 to 14 ("transitional milk")	**Reported Value: 0.3 g/L** Average: not applicable	Kunz *et al.* (2017)
Days 10 to 60 ("mature milk")	**Reported Range: 0.1 to 0.4 g/L** Average: 0.2 g/L	Thurl *et al.* (1996); Thurl *et al.* (2010); Olivares *et al.* (2015); Hong *et al.* (2014); Kunz *et al.* (2017); McGuire *et al.* (2017)
After 2 months ("mature milk")	Not Reported	Not reported

DFL = difucosyllactose.

Using the range of average levels of DFL reported for breast milk over different times of lactation, of 0.2 to 0.5 g/L (see Table 3.1.3.2-1), combined with the estimated formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of DFL from breastfeeding can be estimated at between 52 and 130 mg/kg body weight/day.

3.1.4 History of Commercial Use

- 2'-FL/DFL: 2'-FL/DFL as described herein has no history of commercial use.
- DFL: To the best of our knowledge, DFL on its own has no history of commercial use.
- 2'-FL: 2'-FL produced by fermentation has been commercialized as an ingredient of milk-based infant formulas worldwide. In Spain, two infant formulas under the product name NAN Optipro Supreme containing two HiMOs (2'-FL and LNnT) were launched by Nestlé in July 2017. In Hong Kong, a new growing-up formula milk powder named ILLUMA® Human Affinity Formula was released from Wyeth Nutrition (part of Nestlé S.A.) in November 2017. Between 2017 and 2018, several infant formulas containing 2'-FL have been commercialized by Nestlé under various brand names, including NAN Optipro in selected Middle Eastern countries, Good Start Optipro Supreme in Mexico, and NAN Optipro formulas in European markets (Spain, Poland, Italy, Portugal, and Greece). In the U.S., Gerber (a subsidiary of Nestlé Group) has launched two infant formula powders, namely Good Start Gentle and Good Start Soothe. The examples of commercialized products from selected markets are listed in Table 3.1.4-1 below:

Table 3.1.4-1 Non-Exhaustive List of Commercialized Products Containing 2'-FL Marketed by Nestlé (Glycom)

Product	Description
	**NAN Optipro Supreme 1 HM-0²**, designed for children from 0 months old, containing 2 oligosaccharides designed with a structure identical to those found in human milk: 2'-FL (0.1 g/100 mL) and LNnT (0.05 g/100 mL). Lactic acid bacteria *Lactobacillus reuteri* are added to improve the intestinal flora. Marketed in Spain
	**NAN Optipro Supreme 2 HM-0²**, containing 2 oligosaccharides designed with a structure identical to those found in human milk: 2'-FL (0.1 g/100 mL) and LNnT) (0.05 g/100mL). Lactic acid bacteria *Lactobacillus reuteri* are added to improve the intestinal flora. Marketed in Spain
	**ILLUMA® Human Affinity Formula** (from Wyeth Nutrition, part of Nestlé S.A.), exclusively fortified with important combination of nutrients naturally found in human milk: 2'-FL (0.025 g/100mL) and sn-2 palmitate (0.34 g/100 mL). Marketed in Hong Kong

Table 3.1.4-1 Non-Exhaustive List of Commercialized Products Containing 2'-FL Marketed by Nestlé (Glycom)

Product	Description
	**Good Start 1 Optipro Supreme HM-0** Marketed in Mexico
	**NAN Optipro HM-0** Marketed in the United Arab Emirates
	**Good Start Infant Formula Gentle HM-0, Stage 1** (from Gerber, a subsidiary of Nestlé Group), containing probiotics found in breastmilk and the HMO 2'-FL. Marketed in the U.S.
	**Good Start Infant Formula Soothe HM-0, Stage 1** (from Gerber, a subsidiary of Nestlé Group), containing probiotics found in breastmilk and the HMO 2'-FL. Marketed in the U.S.

2'-FL = 2'-fucosyllactose; HMO = human milk oligosaccharide; LNnT = Lacto-N-neotetraose; U.S. = United States.

In 2016 Abbott Nutrition (Abbott Laboratories) launched two infant formulas containing 2'-FL, namely Similac Pro-Advance™ and Similac Pro-Sensitive™, in the U.S. and Mexico, to offer unique immune-nourishing and infection-reducing benefits. Abbott's infant formula products are also currently available in Hong Kong, the Middle East, and Vietnam. Metagenics launched a dietary supplement for children in the U.S. called MetaKids™ Nutrition Powder (See Table 3.1.4-2 below).

Table 3.1.4-2 Non-Exhaustive List of Commercialized Products Containing 2'-FL Marketed by Abbott Nutrition and Metagenics

Product	Description
	**Abbott Similac Pro-Advance Infant Formula** with the HMO 2'-FL for Immune Support and complete nutrition for baby's first year. Marketed in the U.S.

Table 3.1.4-2 Non-Exhaustive List of Commercialized Products Containing 2'-FL Marketed by Abbott Nutrition and Metagenics

Product	Description
	**Abbott Similac Pro-Sensitive Infant Formula** with the HMO 2'-FL for Immune Support gentle nutrition for fussiness, gas, or mild spit-up. Marketed in the U.S.
	**Abbott Similac HMO Newborn Infant Formula** 1 (0-6 months), containing the HMO 2'-FL. Marketed in Hong Kong
	**Metagenics MetaKids™ Nutrition Powder** provides important foundation nutrition for children 4 to 12 years old. This comprehensive formula features breakthrough prebiotic HMOs (PreBiome 2'-FL™) — structurally much like that found in mother's milk — along with a blend of essential nutrients such as choline, plant sourced DHA, and a proprietary vitamin and mineral blend to help support a healthy diet. MetaKids™ Nutrition Powder contains 0.5 g 2'-FL. Marketed in the U.S.

2'-FL = 2'-fucosyllactose; DHA = docosahexaenoic acid; HMO = human milk oligosaccharide; U.S. = United States.

3.2 Estimated Intake of 2'-FL/DFL

3.2.1 Methods

An assessment of the anticipated intake of 2'-FL/DFL as an ingredient under the intended conditions of use (see Table 1.2-1) was conducted using data available in the 2013-2014 cycle of the U.S. National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Survey (NHANES) (CDC, 2015, 2016, USDA, 2016). A summary 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 2013 to 2014. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (CDC, 2016; USDA, 2016). The NHANES data were employed to assess the mean and 90th percentile intake of 2'-FL/DFL for each of the following population groups:

- Toddlers, ages 1 to 3;
- Children, ages 4 to 10;
- Female teenagers, ages 11 to 18;
- Male teenagers, ages 11 to 18;
- Female adults of childbearing age, ages 19 to 40;

- Female adults, ages 19 to 64;
- Male adults, ages 19 to 64;
- Elderly, ages ≥ 65; and
- Total population (all age and 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 2'-FL/DFL by the U.S. population[11]. Estimates for the daily intake of 2'-FL/DFL represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2013-2014; 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 2'-FL/DFL averaged over all individuals surveyed, regardless of whether they consumed food products in which 2'-FL/DFL is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing 2'-FL/DFL during the 2 survey days). "Consumer-only" intake refers to the estimated intake of 2'-FL/DFL by those individuals who reported consuming food products in which the use of 2'-FL/DFL is currently under consideration. Individuals were considered "consumers" if they reported consumption of 1 or more food products in which 2'-FL/DFL is proposed for use on either Day 1 or Day 2 of the survey.

The estimates for the intake of 2'-FL/DFL 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 2013-2014 NHANES datasets. The results of this assessments are presented in Section 3.2.2.

3.2.2 Intake Estimates for 2'-FL/DFL

A summary of the estimated daily intake of 2'-FL/DFL 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). Intakes are expressed as total wet weight of the ingredient under the conditions of intended use.

The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 92.2% of the population groups consisted of consumers of food products in which 2'-FL/DFL is currently proposed for use (Table 3.2.2-1). Children had the greatest proportion of consumers at 99.0%. 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 (all ages), the mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 1.65 and 3.54 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of 2'-FL/DFL on an absolute basis, at 1.87 and 4.03 g/person/day, respectively. The elderly had the lowest mean consumer-only intake of 1.32 g/person/day, while female teenagers had the lowest 90th percentile consumer-only intakes of 2.75 g/person/day (Table 3.2.2-1).

[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 <INGREDIENT>s added to foods, including contaminants, food additives and novel ingredients. The main input components are concentration (use-level) data and food consumption data. Data sets are combined in the software to provide accurate and efficient exposure assessments.

Table 3.2.2-1 Summary of the Estimated Daily Intake of 2'-FL/DFL from Proposed Food-Uses in the U.S. by Population Group (2013-2014 NHANES Data)*

Toddlers	1 to 3	1.78	3.73	98.5	465	1.80	3.75
Children	4 to 10	1.46	2.91	99.0	986	1.48	2.93
Female Teenagers	11 to 18	1.32	2.74	94.5	572	1.39	2.75
Male Teenagers	11 to 18	1.82	3.24	98.2	570	1.85	3.32
Female Adults of Childbearing Age	19 to 40	1.44	3.12	92.9	826	1.56	3.14
Female Adults	19 to 64	1.47	3.37	92.9	1,764	1.58	3.45
Male Adults	19 to 64	1.73	3.94	92.7	1,522	1.87	4.03
Elderly	65 and up	1.22	2.97	92.2	917	1.32	3.05
Total Population	All ages	1.55	3.44	93.8	7,088	1.65	3.54

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
*Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 34.5 and 67.2 mg/kg body weight/day, respectively. Among the individual population groups, toddlers were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 140.0 and 282.0 mg/kg body weight/day, respectively. The elderly had the lowest mean and 90th percentile consumer-only intakes of 17.5 and 41.2 mg/kg body weight/day, respectively (Table 3.2.2-2).

Table 3.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL/DFL from Proposed Food-Uses in the U.S. by Population Group (2013-2014 NHANES Data)*

Toddlers	1 to 3	138.0	282.0	98.5	460	140.0	282.0
Children	4 to 10	54.7	112.0	98.9	980	55.3	113.0
Female Teenagers	11 to 18	23.3	52.0	94.6	568	24.6	52.8
Male Teenagers	11 to 18	28.7	55.9	98.2	569	29.2	55.9
Female Adults of Childbearing Age	19 to 40	19.9	44.1	92.9	819	21.5	45.6
Female Adults	19 to 64	19.6	45.4	92.9	1,752	21.2	46.4
Male Adults	19 to 64	20.4	47.7	92.7	1,518	22.0	49.8
Elderly	65 and up	16.1	40.9	92.1	906	17.5	41.2
Total Population	All ages	32.3	63.9	93.8	7,045	34.5	67.2

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
*Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

3.2.3 Summary and Conclusions

Consumption data and information pertaining to the individual proposed food-uses of 2'-FL/DFL were used to estimate the *per capita* and consumer-only intakes of 2'-FL/DFL for specific demographic groups and for the total U.S. population. There were a number of assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in both exposure assessments that all food products within a food category contain 2'-FL/DFL 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 2'-FL/DFL will have 100% market penetration in all identified food categories.

In summary, on consumer-only basis, the resulting mean and 90th percentile intakes of 2'-FL/DFL by the total U.S. population from all proposed food-uses, were estimated to be 1.65 g/person/day (34.5 mg/kg body weight/day) and 3.54 g/person/day (67.2 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 1.87 g/person/day (22.0 mg/kg body weight/day) and 4.03 g/person/day (49.8 mg/kg body weight/day), respectively, as identified among male adults. The elderly had the lowest mean consumer-only intakes of 1.32 g/person/day (17.5 mg/kg body weight/day), while female teenagers had the lowest 90th percentile consumer-only intakes of 2.75 g/person/day (52.8 mg/kg body weight/day). When intakes were expressed on a body weight basis, toddlers had the highest mean and 90th percentile consumer-only intake of 140.0 and 282.0 mg/kg body weight/day.

Part 4. Self-Limiting Levels of Use

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

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

Not applicable.

Part 6. Narrative and Safety Information

6.1 Introduction

The subject matter of this GRAS evaluation is 2'-fucosyllactose containing <20% difucosyllactose (*i.e.,* 2'-FL/DFL). 2'-FL/DFL manufactured by Glycom is identical in structure to their natural counterparts secreted into human milk and therefore can be referred to as HiMOs. 2'-FL and DFL are structurally and biologically closely related since DFL is metabolically obtained from 2'-FL by the simple addition of a second fucose unit ("fucosylation"). Consistent with their shared structure and metabolic synthesis routes, 2'-FL and DFL are always found together in human milk. 2'-FL/DFL will be added to infant formula at levels that are consistent with levels of 2'-FL and DFL have been measured in breast milk samples across all lactational stages and therefore the safety of adding 2'-FL and DFL to infant formula is supported by their history of safe consumption by breast-feeding infants. Since infants are a sensitive population group, the safety of dietary ingestion of HiMOs from breast milk consumption can be extended to adults consuming HiMOs at comparable ingestion levels in conventional food products.

Publicly available studies evaluating the toxicity of multiple 2'-FL preparations produced by chemical synthesis and by fermentation include subchronic toxicity studies in neonatal rats and mature rats, and

safety and tolerance studies in neonatal piglets. Several *in vitro* and *in vivo* genotoxicity studies of various 2'-FL preparations have been conducted. The aforementioned studies have been the subject of comprehensive evaluations by qualified experts during previous GRAS evaluations (GRN 546, 571, 650, 735, 749), and by other authoritative bodies (*e.g.*, EFSA). Details on the similarities of the 2'-FL preparations produced by chemical synthesis and by fermentation from these previous GRAS evaluations to Glycom's 2'-FL/DFL is discussed in Section 6.3.1 below. The results of animal toxicity studies have consistently demonstrated that 2'-FL is without evidence of toxicity up to the highest dose tested, findings that are consistent with the safe history of consumption of 2'-FL by breastfed infants. Results of multiple genotoxicity batteries, including the Ames reverse mutation assay, *in vitro* chromosomal aberration assays, and *in vitro*/*in vivo* micronucleus assays were negative for evidence of genotoxicity/mutagenicity. A summary of publicly available toxicity studies of 2'-FL is presented in Section 6.3. A battery of toxicity published studies, including a subchronic repeat-dose 90-day feeding study in neonatal Sprague-Dawley rats, a bacterial reversion assay and an *in vitro* mammalian cell micronucleus assay were conducted using 2'-FL/DFL as manufactured by Glycom (Section 6.3.2). Findings from these studies demonstrated that 2'-FL/DFL is without evidence of toxicity in neonatal rats up to the highest dose tested and is not genotoxic or mutagenic; these results are consistent with observations from previous toxicity evaluations of 2'-FL and therefore further corroborate the safety of the 2'-FL/DFL and the safety of the strain lineage used by Glycom for HiMO production.

Four clinical studies were conducted in which the safety of 2'-FL, either alone or in combination with LNnT or other non-digestible oligosaccharides, was evaluated (see Section 6.4.2). Formula supplemented with 1.0 to 1.2 g 2'-FL (in combination with 0.5 to 0.6 g LNnT) for the first 4 months of life was shown to be well tolerated and supported age-appropriate growth (Puccio *et al.*, 2017), a conclusion that was also reached during the GRAS evaluation of these ingredients and by EFSA NDA Panel during their novel food evaluation (EFSA, 2015a). The outcome of the study by Puccio *et al.* is further corroborated by another clinical trial in which formula supplemented with 0.2 or 1.0 g 2'-FL/L (in combination with 2.2 and 1.4 g GOS/L, respectively) for the first 4 months of life was well tolerated and did not result in any differences in growth or anthropometric measures, when compared to infants receiving a control infant formula containing GOS only, or to infants in the human breast milk reference group (Marriage *et al.*, 2015). A formula supplemented with 2'-FL at 0.2 g/L, in combination with 2.0 g/L scFOS, was well tolerated and did not result in any differences in intake, anthropometric data, or average rank stool consistency compared to the control formula (without any added oligosaccharides) and the breastfed groups when administered up to 35 days of age (Kajzer *et al.*, 2016). In adults, the results of a safety and tolerability study indicate that consumption of 2'-FL at doses of up to 20 g, LNnT at doses of up to 20 g, or their combination at a 2:1 ratio (for total sum of up to 20 g), when taken as a single bolus dose daily for 2 weeks, was well tolerated and did not result in any deviations in laboratory measures of safety (*i.e.*, hematology and blood biochemistry) compared to normal reference values (Elison *et al.*, 2016). These clinical studies support the safety of the 2'-FL for its intended uses.

Glycom evaluated the allergenicity risk of 2'-FL/DFL (Section 6.5). The amino acid sequences of all introduced proteins were evaluated using Allergen Online tool (version 17) hosted by the University of Nebraska's Food Allergen Research and Resource Program (FARRP). No positive alignments between any of the recombinant proteins and known/putative allergen sequences within the database were reported. The production strain used to generate 2'-FL/DFL (*E. coli* K-12 DH1 MDO MAP1001) is a close derivative of *E. coli* K-12 DH1 MDO SCR6 used in the manufactured of 2'-FL described in GRN 650. Strain MAP1001 expresses the same biosynthetic enzymes (colanic acid operon and fucosyltransferase) for 2'-FL production, but has been optimized to remove the plasmids and associated antibiotic resistance markers, and does not require IPTG induction. Information describing the safety of the production organism is discussed in Section 2.2.1. Thus, the production strain contains similar elements as the strain discussed in Section IV.G of GRN 650..

To identify new data pertinent to the safety of 2'-FL/DFL published since the last scientific evaluation of 2'-FL (*e.g.*, GRN 749), Glycom conducted an updated comprehensive search of the published scientific literature from January 2017 to September 2018. The search was conducted using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and Toxfile®. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of 2'-FL/DFL were based all relevant data and information available to Glycom, including both favorable and potentially unfavorable information, that are relevant to the safety of the ingredient for its intended conditions of use. Glycom is not aware of newly published studies to suggest 2'-FL/DFL is unsafe for use as a food ingredient.

Based on the weight of the available evidence on 2'-FL/DFL presented in this dossier, Glycom has concluded that 2'-FL/DFL, as manufactured by Glycom, is GRAS for the specified uses based on scientific procedures.

6.2 Absorption, Distribution, Metabolism and Excretion

2'-FL and DFL present in Glycom's 2'-FL/DFL ingredient are structure-identical to their naturally occurring counterparts in human milk. Absorption, distribution and metabolism, and excretion (ADME) of HMOs have been investigated in a number of studies (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al.*, 2000; Chaturvedi *et al.*, 2001b; Rudolff and Kunz, 2012) demonstrating that HMOs, including 2'-FL and DFL, do not undergo any significant digestion in the upper gastrointestinal tract. Some HMOs have been reported to be absorbed intact following consumption to a small extent, a small portion of which (approximately 1 to 2% of the total amount of HMO ingested) is excreted unchanged in urine. The absorption of 2'-FL/DFL is expected to be highly limited and any level of 2'-FL/DFL that is absorbed would be no different to that occurring in breast-fed infants. For a more comprehensive discussion of the ADME profile of 2'-FL, DFL and other HiMOs the reader is directed to sections IV.B.4 of GRN 546 and IV.D of GRN 650.

6.3 Toxicological Studies

The risk assessment approach for 2'-FL/DFL follows the same procedures used to support the safety of 2'-FL preparations that have been previously concluded to be GRAS. Pivotal data and information supporting the safety of Glycom's HiMO products are based on qualitative and quantitative data establishing that HiMOs manufactured by Glycom are chemically identical to those present within human breast milk and are intended for use in infant formula at levels that are equivalent to mean levels that have been reported for human milk samples across all lactational stages. The safety of non-HiMO constituents originating from the fermentation organism is supported in part by the general history of safe use of *E. coli* K-12 for production of food ingredients (*i.e.*, *E. coli* K-12 is not known to produce undesirable substances) and is further supported by results of animal toxicity studies (neonatal/pre-weaning and post-weaning) and genotoxicity investigations of multiple HiMO preparations produced using Glycom's MDO platform strain. A discussion of toxicity studies conducted on Glycom's 2'-FL preparations is presented below, and a summary of findings from toxicity studies conducted using 2'-FL preparations produced by other manufacturers also is presented. Consistent with expected safety of HiMOs that is established from their history of consumption in human milk, the results of toxicity studies conducted with all HiMOs that have been evaluated to date demonstrate that these ingredients are of very low toxicity potential.

6.3.1 Studies Conducted with Various 2'-FL Preparations

A number of toxicological studies have been reported with 2'-FL. These include genotoxicity assays, 90-day oral toxicity studies and a neonatal piglet feeding study. Considering that 2'-FL is the main component of 2'-FL/DFL (*i.e.*, specified content of at least 75.0%) and that the 2'-FL test articles used in these studies are all highly purified, the available data on 2'-FL are relevant in supporting the safety of 2'-FL/DFL (considering also that all of the authorized 2'-FL sources also contain DFL). The toxicology and safety studies that have been conducted with 2'-FL preparations are summarized in below.

Characteristics of the test articles used in the various toxicology and safety studies that have been conducted with 2'-FL are presented below in Table 6.3.1-1, alongside those of 2'-FL/DFL for comparison. Glycom has analytical data demonstrating that the 2'-FL and DFL components in 2'-FL/DFL, and their 2'-FL obtained by chemical synthesis or microbial fermentation, are all chemically and structurally identical to 2'-FL or DFL that is naturally present in human breast milk. 2'-FL produced by Jennewein Biotechnologie GmbH by microbial fermentation is also a purified preparation consisting largely of 2'-FL (≥90%), with levels of DFL up to 5.0% (GRN 571), while FrieslandCampina's 2'-FL ingredient is also produced by microbial fermentation but with DFL levels of less than 1% (GRN 735). The toxicological data obtained for these 2'-FL preparations are relevant in supporting the safety of 2'-FL/DFL.

Table 6.3.1-1 Test Articles Used in Studies Conducted with 2'-FL Preparations

Parameter	2'-FL Preparations Tested				
	Glycom			**Jennewein**	**Friesland Campina**
References	Coulet *et al*., (2014)	Penard, (2015)	Phipps et al., (2018)	GRN – 571 Hanlon and Thorsrud, (2014)	van Berlo *et al*., (2018)
Production Organism	Not Applicable Chemical synthesis procedure	Modified strain of *E.coli* K-12	Modified strain of *E.coli* K-12	Modified strain of *E. coli* BL21	Modified strain of *E.coli* K-12
Purity (2'-FL assay)	≥ 95%	≥ 94%	≥ 75 %	≥ 90 %	94%
DFL Content	< 1 %	< 1 %	**< 20 %**	**< 5 %**	< 1%
Lactose Content	< 1 %	< 3 %	**< 10 %**	**< 5 %**	< 1%
Fucose Content	< 1 %	< 1 %	< 1 %	< 3 %	< 1%
Other Carbohydrates	< 2 %	< 2 %	< 8 %	< 9 %	
Toxicology/Safety Studies Conducted	• Bacterial reverse mutation test • *In vitro* micronucleus assay • *In vitro* mammalian cell gene mutation test • 90-day oral toxicity study • Human studies (infants, adults)	• Bacterial reverse mutation test • *In vitro* micronucleus assay • 90-day oral toxicity study	• Bacterial reverse mutation test • *In vitro* micronucleus assay • 90-day oral toxicity study	• Ames test • *In vivo* mouse micronucleus test • 90-day feeding study • Piglet feeding study	• Bacterial reverse mutation test • *In vitro* micronucleus assay • 90-day oral toxicity study

2'-FL = 2'-Fucosyllactose; DFL = difucosyllactose; GM = genetically modified; NA = not applicable.

All of the studies described in Table 6.3.1-1 above have been reviewed during previous GRAS evaluations, and by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel). These expert panels have concluded that 2'-FL is safe for its intended uses in infant formula and various conventional food products (U.S. FDA 2015a, 2016a; EFSA, 2015a,b). As such, only a brief description of these studies characterizing the repeat-dose toxicity, genotoxicity and clinical evaluations are discussed below. Studies conducted with 2'-FL/DFL are summarized in detail in Section 6.3.4.

6.3.2 Repeat-Dose Toxicity Studies of 2'-FL

6.3.2.1 90-Day Oral Toxicity Study with Glycom's 2'-FL Produced by Chemical Synthesis

The oral toxicity of 2'-FL has been evaluated in a 90-day study in rats conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Test Guideline No. 408 (OECD, 1998a,b), with an adaptation to include the use of neonatal rats (Coulet *et al*., 2014). The 2'-FL used in the study (Glycom AS, Denmark) was produced by chemical synthesis as described in GRN 546 and had a purity of 99% (by HPLC, on a dry weight basis). Neonatal [post-natal day (PND) 7] Wistar [Crl:WI(Han)] pups[12] were administered 2'-FL by gavage at doses of 0 (water vehicle control), 2,000, 5,000 or 6,000 mg/kg body weight/day from PND 7 to up to 13 weeks of age. A reference control group (15 rats/sex/group) was administered 6,000 mg/kg body weight/day of oligofructose (OF). 2'-FL was well-tolerated at doses of up to 5,000 mg/kg body weight/day for 13 weeks; the authors reported transient lower body weight gain[13] and colored/liquid feces during the first few days of the administration period in the same group. The authors reported three unexplained deaths, 1 male and 1 female in the 6,000 mg/kg group on day 2, and 1 male in the FOS control group on day 12. Glycom notes that no cause of death could be determined following gross or histopathological investigations and were not associated with marked changes in any other safety indices measured at the end of the study. Due to the unexplained deaths, a conservative no-observed-adverse-effect level (NOAEL) of 5,000 mg/kg body weight, was reported by the authors.

The subchronic toxicity study by Coulet *et al.*, (2014) was evaluated by the EFSA NDA Panel during the novel food approval of 2'-FL produced by chemical synthesis (EFSA, 2015a). In their evaluation of the study, the EFSA NDA Panel (EFSA, 2015a) concluded the following:

> "*Based on the decrease in the relative kidney weight in the 2'-FL high-dosed female group, two unexplained deaths in the high-dose 2'-FL group and high-dosed female group, and the significant changes in the haematological and clinical blood parameters in the 2'-FL mid- and high-dosed group, the Panel considers that the no observed adverse effect level (NOAEL) is 2,000 mg/kg body weight per day*".

These effects were not reported in other 90-day oral toxicity studies that have been conducted in rats using 2'-FL obtained by fermentation at doses greater than 2,000 mg/kg body weight/day (Jennewein Biotechnologie GmbH, 2015; Penard, 2015; van Berlo *et al*., 2018). These studies were evaluated by various qualified experts including in the U.S. FDA during previous GRAS evaluations of 2'-FL and these experts unanimously concluded that the study authors' NOAEL determinations were appropriate; further discussion of the conclusions from the NDA panel is presented in Section of IV.E.1 of GRN 650. Accordingly, the NOAEL for 2'-FL is concluded to be 5,000 mg/kg body weight/day.

[12] The control and high-dose groups each consisted of 15 males and 15 females, while the low- and mid-dose groups each consisted of 10 males and 10 females.

[13] No significant difference in food consumption was reported between the groups receiving 2'-FL and the control group.

6.3.2.2 90-Day Oral Toxicity Study with Glycom's 2'-FL - Microbial Source - Modified Strain of Escherichia coli K-12

2'-FL produced by Glycom *via* fermentation was evaluated in an adapted sub-chronic (90-day) oral toxicity study in 7-day-old Wistar [Crl:WI(Han)] rats (See Section IV.E.1 of GRN 650; Penard, 2015) The study was conducted in accordance with OECD principles of GLP (OECD, 1998a) and OECD Test Guideline 408 (OECD, 1998b), with an adaptation to include the use of neonatal rats. The studies were conducted in rats starting on PND 7 to adequately cover the developmental window during which infants will be exposed. Fructo-oligosaccharide (FOS; tradename Orafti® P95), was used as the reference control on the basis that it is currently authorized as an ingredient in infant and follow-on formulae. Rats (10/sex/group) were gavaged at doses of 0 (water vehicle control), 2,000, 4,000, or 5,000 mg/kg body weight/day of 2'-FL (97.6% purity) or the reference compound, FOS, at 5,000 mg/kg body weight/day for 90 or 91 days. Additional groups of 5 males and 5 females were given the control, 2'-FL, or FOS doses for 90 days and were terminated after a 28-day recovery period. The study design was similar to that described by Coulet *et al*., (2014), and for further discussion of the study methodology and results the reader is directed to Section IV.E.1 of GRN 650.

No test article-related mortalities occurred during the study. The majority of animals receiving the reference compound presented with liquid feces, which was also reported in mid- and high-dose animals receiving 2'-FL. Mid- and high-dose animals receiving 2'-FL also had soiled urogenital regions. No remarkable effects in body weight, body weight gain, or food consumption were reported. No toxicologically relevant effects in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (as evaluated in the Morris water maze), exploratory behavior, or general movement (as evaluated in the open-field test) were reported at any dose. Administration of 2'-FL resulted in statistically significant changes in some of the hematological parameters when compared to the negative control; however, these changes were small in magnitude, remained within the normal historical control data range, and were reported in the animals administered the FOS reference control. Thus, they were not considered to be toxicologically relevant. No test article-related differences in urinalysis parameters were reported between treatment groups and the water control or reference compound. Minor differences in mean specific gravity or mean urinary pH were not considered of toxicological significance as they were within the normal historical control data range and were small in magnitude. No treatment-related differences in organ weights or macroscopic observations were reported between rats receiving 2'-FL and the control and reference groups. No evidence of treatment-related effects in histological observations was report ed in animals receiving 2'-FL compared to control and reference groups. A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was determined.

6.3.2.3 90-Day Oral Toxicity Study with Jennewein's 2'-FL Microbial Source – Modified strain of Escherichia coli BL21

A 90-day feeding study was conducted with Jennewein's 2'-FL ingredient (94.1% purity). The study was conducted in accordance with GLP and with consideration of OECD Test Guideline 408 as a limit test (OECD, 1998a,b). A summary of the study results, as well as full study report, are publicly available for review in GRN 571 (Jennewein Biotechnologie GmbH, 2015; U.S. FDA, 2015b). In brief, the study was conducted using groups of ten 4-week-old male and female CD® Crl:CD Sprague-Dawley rats randomized to 1 of 2 treatment allocations administered 2'-FL in the diet at concentrations of 0 or 10% for 90 days. Additional groups of 3 and 9 animals per sex were included in the control (0%) and treatment (10%) groups, respectively, and used exclusively for blood sampling. 2'-FL was well tolerated by the test animals, with the only notable effects being sporadic pale coloration of the feces in the treatment group, which was attributed to the presence of undigested 2'-FL. No animal deaths were reported. No test article-related effects in body weight, body weight gain, food consumption, water consumption, neurological parameters,

hematological and blood biochemical parameters, urinalysis, ophthalmological observation, organ weights, or macroscopic or histopathological findings were reported. The NOAEL was concluded by Jennewein to be 10% dietary concentration of 2'-FL (the only dose tested), which is equivalent to 7,660 mg/kg body weight/day in males and 8,720 mg/kg body weight/day in females.

6.3.2.4 90-Day Oral Toxicity Study with FrieslandCampina's 2'-FL - Microbial Source - Modified Strain of Escherichia coli K-12

FrieslandCampina's 2'-FL ingredient has been evaluated in an adapted sub-chronic (90-day) oral toxicity study in 25-day-old Wistar [Crl:WI(Han)] rats (van Berlo *et al*., 2018). The study was conducted in accordance with OECD principles of GLP (OECD, 1998a) and OECD Test Guideline 408 (OECD, 1998b) altered to accommodate the use of juvenile animals. The studies were conducted in rats starting on PND 25 to be equivalent to the immune development of the human neonate and ended on PND 115 when immune and sexual maturity is attained in rats. No reference control was used. Rats (10/sex/group) were exposed to 2'-FL in animal feed (VRF1 finely ground) at concentrations of 0%, 3%, 6%, or 10% (w/w) for 13 weeks. The prepared diets were confirmed for stability, homogeneity and dose levels. The overall mean intake of 2'-FL was 2,170, 4,270, and 7,250 mg/kg body weight/day for males and 2,450, 5,220, and 7,760 mg/kg body weight/day for females from the low-, mid- and high-dose group, respectively. This study has been described previously on page 54 of GRN 735.

No test article-related mortalities occurred during the study. One female from the mid-dose group died in the 4th week post-exposure but was deemed to be unrelated to test article administration and bears no toxicological relevance. There were no significant differences in body weights between the control and treatment groups. There were no significant differences in food consumption in male rats across groups, however a slight but significant decrease in food consumption in females of the high-dose group was reported. No significant difference in water consumption across groups, except for a slight but significant increase in males of the high dose group on Days 35 to 36 and females of the high-dose group on Days 38 to 39. Administration of 2'-FL resulted in statistically significant changes in a small number of the hematological, clinical chemistry, and urinalysis parameters when compared to the control; however, these changes were small in magnitude and considered a chance finding. Thus, they were not considered to be toxicologically relevant. The relative weight of the liver was significantly increased in males in the high - dose group. This increase was not associated with histopathological evidence and therefore is not considered adverse. The absolute and relative weights of the filled and empty cecum were significantly increased in the mid- and high-dose group in male and female rats, as well as the absolute weight of the filled cecum in low-dose males. The cecal enlargement was ascribed to the fact that the test substance is a non-digestible carbohydrate and considered a physiological response rather than a toxic effect. No treatment-related differences in macroscopic observations were reported between rats receiving 2'-FL and the control group. No evidence of treatment-related effects in histological observations was reported in animals receiving 2'-FL compared to the control group.

A NOAEL of 7,250 mg/kg body weight/day for males and ≥7,760 mg/kg body weight/day for females, the highest dose tested for each sex, was determined.

6.3.2.5 Other Studies

6.3.2.5.1 Piglet Feeding Study with Jennewein's 2'-FL Microbial Source – Modified Strain of *Escherichia coli* BL21

The safety of 2'-FL produced by Jennewein was evaluated in a neonatal piglet model (Hanlon and Thorsrud, 2014). The study was conducted using 2'-FL manufactured by Jennewein, and it was in compliance with the OECD and FDA's principles of GLP. Domestic Yorkshire Crossbred farm piglets received liquid diets[14] containing 0 (control), 200, 500, or 2,000 mg/L of 2'-FL, corresponding to doses of 0, 29.37, 72.22, or 291.74 mg/kg body weight/day in males and 0, 29.30, 74.31, and 298.99 mg/kg body weight/day in females, respectively. The 2'-FL was produced by fermentation as described within GRN 571 and was characterized as the following: 97.9% 2'-FL, 4.2% water, 0.37% ash, 3.3% difucosyllactose, 1.9% fucosyl-galactose, and <50 ppm protein (Jennewein, 2015; U.S. FDA, 2015b). Piglets were administered the liquid diet for the first 3 weeks of life. The digestive systems of neonatal piglets have many similarities to those of human infants, particularly with regards to presence of specific digestive enzymes, nutrient absorption, gut closure, dietary requirements, microbial population, and gut transit time. As such, neonatal piglets are considered a good animal model for assessing the safety of dietary compounds on human infants. Detailed discussions of the study methodology and results are incorporated by reference to Section IV.E.2 of GRN 650.

Dietary administration of 2'-FL was reported by the study investigators to be "well tolerated". All animals survived to the scheduled necropsy. Clinical observations included the following: watery feces were noted in 2 low-dose males and 2 low-dose females, 1 mid-dose male and 2 mid-dose females, and 3 high-dose males and 2 high-dose females. One high-dose male and 2 high-dose females exhibited a lack of appetite on 1 day. Lastly, 1 low-dose female exhibited a lack of appetite for 2 days during the study. These observations in appetite were not considered to be toxicologically relevant as there was no dose relationship and there were no differences in body weight reported between treated piglets and controls. No differences in food consumption were reported between treatment groups. No test-article related effects in hematological, coagulation, or blood biochemical parameters were reported. A statistically significant increase (125%) in alanine aminotransferase concentration was reported in high-dose males; however, there were no significant differences in the other clinical chemistry markers for toxicity and no differences in absolute and relative liver weight were reported compared to controls. Furthermore, there were no histopathological correlates and thus this observation was deemed to be not test article-related. Similarly, no test article-related effects on urinalysis parameters were reported. No gross or histopathological findings were associated with the test article. Animals in all treatment groups and the controls had variable minimal-to-mild focal acute inflammation within the keratinized portion of the non-glandular stomach. There was no clear dose dependence and in the absence of similar findings in the stomach, this observation was not definitively linked to the test article. It was further noted by the study investigators that the incidence matched historical control data from the same facility. Although there was a statistically significant increase in the absolute weights of the heart and kidneys for low-dose males, there was not a difference in the relative (to body weight) organ weights and thus were not considered to be test article-related.

It was thus concluded that the addition of 2'-FL to milk replacer at concentrations of up to 2,000 mg/L was well tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth at doses equivalent to 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females.

[14] Land O'Lakes® ProNurse® Specialty Milk Replacer from Purina Animal Nutrition, Gray Summit, Missouri.

6.3.3 Genotoxicity

Genotoxicity studies of 2'-FL obtained by chemical synthesis, including a bacterial reverse mutation assay, an *in vitro* mammalian cell gene mutation test, and an *in vitro* micronucleus assay have been the subject of previous comprehensive reviews by various qualified Experts including the U.S. FDA and EFSA (U.S. FDA, 2015a; 2016a; EFSA, 2015a). Details of the bacterial reverse mutation assay and *in vitro* mammalian cell gene mutation test were published by Coulet *et al.* (2014); the *in vitro* micronucleus assay is described in Section IV.E.4 of GRN 650 (Verbaan, 2015a). These studies were all conducted in accordance with OECD test guidelines. Based on the results of these studies it has been concluded that 2'-FL does not pose any safety concerns with respect to genotoxicity.

6.3.3.1 Studies Conducted with Glycom's 2'-FL Microbial Source – Modified Strain of Escherichia coli K-12

Bacterial Reverse Mutation Assay

The mutagenicity of 2'-FL (97.6% 2'-FL; Glycom) was evaluated in a bacterial reverse mutation assay in *Salmonella* Typhimurium (*S.* Typhimurium) strains TA98, TA100, TA1535, and TA1537 and in *E. coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation and pre-incubation methods (See Section IV.E.4 of GRN 650; Verspeek-Rip, 2015). The study was conducted in accordance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for different strains in assays conducted in the absence of S9: 2-nitrofluorene (TA98, TA1537, pre-incubation assay), methylmethanesulfonate (TA100), sodium azide (TA1535), ICR-191 (TA1537, direct plate assay), 9-aminoacridine (TA1537), or 4-nitroquinoline n-oxide (WP2uvrA). For assays conducted in the presence of S9, 2-aminoathracene was employed as the positive control.

Using the plate incorporation method, bacterial strains were treated with 2'-FL at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate. For the pre-incubation method, bacterial strains were incubated with 2'-FL at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg/plate. No cytotoxicity or precipitation was reported in any strain treated with 2'-FL in the presence or absence of S9. Treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration in both experiments either in the presence or absence of S9. In contrast, positive control agents substantially induced an increase in the number of revertant colonies compared to the negative control. 2'-FL was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

In vitro Micronucleus Assay

The genotoxicity 2'-FL (97.6% 2'-FL) manufactured by Glycom was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (See Section IV.E.4 of GRN 650; Verbaan, 2015b). This study also was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2014). Mitomycin C and cyclophosphamide were used as the positive controls in the absence and presence of S9, respectively, and water was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for 24 hours in

the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition.

In both experiments, there were no signs of precipitation or cytotoxicity (as determined by the cytokinesis block proliferation index) reported in cells treated with 2'-FL at any concentration. No statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei were reported in cells treated with 2'-FL, in both experiments. 2'-FL was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

6.3.3.2 Studies Conducted with Jennewein's 2'-FL Microbial Source – Modified Strain of Escherichia coli BL21

Jennewein conducted a bacterial reverse mutation assay and an *in vivo* micronucleus assay with their 2'-FL preparation (containing up to 5.0% DFL). These studies are unpublished, but the full study reports are publicly available in the GRAS notice that was submitted to the U.S. FDA (GRN 571).

Bacterial Reverse Mutation Test

In brief, the bacterial reverse mutation test was conducted in accordance with OECD Test Guideline 471 and GLP. In the plate incorporation test and the pre-incubation test, 2'-FL was administered at concentrations of 31.6, 100, 316, 1,000, 3,160, and 5,000 µg/plate in *S.* Typhimurium strains TA 98, TA 100, TA 102, TA 1535, and TA 1537 in the absence and presence of metabolic activation. No evidence of cytotoxicity was reported. Treatment with 2'-FL did not increase the number of revertant colonies when compared to the negative control in any of the test strains, while mutagenic responses were reported for the positive controls. Based on these results, Jennewein concluded that 2'-FL is not mutagenic under the conditions of the assay.

In vivo Mammalian Micronucleus Test

The *in vivo* mammalian micronucleus test was conducted in accordance with OECD Test Guideline 474 and GLP. In this study, Crl:CD(SD) rats (5/sex/group) were administered a single dose of 2'-FL at 500, 1,000, or 2,000 mg/kg body weight by gavage. A group of animals received the vehicle (0.8% aqueous hydroxypropylmethylcellulose) as a negative control, while another group received cyclophosphamide as the positive control. The animals were terminated at 24 and 48 hours following the administration of the test articles, and the bone marrow smears were immediately prepared. Two thousand erythrocytes were evaluated per animal. No evidence of systemic toxicity was reported in any of the animals. Administration of 2'-FL did not increase the incidence of micro-nucleated polychromatic erythrocytes (PCEs) at any of the doses tested when compared to the negative control, while a significant increase was reported for the positive control. Based on these results, Jennewein concluded that 2'-FL is not genotoxic under the conditions of the assay.

6.3.3.3 Studies Conducted with FrieslandCampina's 2'-FL Microbial Source – Modified Strain of Escherichia coli K-12

Bacterial Reverse Mutation Assay

The mutagenicity of 2'-FL (94% 2'-FL; <1% DFL; FrieslandCampina) was evaluated in a bacterial reverse mutation assay in *S.* Typhimurium strains TA1535, TA1537, TA98, and TA100 and in *E. coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation method (van Berlo *et al.*,

2018). The study was conducted in accordance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997). The phosphate buffered saline (PBS) vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for different strains in assays conducted in the absence of S9: sodium azide (TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), sodium azide (TA100), or N-ethyl-N-nitrosourea (WP2uvrA). For assays conducted in the presence of S9, 2-aminoathracene was employed as the positive control for all except benzo(a)pyrene for TA1537.

Using the plate incorporation method, bacterial strains were treated with 2'-FL at 62, 195, 556, 1667, or 5,000 µg/plate, with 5,000 µg/plate being the recommended limit dose for this test system. No cytotoxicity was reported in any strain treated with 2'-FL in the presence or absence of S9. Treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration either in the presence or absence of S9. In contrast, positive control agents substantially induced an increase in the number of revertant colonies compared to the negative control. Thus, 2'-FL was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

In vitro Micronucleus Assay

The genotoxicity of 2'-FL (94% 2'-FL; <1% DFL; FrieslandCampina) was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (van Berlo *et al*., 2018). This study also was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2014). Cyclophosphamide and vinblastine were used as the positive controls in the presence and absence of S9, respectively, and culture medium was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with 2'-FL at concentrations of 500, 1,000, or 2,000 µg/mL for 4 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 20 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 500, 1,000, or 2,000 µg/mL for 24 hours in the absence of S9. At least 2,000 bi-nucleated cells per concentration (1,000 per culture) were examined for the presence of micronuclei. In both experiments, there were no signs of cytotoxicity reported in cells treated with 2'-FL at any concentration. No statistically or biologically significant increases in the frequency of binucleated cells with micronuclei were reported in cells treated with 2'-FL, in both experiments. 2'-FL was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

6.3.4 Toxicology Studies Conducted with Glycom's 2'-FL/DFL Ingredient

Glycom's 2'-FL/DFL was tested in a comprehensive series of toxicological studies, including a bacterial reverse mutation assay, an *in vitro* mammalian cell micronucleus test in human lymphocytes and an adapted subchronic (90-day) oral toxicity study in rats. These studies were all conducted with a test article that is representative of the material intended to be commercially marketed and they were performed in accordance with the Organization for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) and OECD test guidelines for the toxicity testing of chemicals. Detailed descriptions of these studies are presented in below.

6.3.4.1 Repeat Dose Toxicity

6.3.4.1.1 14-Day Toxicity Study in the Neonatal Rat

A 14-day range findings toxicity study was conducted in rats to evaluate the potential short-term toxicity of 2'-FL/DFL and select dose levels for the subsequent 90-day study [Flaxmer, 2017].

Groups of 8 male and 8 female neonatal rats were dosed with 0 (water for irrigation), 4,000 or 5,000 mg/kg body weight/day 2'-FL/DFL, by gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 2'-FL/DFL were corrected to account for "other carbohydrates" within the test article batches.

All animals were reported daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

There were no test item-related deaths. One male receiving 5,000 mg/kg body weight/day was found dead on Day 15 (just prior to scheduled necropsy). The precise cause of death is unclear; no clinical signs had been reported for this animal, the animal gained a similar amount of weight to other males throughout the study and macroscopic examination revealed no abnormalities, including no evidence of dosing trauma. However, in the absence of any other deaths during the study, this isolated death was considered incidental and unrelated to the test item.

Transient clinical signs (red and/or yellow staining around the anus was reported for some 2'-FL/DFL-treated animals) were absent by the end of the treatment period and were considered non-adverse. There were no biologically relevant differences in body weight between test item-treated groups and controls. No test item-related macroscopic abnormalities were reported.

In the absence of any test item-related adverse findings, 5,000 mg/kg body weight/day (the maximum tolerated dose, based on data for similar compounds) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high-dose for the 90-day study.

6.3.4.1.2 90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 2'-FL/DFL when administered by gavage, to neonatal rats from Day 7 of age [Phipps *et al*., 2018]. The study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals. This study utilized the same study design used for the toxicity evaluation of 2'-FL described by Coulet *et al*., (2014).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day 2'-FL/DFL, by gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose 2'-FL/DFL group and identify any effects related to the general fiber-like characteristics of the reference material. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept undosed for 4 weeks, to assess the reversibility of any reported effects.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment 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 taken for hematology, blood chemistry, and coagulation during Week 13 and at the end of the treatment-free period; urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period.

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

All animals at the end of the treatment and recovery periods were subjected to a gross macroscopic necropsy, where selected organs (adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, and uterus with cervix) were weighed and fixed. The following tissues were examined microscopically: adrenals, brain, femur, heart, kidneys, liver, lungs, nasal turbinates, spinal cord, sternum, stomach, thyroid, and uterus) for animals in the vehicle control and high dose 2'-FL/DFL groups.

There were no deaths and no test item-related clinical signs or ocular changes. Animals given 2'-FL/DFL gained similar amounts of weight and ate similar amounts of food compared with controls. There was no effect of 2'-FL/DFL administration on pre-weaning development [as evaluated by the age of attainment of the surface and air righting reflexes, and the pupil reflex and startle response tests conducted on Day 14 of treatment (Day 20 of age)]. Ulna length and growth were similar between 2'-FL/DFL-treated groups and controls. No test item-related differences in behavior of the animals during the in-hand and arena observations in Week 11 of treatment (Day 81 to 83 of age) were reported. Mean activity count for females given 5,000 mg/kg body weight/day was slightly lower than that of vehicle controls, but as this was not seen for males and there was no dose response-relationship, it was considered unrelated to administration of 2'-FL/DFL; the mean value (19.8) was also similar to the value for reference controls (20.6). Morris maze performance was also unaffected by administration of 2'-FL/DFL, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries and failed trials.

There were no test item-related differences for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening for males and females, respectively). Mean age and body weight for balano-preputial skinfold separation were slightly higher for 2'-FL/DFL-treated males, compared with vehicle controls. However, the differences were minor and considered to be due to aberrantly low vehicle control values, rather than any effect of the test item; 4 individual vehicle control values for age and 4 for body weight were below the 5% confidence historical control data (HCD) limits, compared to only 1 individual high-dose 2'-FL/DFL value (for either parameter) being outside the respective HCD ranges. Furthermore, the 5,000 mg/kg body weight/day 2'-FL/DFL values were comparable to reference control values.

No test item-related or biologically relevant differences in hematology parameters between 2'-FL/DFL-treated groups and controls were reported. The statistically significant differences reported at the end of the treatment period were not associated with a dose response [increased hematocrit, hemoglobin and red blood cells for males (all 2'-FL/DFL groups) and females (1,000 or 3,000 mg/kg body weight/day); increased mean cell volume for both sexes given 1,000 or 3,000 mg/kg body weight/day; decreased mean corpuscular

hemoglobin and mean corpuscular hemoglobin concentration for males (all groups) and females (1,000 or 3,000 mg/kg body weight/day); increased mean corpuscular volume for males and females at 3,000 mg/kg body weight/day; decreased red blood cell distribution width for males given 1,000 or 3,000 mg/kg body weight/day; increased basophils and large unstained cells for males (all 2'-FL/DFL groups); increased basophils for females (all 2'-FL/DFL groups); decreased white blood cell count, lymphocytes and eosinophils for females receiving 1,000 or 3,000 mg/kg body weight/day; decreased monocytes for females (all 2'-FL/DFL groups)]. Values for all of these parameters were also within the respective HCD ranges. Statistically significantly increased prothrombin time for males in all 2'-FL/DFL groups was also not associated with a dose response and was not seen for females. Furthermore, the value for the high dose group (22.2 seconds) was similar to that for reference controls (22.9 seconds). However, some of the individual values were above the HCD upper limit (6, 5 and 3 out of 10 at 1,000, 3,000, or 5,000 mg/kg body weight/day, respectively). Although this was likely attributable to the vehicle controls having relatively low values (2 out of 10 were below the HCD lower limit), this parameter was re-evaluated at the end of the treatment-free period and values were similar between all groups.

There were also no test item-related or biologically relevant differences in blood chemistry parameters between 2'-FL/DFL-treated groups and controls. Statistically significant differences compared with vehicle controls were either not associated with a dose-response relationship [increased aspartate aminotransferase (AST) for males given 1,000 or 3,000 mg/kg body weight/day and females given 1,000 mg/kg body weight/day; reduced albumin for males given 3,000 mg/kg body weight/day and females in all 2'-FL/DFL-treated groups] or the differences were inconsistent between the sexes (decreased creatinine for males given 5,000 mg/kg body weight/day, increased urea for females receiving 1,000 or 3,000 mg/kg body weight/day and increased calcium and phosphorus for females given 5,000 mg/kg body weight/day). Values for all of these parameters were within the respective HCD ranges.

Urinalysis parameters were unaffected by 2'-FL/DFL administration. Urinary pH was statistically significantly higher for females receiving 5,000 mg/kg body weight/day 2'-FL/DFL and total creatinine was statistically significantly lower, compared with vehicle controls. However, as the differences were not seen in males and values for both parameters were within the respective HCD ranges, these findings were considered biologically irrelevant and unrelated to administration of 2'-FL/DFL.

There were no test item-related differences in organ weights between 2'-FL/DFL-treated groups and vehicle controls at the end of the treatment or treatment-free periods. Where statistically significant differences were reported, the differences were clearly unrelated to 2'-FL/DFL administration (increased body weight adjusted kidney and seminal vesicle weights for males given 1,000 mg/kg body weight/day and increased thymus weights for all male 2'-FL/DFL-treated groups at the end of the treatment period were not dose-related; increased body weight adjusted pituitary weights for females given 5,000 mg/kg body weight/day were only seen at the end of the recovery period and were not evident immediately after cessation of dosing).

Macroscopic examinations (at the end of the treatment and treatment-free periods) and histopathological examinations (at the end of the treatment period only) revealed no test item-related findings. The only findings reported were incidental and generally consistent with changes encountered in Sprague-Dawley rats of this age kept under laboratory conditions.

In absence of any test item-related adverse effects, the NOAEL was concluded to be 5,000 mg/kg body weight/day 2'-FL/DFL, the highest dose tested and maximum tolerated dose, based on data for similar

compounds.

6.3.4.2 Genotoxicity

6.3.4.2.1 Bacterial Reverse Mutation Test

Phipps et al., (2018) reported findings on the potential mutagenicity of 2'-FL/DFL was in a bacterial reverse mutation test (Ames test), which was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[15] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998), and FDA Redbook IV.C.1.a. (U.S. FDA, 2000).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *S.* Typhimurium) strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with 2'-FL/DFL at concentrations of up to 5,000 µg/plate (the regulatory maximum dose level) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for 2'-FL/DFL 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) 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 in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced significant 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 that 2'-FL/DFL is non-mutagenic at concentrations up to 5,000 µg/plate (the regulatory maximum dose level).

6.3.4.2.2 *In Vitro* Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of the 2'-FL/DFL was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes, in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2016) [Phipps *et al*., 2018].

An initial preliminary cytotoxicity test was conducted using 2'-FL/DFL at concentrations of 0 to 2,000 µg/mL (the regulatory maximum dose level), in the presence (3-hour treatment) and absence (3 and 24-hour treatments) of S9 metabolic activation, where there was no evidence of cytotoxicity reported at any dose level. Cytotoxicity was assessed again in the main experiment, where there was no evidence of cytotoxicity at any dose level under any of the experimental conditions.

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

[15] 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.

significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded that 2'-FL/DFL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the regulatory maximum dose level), in the absence and presence of metabolic activation.

6.4 Human Studies

6.4.1 Safety and Tolerance of 2'-FL in Combination with LNnT in Infants and Adults (Puccio *et al.*, 2017; Alliet *et al.,* 2016; Steenhout *et al*., 2016; Elison *et al.*, 2016)

Two published clinical studies (Puccio *et al*., 2017; Elison *et al*., 2016) conducted in infants and adults administered 2'-FL manufactured by Glycom using chemical synthesis processes have been previously evaluated during the company's GRAS evaluations and are summarized in GRN 546 and 650. These studies also were evaluated by the EFSA NDA Panel during the Novel Food evaluation of 2'-FL (EFSA, 2015a). The study by Puccio *et al.*, (2017) was a randomized, double-blinded, controlled, multi-center, parallel-design study where healthy, full-term infants were provided a standard term infant formula supplemented with 2'-FL (providing 1.0 to 1.2 g 2'-FL/L of reconstituted formula) in combination with LNnT (providing 0.5 to 0.6 g LNnT/L reconstituted formula) (Puccio *et al.*, 2017). A comparator group receiving a standard whey-predominant starter infant formula without HiMOs was included as a control. Infants were aged 0 to 14 days at enrolment, and clinic visits were scheduled at 1, 2, 3, 4, 6, and 12 months of age. The infants were exclusively fed the test or control formulas for the first 4 months of age, with complementary foods allowed to be introduced thereafter. At 6 months of age, the infants in both study groups (test and control formula) were switched to an intact protein, cow milk–based follow-up formula without HiMOs for feeding through to 12 months of age. Endpoints related to growth (body weight gain, body weight, body length, and head circumference), as well as gastrointestinal tolerance, behavioral patterns, and morbidity were collected through to 12 months of age. Additionally, the effects of 2'-FL supplementation on the intestinal microbiota profile were also assessed in the infants (Alliet *et al.,* 2016; Steenhout *et al*., 2016). The EFSA NDA Panel (EFSA, 2015a) made the following conclusion regarding this study:

> "*The Panel notes that this study showed no difference in growth in infants who consumed a formula added with the combination of 2'-FL and LNnT (at the concentrations tested in the study), compared with the control formula infants, and that the growth curves were comparable to the WHO standard curves. The Panel also notes that the results on stool and microbiota endpoints do not raise safety concerns*".

The safety and tolerability of high intake levels of 2'-FL alone or in combination with LNnT was evaluated in adults by Elison *et al*., (2016). The study was a randomized, placebo-controlled, double-blind, parallel-design study involving healthy adult volunteers. The participants were provided either 2'-FL or LNnT alone (at doses of 5, 10, or 20 g per day of 2'-FL or LNnT), or a combination of 2'-FL and LNnT (5, 10, or 20 g per day as the combined amount of 2'-FL and LNnT at a ratio of 2:1). A comparator group received glucose only as a placebo control. All test articles were consumed as single daily bolus doses for 2 weeks. The EFSA NDA Panel recognized that when compared to the placebo control, the high dose of 2'-FL (20 g) resulted in an increase in nausea, rumbling, bloating, passing gas, diarrhea, loose stools, and urgency, as indicated by the Gastrointestinal Symptom Rating Scale (EFSA, 2015a). However, these effects were minor, generally being

rated at a discomfort level of "mild" or less. It is also unlikely that the consumer will experience such high levels of bolus intake under the proposed conditions of use for 2'-FL in foods. The EFSA NDA Panel noted that the highest intake of 2'-FL was estimated to be 8 g/day among women of child-bearing age, based on a 95th percentile intake of 5 g/day, plus the maximum intake of 3 g/day from food supplements. Considering that 2'-FL/DFL is intended to replace the uses of 2'-FL in foods, the intakes of 2'-FL from 2'-FL/DFL are not expected to be additive to those of 2'-FL from its existing authorized uses in the EU.

6.4.2 2'-FL in Combination with Galacto-Oligosaccharides (GOS) in Infant Formula (Marriage *et al.*, 2015; Goehring *et al.*, 2016)

The safety of 2'-FL was evaluated in a published randomized, controlled, double-blinded, prospective study conducted in healthy, full-term, singleton infants (Marriage *et al.,* 2015). Infants were enrolled within Day of Life (DOL) 5 and were provided 1 of 3 formulae: i) a standard, milk-based, commercially available infant formula containing 2.4 g galacto-oligosaccharides (GOS)/L (control formula); ii) the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L; or iii) the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L. All formula had a caloric density of 64.3 kcal/dL (comparable to human milk) and contained a total of 2.4 g/L of non-digestible oligosaccharides. The mothers of infants receiving formulae were instructed to feed the study formulae as their infant's sole source of nutrition until DOL 119. A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) also was included. Infants belonging to the breastfed reference group were not to receive greater than 240 mL of infant formula per week. The primary endpoint was body weight gain from DOL 14 to DOL 119. Secondary endpoints included measures of tolerance and other anthropometric parameters. The presence of 2'-FL also was evaluated in blood and urine collected from a subset of infants at DOL 42 and 119 and in the comparator group at DOL 42.

A total of 338 infants completed the study; the number of infants discontinuing the study formulae was not different among the formula-fed groups. No significant differences in body weight gain, body weight during clinical visits, length, or head circumference were report ed between the formulae groups and the human milk reference group. The mean daily volume of study formula consumed during the study period was similar between the control and test formula groups; however, between enrolment and DOL 28, the control group consumed significantly more formula than the group receiving the formula supplemented with 0.2 g 2'-FL (661 mL/day compared to 614 mL/day, $p = 0.024$). The mean consumption values for other time points and for the other formula group were not disclosed by the authors. All formulae were well tolerated, and no significant differences in the overall percentage of infants with adverse events or serious adverse events were reported between infants receiving the experimental formulae and the standard formula. Average stool consistency, number of stools per day, and the percent of feedings associated with spit-up or vomit were comparable between all groups. Overall, the investigators concluded that the feeding of infant formula with a caloric density similar to that of human milk resulted in comparable growth rates to that of human milk-fed infants and that formulae supplemented with 2'-FL were well tolerated (Marriage *et al.*, 2015).

2'-FL was detected in the plasma and urine of infants provided 2'-FL in formula and in infants consuming human milk. The plasma concentrations of 2'-FL on DOL 42 reflected the amount of 2'-FL present in the feeds (*i.e.*, human milk > formula containing 1.0 g 2'-FL/L > formula containing 0.2 g 2'-F/L); however, the mean plasma concentrations were not similarly correlated for samples obtained on DOL 119. This finding was hypothesized by the study investigators to be due to developmental changes in the structure and function of the gastrointestinal tract mucosa, leading to a less permeable gut, as well as compositional changes in the intestinal microbiota, leading to better utilization of 2'-FL by the microflora. No 2'-FL was detected in the plasma of infants fed the standard milk-based commercial formula containing GOS only.

Mean urine concentrations of 2'-FL were greatest in infants fed human milk and the formula containing 1.0 g 2'-FL/L, followed by infants fed the formula containing 0.2 g 2'-FL/L. The relative excretion was calculated to be 1.44 and 1.12% for the group receiving the 0.2 g 2'-FL/L and 1.0 g 2'-FL/L formulae, respectively.

The effects of 2'-FL supplementation on biomarkers of immune function among infants was also evaluated in this study; these results have been separately reported in a publication by Goehring *et al.* (2016). At 6 weeks of age, 2 to 3 mL of non-fasting venous blood was drawn and analyzed for immunophenotypic markers (by flow cytometry) for the following cell surface markers: CD4, CD8, CD20, and CD56. Plasma samples were analyzed for cytokines: IFN-α2, IFN-δ, IL-10, IL-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, IFN-δ–induced protein 10, RANTES (regulated upon activation, normal T cell expressed and secreted), and TNF-α. RNA from peripheral blood mononuclear cells were quantified and used to detect a respiratory syncytial virus (RSV)-specific gene product, NS1, to quantify viral load. The control formula group exhibited lower percentages of circulating T lymphocytes and CD8+ lymphocytes compared to the breastfed group; however, no significant differences in any cell type were reported between the 2'-FL supplemented groups and the control and breastfed groups with the exception of a lower CD8+ population in infants receiving 1.0 g 2'-FL/L compared to the breastfed group. The inflammatory cytokine profiles revealed a statistically significant higher concentration of circulating inflammatory cytokines IFN-α2, IL-1β, IL-6, TNF-α and IL1ra in the control formula group when compared to be breastfed group. However, no statistically significant differences were reported in both groups receiving the experimental formulas containing 2'-FL, when compared to the breastfed group. No significant differences in the other plasma cytokines or RANTES were reported between any of the groups. No significant differences in RSV NS1 viral load were reported between any groups. In *ex vivo* RSV-induced peripheral blood mononuclear cell (PMBC) culture, cytokine production in the breastfed group did not significantly differ from the groups receiving formula containing 2'-FL; however, TNF-α and IFN-γ were significantly lower, and a non-significant trend towards reduced IL-1ra and IL-6 was reported, in the breastfed group compared to the control formula. The study authors concluded that infants provided 2'-FL fortified formulas exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group. In contrast, such effects were not reported among infants administered the control formula containing GOS only.

6.4.2.1 2'-FL in Combination with Short-Chain Fructo-Oligosaccharides in Infant Formula (Kajzer et al., 2016)

The gastrointestinal tolerance of an infant formula containing 2'-FL in combination with short-chain fructo-oligosaccharides (scFOS) was evaluated in a prospective, randomized, multi-center, double-blinded controlled 2-arm study in full-term, singleton infants (Kajzer *et al.*, 2016). A total of 121 infants were enrolled between 0 to 8 days of age and were either breastfed (reference group) or assigned to receive 1 of 2 experimental milk-based formulas containing a caloric density of 643 kcal/L. The control formula did not contain oligosaccharides (composition not further detailed), and the test formula was supplemented with 0.2 g/L 2'-FL combined with 2.0 g/L scFOS. A record of milk/formula intake, stool patterns (including average mean rank stool consistency), anthropometrics, were obtained until 35 days of age and parental questionnaires (not further detailed) were also collected.

No significant differences in mean rank stool consistency were reported between groups. No significant differences among stool consistency were reported between groups, although the average number of stools per day was significantly higher in the breastfed group compared to both formula groups. No significant differences in formula intake, number of study formula feedings per day, anthropometric data, or percent feedings with spit up/vomit were reported by the investigators. The study authors concluded that the test

formula was well tolerated, with anthropometric data and feeding characteristics similar to infants fed control formula or breast milk.

6.5 Allergenicity

As explained in Section 2.2.3, the purification steps involved in the manufacture are proven to remove protein (*i.e.*, potential allergen) to a level of <0.0017 % (w/w). Glycom's supplier management procedure ensures that all suppliers of raw materials and processing aids must declare and demonstrate that their materials do not contain any of the allergens specified under Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers[16].

In addition, Glycom has assessed the allergenic potential of the recombinant proteins expressed by the production strain using bioinformatic analyses. The amino acid sequences of the recombinant proteins were assessed using BLAST search algorithms of Allergen Online (version 17) against a curated database of known and putative allergens hosted by the Food Allergen Research and Resource Program (FARRP) of the of the University of Nebraska (FARRP, 2017). The January 18, 2017 update to the database was used for the analyses and contained sequences of 2035 putative allergens. The online tool allows search by 3 different search algorithms each with its own alert limit for potential allergenicity: (i) full sequence length (FASTA) comparison with an alert limit of minimum 50% sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of minimum 35% sequence similarity to hint for potential allergenic potential; (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified, and therefore, it is anticipated that the 2'-FL/DFL product will not pose an allergenic risk to the consumer.

6.6 Expert Panel Evaluation

Glycom has concluded that 2'-FL/DFL 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 2'-FL/DFL, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, convened by Glycom, independently and critically evaluated all data and information presented herein, and also concluded that 2'-FL/DFL 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 Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of 2'-FL/DFL is presented in Appendix A.

[16] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. OJ L 304, 22.11.2011, p. 18–63.

6.7 Conclusion

Based on the above data and information presented herein, Glycom has concluded that the intended uses of 2'-FL/DFL 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 Panel of Experts, qualified by experience and scientific training, to evaluate the use of 2'-FL/DFL in infant formula and conventional food, who similarly concluded that the intended use of 2'-FL/DFL in infant formula and conventional food as described herein is GRAS.

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

Part 7. §170.255 List of Supporting Data and Information

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APPENDIX A

EXPERT PANEL CONSENSUS STATEMENT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED INFANT FORMULA AND CONVENTIONAL FOOD USES OF 2'-FUCOSYLLACTOSE/DIFUCOSYLLACTOSE (2'-FL/DFL)

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL) for Uses in Infant Formula and Conventional Food Products

August 14, 2018

INTRODUCTION

Glycom A/S (Glycom) convened a panel of independent scientists (the "Expert Panel"), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of data and information pertinent to the safety of the company's human-identical milk oligosaccharide mixture of 2'-fucosyllactose/difucosyllactose (2'-FL/DFL), produced by fermentation using a modified strain of *Escherichia coli* K-12 DH1, and to determine whether the intended uses of 2'-FL/DFL in non-exempt term infant formula and various conventional food and beverage products as described in Table A-1, would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, independently and collectively, critically examined a comprehensive package of publicly available scientific information and data compiled from the literature and presented to the Panel in a dossier titled "*GRAS Status of 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)*" [dated August 7, 2018], which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of 2'-FL/DFL. This information was prepared in part from a comprehensive search of the scientific literature performed by Glycom and also included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, the history of consumption from human breast milk, and the safety of 2'-FL/DFL.

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

SUMMARY AND BASIS FOR GRAS

Glycom A/S (Glycom), a manufacturer of human-identical milk oligosaccharides (HiMOs), intends to introduce a 2'-fucosyllactose (2'-FL) preparation containing <20% difucosyllactose (DFL) to the United States (U.S.) marketplace as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products (Table A-1). 2'-FL/DFL comprises 2 structurally and biologically related HiMOs produced as a mixture by fermentation using a modified strain of *Escherichia coli* (*E. coli*) K-12. The mixture of

2'-FL and DFL (2'-FL/DFL) is a powder that is produced by fermentation which contains not less than 85% 2'-FL and DFL in a respective ratio similar to the range naturally present in human milk.

The main constituent, 2'-FL, is a neutral trisaccharide that is secreted naturally in large amounts into human milk, but it is also found at minor amounts in some other mammalian milks. 2'-FL is the most abundant human milk oligosaccharide (HMO) in human milk (~2.5 g/L). 2'-FL is comprised of L-fucose, D-galactose, and D-glucose. Alternatively, the molecular constitution can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an α(1→2) bond to form the trisaccharide. 2'-FL is a chemically defined trisaccharide that occurs only as one specific constitutional isomer. The second component, DFL, is a derivative of 2'-FL where a second fucose sugar has been added to the 3-glucose position of 2'-FL. It is a neutral tetrasaccharide and is also present in human milk at significant amounts (~0.5 g/L). DFL is composed of two units of L-fucose, one unit of D-galactose, and one unit of D-glucose.

2'-FL and DFL obtained from microbial fermentation are chemically and structurally identical to 2'-FL and DFL that are secreted into human breast milk, as confirmed by 1H- and 2D-nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry. Therefore, both 2'-FL and DFL have an established long history of safe consumption as a nutritive component of human breast milk in infants on the basis that the 2'-FL/DFL manufactured by Glycom is chemically identical to 2'-FL and DFL present in human breast milk.

The Expert Panel critically reviewed details of the manufacturing process for 2'-FL/DFL. The ingredient is manufactured in compliance with cGMP and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacturing process can be broadly divided into 2 stages. In Stage 1 [upstream processing (USP)], D-lactose is converted to 2'-FL and DFL by a sequential series of fucosylation steps using the adapted cellular metabolism of the production microorganism, which uses D-glucose (or optionally D-glycerol or D-sucrose) as an exclusive energy and carbon source and D-lactose as a substrate for 2'-FL and DFL biosynthesis. Nitrogen sources were provided by ammonium salts. The production microorganism is a derivative of *E. coli* K-12 DH1, which is a safe laboratory strain with a well-characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). *E. coli* K-12 DH1 was optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various carbohydrates (identical to those described in GRN 650), and then transformed by homologous recombination at three targeted loci within the genome using three working plasmids carrying the required genes for 2'-FL/DFL biosynthesis. Recombinant genes introduced into the production organism included the colanic acid operon from *E. coli* and a fucosyltransferase gene from *Helicobacter pylori* (these genes are also identical to those described in GRN 650). The resulting strain was designated MAP1001. The Expert Panel noted that the introduced genes were 'cloned' from the donor organisms using *de novo* DNA synthesis of published gene sequences avoiding the introduction of unwanted DNA sequences to the production organism. During fermentation, both 2'-FL and DFL are efficiently excreted into the fermentation broth and the microbial biomass containing the production organism is removed from the culture supernatant by ultrafiltration. The Expert Panel noted that the introduced genetic modifications were well characterized, and the biosynthetic functions of the expressed enzymes are well defined and would not introduce unexpected pleiotropic effects to the organism. The recombinant proteins were further characterized using bioinformatic tools and were demonstrated not to be homologous to amino acid sequences of known or putative toxins or allergens.

In Stage 2 [downstream processing (DSP)], a series of purification, isolation, and concentration steps are used to generate the final high-purity 2'-FL/DFL ingredient. No solvents are used during manufacturing and Glycom confirms that all processing-aids and food contact articles used in Stage 1 and 2 are used in accordance with an appropriate federal regulation, have been previously determined to be GRAS or have been the subject of an effective food contact notification. Quality control measures are in place during the entire purification and

isolation process to ensure that the final batches of 2'-FL/DFL released conform with the product specifications. The 2'-FL and DFL produced by fermentation are chemically identical to those present in human milk from lactating women. There have been no modifications to the molecular structure of 2'-FL or DFL during their manufacture from that of the 2'-FL and DFL present in human milk.

Glycom has established food-grade specifications for 2'-FL/DFL. The specifications for 2'-FL/DFL include parameters related to physical properties, purity, water, ash content, and microbiological contaminants. As a simple carbohydrate mixture, the composition of 2'-FL/DFL is specified by a combination of parameters but foremost by the sum of 2'-FL and DFL which makes up at least 85.0% of the dry powder. The specified purity of 2'-FL is set at a minimum assay value of at least 75.0% on a dry weight basis, and DFL at no more than 20.0% on a dry weight basis. Small quantities of D-lactose (Max. 10.0% w/w), L-fucose (Max. 1.0% w/w), and 2'-fucosyl-D-lactulose (Max. 2.0% w/w) originating from the fermentation media are also detectable in the final ingredient. As these carbohydrates are naturally present in human breast milk, the total human milk saccharide content of the ingredient is at least 92.0%. Specifications have been established for carbohydrate-type compounds and residual proteins originating from the fermentation and downstream purification processes. All analytical methods are internationally recognized or have been validated internally. The Expert Panel reviewed the results from 5 batches of 2'-FL/DFL demonstrating that the manufacturing process produces a consistent material in conformance with the product specifications.

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

The Expert Panel reviewed the data supporting the bulk stability of 2'-FL/DFL, produced by fermentation as described herein, under real-time conditions of 25°C and 60% relative humidity (RH), as well as accelerated conditions of 40°C and 75% RH, both over a 9-month storage period. 2'-FL/DFL was stable throughout the storage period with no appreciable changes in organoleptic properties, degradation of the material, or alterations in impurity profiles. Stress/forced stability tests of 2'-FL/DFL powder in aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively, to identify the likely degradation products of 2'-FL/DFL at acidic (<5.0) and neutral pH (>6.0). The results of these studies revealed two potential pH-dependent chemical degradation pathways of 2'-FL/DFL, namely hydrolysis of 2'-FL (to fucose and lactose, and slightly to 2-fucosyl-galactose and glucose) at pH <5.0, and isomerization of 2'-FL/DFL (to fucosyl-lactulose and difucosyl-lactulose) at pH >6.0. The stability of 2'-FL/DFL described herein has also been evaluated in a commercially-representative infant formula, with data supporting that 2'-FL and DFL are stable in infant formula at up to 12 months of storage.

Glycom provided a summary on the stability of chemically synthesized 2'-FL, which was evaluated in various food matrices, including a commercially-representative infant formula, yogurts, ready-to-drink flavored milk, and citrus fruit beverages (the results of which were provided by reference to GRN 546). As 2'-FL produced by fermentation is compositionally comparable to 2'-FL manufactured by chemical synthesis, there are no anticipated differences in their stabilities in food matrices and thus, 2'-FL has been sufficiently demonstrated to be stable in infant formula and representative food and beverages under the conditions of these studies.

2'-FL/DFL is intended to be added as a food ingredient to foods targeted to infants and young children, including non-exempt term infant formula, and in specific conventional food and beverage products used by

the general population (Table A-1). The maximum use-levels in term infant formulas are proposed on the basis of providing similar levels of 2'-FL and DFL on a body weight basis as those consumed by breast-fed infants. In the U.S., Glycom's 2'-FL obtained by fermentation has been concluded to be GRAS for use in term infant formula at a maximum level of 2.4 g/L and for use in various other conventional foods and beverages at maximum levels ranging from 0.084 to 2.4 g/serving (GRN 650). The DFL content of this 2'-FL preparation has been specified as less than 1.0%. 2'-FL preparations obtained by fermentation by other manufacturers have also been concluded to be GRAS for use in the U.S. in infant formulas at levels ranging from 2.0 to 2.4 g/L (GRN 749, 735, 571), and in various other foods and beverages at levels ranging from 0.28 to 1.2 g/serving (GRN 735). The DFL content of the 2'-FL preparations described in GRN 571 and 749 is up to 5% and up to 7%, respectively. Glycom's 2'-FL obtained by chemical synthesis has been concluded to have GRAS status in term infant formula in the U.S., at a maximum level of 2.4 g/L, and in various other foods at maximum levels ranging from 0.084 to 2.4 g/serving. 2'-FL produced from both synthetic and microbial sources has been approved for use in infant and follow-on formula (at levels up to 1.2 g/L) and various other foods and beverages in the EU, and 2'-FL obtained from fermentation has been approved for use in various infant and follow-on formulas and/or growing up milk in Israel (at levels up to 2.0 g/L) and Singapore (at levels up to 1.2 g/L).

The Expert Panel critically evaluated the published data and information characterizing the safety of 2'-FL and DFL. Human milk contains, as its third largest solid component, a fraction consisting of a complex family of structurally-related oligosaccharides, known as human milk oligosaccharides (Kuhn, 1952; Malpress and Hytten, 1958; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). 2'-FL and DFL belong to the fucosylated sub-fraction of HMOs in human milk, oligosaccharides that contain the sugar fucose and which is reported to constitute on average around 70% of the total HMO fraction. Approximately 80% of the global population of mothers express 2'-FL and DFL into their milk (termed "Secretors"). In milk from Secretor mothers, 2'-FL has been reported to be the major milk oligosaccharide, followed by lacto-N-fucopentaose I (LNFP I) and DFL (Sprenger *et al.*, 2017). Mean values of 2'-FL and DFL in milk from Secretor mothers has been reported to be 2.74 and 0.42 g/L, respectively (Thurl *et al.*, 2017). 2'-FL and DFL therefore have an established history of safe consumption by infants consuming human milk.

Publications from independent research groups have reported on the concentration of 2'-FL and DFL in pooled human milk (Chaturvedi *et al.*, 1997, 2001a; Nakhla *et al.*, 1999; Erney *et al.*, 2000, 2001; Morrow *et al.*, 2004; Musumeci *et al.*, 2006; Asakuma *et al.*, 2008, 2011; Smilowitz *et al.*, 2013; Spevacek *et al.*, 2015; Austin *et al.*, 2016; McGuire *et al.*, 2017), and milk from Secretor mothers (Thurl *et al.*, 1996, 2010; Coppa *et al.*, 1999, 2011; Leo *et al.*, 2009; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013; Hong *et al.*, 2014; Olivares *et al.*, 2015; Kunz *et al.*, 2017; Sprenger *et al.*, 2017; McGuire *et al.*, 2017) across a variety of demographic groups, Lewis body genotypes, and lactational stages. Based on these publications, the average concentration of 2'-FL in pooled human breast milk after full-term birth is highest in colostrum (3.2 g/L), followed by transitional milk (2.5 g/L) and continue to decline slowly in mature milk (2.2 g/L) and mature milk from a lactation stage later than 2 months (1.9 g/L). In milk from Secretor mothers, the corresponding levels are significantly higher, with average levels reported at 4.0 g/L in colostrum, 3.3 g/L in transitional milk, 3.0 g/L in mature milk and 2.4 g/L in mature milk from a lactation stage later than 2 months. Concentrations of 2'-FL between different mothers can be highly variable, with reported levels reaching as high as 8.4 g/L. There are negligible differences in average 2'-FL concentrations in Asia, China, Europe, and the U.S., while concentrations are higher in Mexico, Peru, and Hispanic populations of the U.S due to higher Secretor frequency. Using the range of average levels of 2'-FL reported for breast milk over different times of lactation, of 1.9 to 4.0 g/L, combined with the estimated formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of 2'-FL from breastfeeding can be estimated at between 494 to 1,040 mg/kg body weight/day.

The average content of DFL in pooled human breast milk after full-term birth is highest in colostrum (0.5 g/L), followed by transitional milk (0.4 g/L) and continues to decline slowly in mature milk (0.3 g/L). However, the reported ranges of DFL in human milks widely vary from 0.1 to 1.0 g per liter of milk. Using the range of average levels of DFL reported for breast milk over different times of lactation, of 0.2 to 0.5 g/L, combined with the estimated formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of DFL from breastfeeding can be estimated at between 52 and 130 mg/kg body weight/day.

The Expert Panel reviewed the estimated dietary exposures to 2'-FL/DFL based on an assessment of the anticipated intake of 2'-FL/DFL as an ingredient under the intended conditions of use provided by Glycom. Intakes estimates were conducted using the recently published food consumption data from the U.S. National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Survey (NHANES). Among the total population (all ages), the mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 1.65 and 3.54 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of 2'-FL/DFL on an absolute basis, at 1.87 and 4.03 g/person/day, respectively. The elderly had the lowest mean consumer-only intake of 1.32 g/person/day, while female teenagers had the lowest 90th percentile consumer-only intakes of 2.75 g/person/day. On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 34.5 and 67.2 mg/kg body weight/day, respectively. Among the individual population groups, toddlers were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 140.0 and 282.0 mg/kg body weight/day, respectively. The elderly had the lowest mean and 90th percentile consumer-only intakes of 17.5 and 41.2 mg/kg body weight/day, respectively.

Reviews of published data and information characterizing the absorption, distribution, metabolism, and excretion (ADME) of HiMOs, including 2'-FL and DFL, have been the subject of previous comprehensive evaluations, and this information is incorporated herein by reference to GRN 546 (Section IV.B.4) and GRN 650 (Section IV.D). Briefly, 2'-FL and DFL present in Glycom's 2'-FL/DFL ingredient are structurally-identical to their naturally occurring counterparts in human milk. A number of ADME studies have demonstrated that HMOs, including 2'-FL and DFL, do not undergo any significant digestion in the upper gastrointestinal tract, and are transported intact to the large intestine where they are subjected to fermentation by microbiota populations that are indigenous to this site (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al*., 2000; Chaturvedi *et al*., 2001b; Rudolff and Kunz, 2012). A small portion of HMO's have been reported to be absorbed (approximately 1 to 2% of the total amount of HMO ingested), which are excreted unchanged in urine. Therefore, the absorption of 2'-FL/DFL is expected to be highly limited and any level of 2'-FL/DFL that is absorbed would be no different to that occurring in breast-fed infants.

The Expert Panel previously evaluated a number of toxicological studies that were conducted with 2'-FL obtained by fermentation or chemical synthesis, including 90-day oral toxicity studies, a neonatal piglet feeding study, and genotoxicity assays (*i.e*., GRN 546 and 650). Considering that 2'-FL is the main component of 2'-FL/DFL (*i.e.*, specified content of at least 75.0%) findings from these studies are relevant to the GRAS status of 2'-FL/DFL. Comprehensive discussions of the published toxicity studies as they apply to the safety of 2'-FL for use in infant formula and conventional food and beverage products were incorporated by reference to GRN 546, GRN 571, GRN 650, GRN 735, GRN 749. The Expert Panel noted that all HiMOs manufactured by Glycom are produced from a production organism originating from the same MDO lineage strain and that all HiMOs are produced by fermentation using defined carbon and nitrogen sources. Toxicology studies of 2'-FL produced by fermentation support the safety of the MDO lineage, and that the introduced genetic modifications produce a predictable pattern of metabolites and intended fermentation products without concern for unexpected pleiotropic effects. The Expert Panel noted that there have been no toxicologically significant findings in any

reported toxicity study that suggests that 2'-FL, or other HiMOs produced using Glycom's MDO strain lineage, display toxicity profiles in animals that would be of concern for use as an ingredient in formula or other conventional food products. During Glycom's updated literature search new toxicity studies pertinent to the toxicity of 2'-FL included a subchronic toxicity study conducted by van Berlo *et al*. (2018). The 2'-FL test article evaluated by the authors was produced by FrieslandCampina and the ingredient has GRAS status as described in GRN 735. The ingredient was produced by microbial fermentation from a modified strain of *E. coli* K-12 and was evaluated using a modified sub-chronic (90-day) oral toxicity study in 25-day-old Wistar [Crl:WI(Han)] rats (van Berlo *et al*., 2018). The study was conducted in accordance with OECD principles of GLP (OECD, 1998a) and OECD Test Guideline 408 (OECD, 1998b) modified to accommodate the use of juvenile animals. Rats (10/sex/group) were exposed to 2'-FL in animal feed (VRF1 finely ground) at concentrations of 0, 3, 6, or 10% (w/w), equivalent to doses of 0, 2,170, 4,270, and 7,250 mg/kg body weight/day for males and 0, 2,450, 5,220, and 7,760 mg/kg body weight/day for females, for 13 weeks [from post-natal day (PND) 25 to PND 115]. No test article-related effects on mortality, neurotoxicity, ophthalmology, body weight and food consumption (in male rats only) were reported. A slight but significant decrease in food consumption in females of the high-dose group was reported. No significant difference in water consumption across groups, except for a slight but significant increase in males of the high dose group on Days 35 to 36 and females of the high-dose group on Days 38 to 39 was reported. Administration of 2'-FL resulted in statistically significant changes in a small number of the hematological, clinical chemistry, and urinalysis parameters when compared to the control; however, these changes were minor and not considered to be toxicologically relevant as they were not dose-dependent, not observed in both sexes, or were within the normal historical control range. The relative-to-body weight of the liver was significantly increased in males in the high-dose group; however, this was not associated with histopathological evidence and therefore is not considered adverse. The absolute and relative-to-body weights of the filled and empty cecum were significantly increased in the mid- and high-dose group males and females; the absolute weight of the filled cecum in low-dose males was also increased. The cecal enlargement was reported to be a physiological response due to the non-digestibility of the 2'-FL and is therefore not a toxic effect. No treatment-related differences in macroscopic or microscopic (histological) findings were reported between rats receiving 2'-FL and the control group. A NOAEL of 7,250 mg/kg body weight/day for males and 7,760 mg/kg body weight/day for females, based on the highest concentration tested (10%) for each sex. This study confirms the low toxicity of 2'-FL.

The Expert Panel noted that 2'-FL and other HiMOs are natural constituents of human breast milk and therefore are not genotoxic. However, various 2'-FL preparations have been evaluated in published and unpublished genotoxicity studies, including the bacterial reverse mutation assay, and an *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells[1], *in vitro* micronucleus assays conducted in cultured peripheral human lymphocytes, and *in vivo* micronucleus studies. These studies are incorporated by reference to previous GRAS notifications (*e.g*., GRN 650). The results from these studies confirmed that 2'-FL does not have mutagenic or genotoxic potential, and that the manufacturing process did not introduce undesirable substances with potential mutagenic/genotoxic potential.

As part of its Product Stewardship Program, Glycom commissioned a series of toxicological studies on its 2'-FL/DFL ingredient described herein which is the same ingredient to be commercially marketed. These studies include a modified subchronic (90-day) oral toxicity study in rats, a bacterial reverse mutation assay, and an *in vitro* mammalian cell micronucleus test in human lymphocytes.

The Expert Panel critically evaluated the results of a 90-day oral toxicity study on 2'-FL/DFL manufactured by Glycom from fermentation as described herein (Phipps *et al*., 2018). The study was conducted in compliance

[1] This study was conducted using 2'-fucosyllactose manufactured by chemical synthesis

with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals. Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 0 (oligofructose powder as reference control), 1,000, 3,000, or 5,000 mg/kg body weight/day 2'-FL/DFL, by gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days. Dosing was initiated on PND 7. There were no consistent, statistically significant and dose-dependent adverse effects reported on survivability, morbidity, body weight, body weight gain, food consumption, pre-weaning development or day at which physical signs of sexual maturation were attained, behavior and activity, clinical chemistry, urinalysis, macroscopic (including organ weights and ratios) and histopathological findings.

The NOAEL was concluded to be 5,000 mg/kg body weight/day 2'-FL/DFL, the highest dose tested.

The potential mutagenicity of Glycom's 2'-FL/DFL as described herein was evaluated in a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test, which were performed in accordance with OECD Test Guidelines 471 (OECD, 1997) and 487 (OECD, 2016), respectively (Phipps *et al*., 2018). The results of these studies demonstrated that 2'-FL/DFL is non-mutagenic, non-clastogenic, and non-aneugenic.

The Expert Panel was provided the summaries from published clinical studies conducted in infants and adults in which the safety of 2'-FL, either alone or in combination with lacto-*N*-neotetraose (LNnT) or other non-digestible oligosaccharides, was evaluated (Marriage *et al*., 2015; Alliet *et al.,* 2016; Steenhout *et al*., 2016; Elison *et al*., 2016; Kajzer *et al.*, 2016; Puccio *et al*., 2017). Details of two of these studies (Puccio *et al*., 2017; Elison *et al*., 2016) were incorporated by reference to GRN 546 and 650. The Expert Panel noted that all these studies were previously reviewed by the Expert Panel during previous GRAS evaluations and by other qualified Experts including the EFSA NDA Panel during the Novel Food evaluation of 2'-FL (EFSA, 2015). Findings from these studies have consistently demonstrated that administration of 2'-FL and other HiMOs at concentrations that are representative of levels in human breast milk is safe and well tolerated.

Glycom evaluated the allergenic potential of the recombinant proteins expressed by the production strain using bioinformatic analyses. The amino acid sequences of the recombinant proteins were assessed using BLAST search algorithms of Allergen Online (version 17) against a curated database of known and putative allergens hosted by the Food Allergen Research and Resource Program (FARRP) of the of the University of Nebraska (FARRP, 2017). The online tool allows search by 3 different search algorithms each with its own alert limit for potential allergenicity: (i) full sequence length (FASTA) comparison with an alert limit of minimum 50% sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of minimum 35% sequence similarity to hint for potential allergenic potential; (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified. In addition, the purification steps involved in the manufacture of 2'-FL/DFL are proven to remove protein (*i.e.*, potential allergen) to a level of <0.0017 % (w/w). Based on the purification process utilized during the manufacturing process and absence of detectable protein in the ingredient, the Expert Panel considered the risk of allergenicity to be very low. The Expert Panel noted that as lactose is used as a substrate during fermentation that food products containing 2'-FL/DFL would require labeling "contains milk" in accordance with the Food Allergen Labeling and Consumer Protection Act (FALCPA).

Following its independent and collective critical evaluation of the available information of 2'-FL and DFL, including preclinical and clinical studies, the Expert Panel concluded that the data basis supports the conclusion presented on the next page.

CONCLUSION

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

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

[redacted]

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20 August 2018
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ATTACHMENT A1: INTENDED FOOD USES AND USE LEVELS FOR 2'-FL/DFL IN THE UNITED STATES

Table A-1 Summary of the Individual Proposed Food Uses and Use-Levels for 2'-FL/DFL in the U.S.

Food Category	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use-Level (g/RACC)	Proposed Maximum Use-Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.96	4.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.72	2.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.16	1.6
	Toddler Formulas	100 mL[c]	0.12	1.2
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.07 to 1.70	10
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	1.6	40
	Cereal and Granola Bars	40 g	0.8	20
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.48	2.0
Milk Products	Buttermilk*	240 mL	0.48	2.0
	Flavored Milk	240 mL	0.48	2.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.96	4.0
	Yogurt*	170 g	3.4	20

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose; CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2018). 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] Includes ready-to-drink and powder forms.

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

*2'-FL/DFL is intended for use in unstandardized products when standards of identity do not permit its addition.

Glycom A/S Kogle Allé 4 2970 Hørsholm. Denmark

20 December 2018

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

RECEIVED
JAN 28 2019
OFFICE OF
FOOD ADDITIVE SAFETY

Dear Dr. Gaynor:

Re: Amendment to GRAS Notice for 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)

Glycom A/S (Glycom), a manufacturer of human-identical milk oligosaccharides (HiMOs), has previously concluded that the company's 2'-fucosyllactose (2'-FL) preparation containing up to 20% difucosyllactose (DFL) (henceforth 2'-FL/DFL) has Generally Recognized as Safe (GRAS) status for use as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products across multiple categories. Glycom's GRAS conclusion was notified to the offices of the United States Food and Drug Administration (U.S. FDA) on September 20th, 2018 and was filed by the agency under GRN No. 815. In accordance with 21 CFR §170.260 – *Steps you may take before FDA responds to your GRAS notice*, Glycom hereby submits the following minor amendment to GRN 815, which includes a corrected pH range and revised specifications for microbiological parameters listed in Table 2.3.1-1 of GRN 815. Glycom's revised specification includes minimum limits for the use of 2'-FL/DFL during the wet-blending stage of infant formula manufacturing and non-infant applications. Further description of this amendment and a narrative supporting that these proposed revisions would be generally recognized safe are discussed in the attached amendment.

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

Sincerely,

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

GRAS STATUS OF 2'-FUCOSYLLACTOSE/ DIFUCOSYLLACTOSE (2'-FL/DFL)

AMENDMENT TO GRN 815

PREPARED FOR:
Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

DATE:
19 December 2018

GRAS Status of 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)

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List of Figures and Tables

GRAS Status of 2'-Fucosyllactose/Difucosyllactose (2'-FL/DFL)

Part 1. Introduction

Glycom A/S (Glycom), a manufacturer of human-identical milk oligosaccharides (HiMOs), has previously concluded that the company's 2'-fucosyllactose (2'-FL) preparation containing up to 20% difucosyllactose (DFL) (henceforth, 2'-FL/DFL) has Generally Recognized as Safe (GRAS) status for use as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products across multiple categories. Glycom's GRAS conclusion was notified to the offices of the United States Food and Drug Administration (U.S. FDA) on September 20th, 2018 and was filed by the agency under GRN No. 815. In accordance with 21 CFR §170.260 – Steps you may take before FDA responds to your GRAS notice, Glycom hereby submits the following amendment to GRN 815, which includes a revised specification for microbial contaminants in Table 2.3.1-1 that applies to 2'-FL/DFL used exclusively for infant formula and toddler nutrition products subjected to heat treatments (*e.g.*, as applied during the wet-blending processing stage of infant formula manufacture), and for use in conventional non-infant formula food uses. Further description of this amendment and a narrative supporting that these proposed revisions would be generally recognized safe are discussed below in Part 2 of this supplement.

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

2.1 Amendment to Specifications

The revised specifications for 2'-FL/DFL are presented in Table 2.1-1 and include revised microbial specification limits for the addition of 2'-FL/DFL to infant and toddler formula products during the wet blending stage of the manufacturing process and therefore are subjected to heat-treatment[1] microbial kill steps: Aerobic mesophilic total plate count (<1000 CFU/g), *Enterobacteriaceae* (<10 CFU/g), *Salmonella* spp. (Absent in 25 g), Yeast (100 CFU/g), Molds (100 CFU/g). The pH range has been also adjusted from 3.5 to 5.4 to 4.0 to 6.0. This revised specification also is considered suitable use in conventional food products targeted to the general population (*i.e.*, non-infant formula applications).

[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 *Salmonella* or *Enterobacteriaceae*, including *E. 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.

Table 2.1-1 Specifications for 2'-FL/DFL

Definition

2'-Fucosyllactose/difucosyllactose (2'-FL/DFL) is a purified white to off-white powder that is produced by a microbial process.

Source

A modified strain of *Escherichia coli* K-12 DH1.

Parameter	Specification	AVE	±	SD	Method
Appearance	Powder or agglomerates	Complies			ISO 6658:2007
Color	White to off white	Complies			ISO 6658:2007
Identification (2'-FL/DFL)	RT of standard ± 3%	Complies			Glycom method HPAEC-HMO-011
Assay (water free) – Sum of HiMS[a]	Not less than 92.0 w/w %	94	±	0.4	Glycom method HPAEC-HMO-011,012
Assay (water free) – Sum of 2'-FL and DFL	Not less than 85.0 w/w %	92	±	1	Glycom method HPAEC-HMO-012
Assay (water free) – 2'-FL	Not less than 75.0 w/w %	81	±	2	Glycom method HPAEC-HMO-012
Assay (water free) – DFL	Not more than 20.0 w/w %	11	±	1	Glycom method HPAEC-HMO-012
D-Lactose	Not more than 10.0 w/w %	1.9	±	1.3	Glycom method HPAEC-HMO-011
L-Fucose	Not more than 1.0 w/w %	0.1	±	0.0	Glycom method HPAEC-HMO-011
2'-Fucosyl-D-lactulose	Not more than 2.0 w/w %	0.9	±	0.1	Glycom method HPAEC-HMO-011
Sum of other carbohydrates	Not more than 6.0 w/w %	2.2	±	0.1	Glycom method HPAEC-HMO-011
pH (20°C, 5% solution)	4.0 to 6.0	4.5	±	0.3	Ph. Eur. 9.2 2.2.3 (07/2016:20203)
Water	Not more than 6.0 w/w %	0.4	±	0.0	Glycom method KF-001
Ash, sulphated	Not more than 0.8 w/w %	0.04	±	0.03	Ph. Eur. 9.2 2.4.14 (04/2010:20414)
Residual protein by Bradford assay	Not more than 0.01 w/w %	< 0.0017			Glycom method UV-001
Microbiological Parameters[b]					
Aerobic mesophilic total plate count	Not more than 500 CFU/g	< 10			ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	Complies			ISO 21528-1:2004, ISO 21528-2:2004
Salmonella spp.	Absent in 25 g	Complies			ISO 6579:2006
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies			ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	Complies			ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	< 10			ISO 7932:2005
Yeasts	Not more than 10 CFU/g	< 10			ISO 7954:1999
Molds	Not more than 10 CFU/g	< 10			ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	0.02	±	0.03	Eur. Ph. 2.6.14

2'-FL = 2'-fucosyllactose; AVE = average; CFU = colony-forming units; DFL = difucosyllactose; EPA = Environmental Protection Agency; EU = endotoxin units; Eur. Ph. = European Pharmacopoeia; HiMS = Human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; MPN = most probable number; RT= retention time.

[a] HiMS = Sum of 2'-FL, 3-fucosyllactose, DFL, lactose and fucose.

[b]The following microbial specifications represent minimum limits for 2'-FL/DFL that is added to infant formula and toddler formula products during the wet-mix stage of the formula manufacturing process, and also is suitable for conventional food products used by the general population (*i.e.*, non-infant formula and toddler formula food products): Aerobic mesophilic total plate count (<1000 CFU/g), *Enterobacteriaceae* (<10 CFU/g), *Salmonella* spp. (Absent in 25 g), Yeast (100 CFU/g), Molds (100 CFU/g).

2.2 Product Analysis – Justification for Revised Specification

The microbiological parameters of 2'-FL/DFL have been revised to account for occasional low-level detection of *Enterobacteriaceae* in some production batches. *Enterobacteriaceae* are part of the normal microbiota in food processing environments, food factories, and as it is not possible to completely eliminate

the presence of this group of microorganisms from the environment; these organisms sporadically gain access to the processing lines and products during post-sterilization manufacturing stages (*e.g.*, spray-drying) (WHO, 2006). As discussed by Cordier (2006) low level presence of *Enterobacteriaceae* in food is accepted and does not represent a direct safety concern; specification limits for *Enterobacteriaceae* are therefore typically established based on the type of food and the consumer. The establishment of strict control measures for members of the *Enterobacteriaceae* is thus not frequently applied during the manufacture of most conventional food products; however, an exception to this rule relates to *Salmonella* sp. and *Cronobacter sakazakii* contamination of powdered infant formula preparations that may be consumed by premature infants or term infants during the first few weeks of life.

The manufacture of infant formulae can be subdivided in two separate parts, a wet and a dry blending process. During wet-blending the raw materials used to manufacture the formulae such as lactose, liquid whey or dissolved dry ingredients (*e.g.*, 2'-FL/DFL) are heat-treated. Heat-treatment during wet blending can achieve reductions in excess of 8 to 12 log units for vegetative microorganisms such as *Salmonella* or *C. sakazakii*, and therefore heat-treatment is a highly effective process control for microbial contamination during wet blending (Cordier, 2006). The major risks for microbial contamination of infant formula occurs during the dry blending step where heat sensitive ingredients (vitamins, probiotic organisms) are blended into the powder formula. Accordingly, microbial specification limits for *Salmonella* sp. (absent in 25 g) and *Cronobacter* sp. (absent in 10 g) are required for ingredients that are added to infant formula during dry blending to meet the microbial requirements for infant formula as defined under 21 CFR §106.55 (U.S. FDA, 2018). During the dry blending stage of manufacturing (drying and cooling steps, intermediate storage steps, mixing of ingredients up to the filling of the product in its final container) it is essential to avoid or minimize post-process contamination; these processing steps are therefore located in high hygiene areas physically separated from the rest of the processing areas, including the wet blending steps (Cordier, 2006).

In consideration of the generally recognized view that *Enterobacteriaceae* are highly sensitive to heat processing and can be effectively controlled *via* thermal killing steps (WHO, 2006), Glycom has revised the microbial specifications for 2'-FL/DFL described in Table 2.3.1-1 of GRN 815 to include the following minimum microbial limits that can be applied for use of the ingredient during the wet blending stage of infant and toddler nutrition products manufacturing and for all non-infant formula food applications: Aerobic mesophilic total plate count (<1000 CFU/g), *Enterobacteriaceae* (<100 CFU/g), *Salmonella* spp. (Absent in 25 g), Yeast (100 CFU/), Molds (100 CFU/g). These specification limits are comparable to those concluded to be GRAS for use of galacto-oligosaccharides in infant formula during wet-blending as described in GRN 620 (U.S. FDA, 2016).

The proposed microbiological criteria for uses of 2'-FL/DFL in non-infant formula applications, which may not be subjected to thermal processing are aligned with international standards for microbiological examination of ready-to-eat foods including non-infant formula foods for infants and children (NRC, 1985; FSANZ, 2001, 2018).

The proposed new pH range of 4.0 to 6.0 (instead of from 3.5 to 5.4) for 2'-FL/DFL, is a correction of a mistake, where the pH ranges has been accidentally copied from the specification of other previously approved crystalline HMO to the specification of 2'-FL/DFL product. The new pH range (4.0 to 6.0) assures improved stability and significantly limits the degradation of amorphous, spray-dried 2'-FL/DFL under recommended storage conditions.

Part 3. List of Supporting Data and Information

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From:	Christoph Röhrig
To:	Anderson, Ellen
Subject:	RE: Amendment to GRAS notice for 2"-fucosyllactose/difucosyllatose
Date:	Thursday, January 31, 2019 5:11:32 AM
Attachments:	image001.png

Dear Ellen,

Thanks for confirming receipt.

Kind regards,
Christoph

Christoph H. Röhrig
PhD, Senior Scientist
Head of Regulatory & Scientific Affairs

_____
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From: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Sent: 31. januar 2019 03:56
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: Amendment to GRAS notice for 2'-fucosyllactose/difucosyllatose

Hello Dr. Röhrig,

This email is to confirm the receipt of your amendment to GRN 815 by our office on January 28, 2019.

Sincerely,
Ellen

Ellen Anderson
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309

ellen.anderson@fda.hhs.gov

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

13 March 2019

Ellen Anderson
Consumer Safety Officer
Division of Petition Review, HFS-265
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740-3835 USA

Re: GRAS Notice No. GRN 000815

Dear Dr. Anderson,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 26 February 2019 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for 2'-fucosyllactose/difucosyllactose (2'-FL/DFL) filed by the Agency under GRN 815.

FDA.1. In Table 1.3-1, the use level is indicated as 1.6 g/L of 2'FL/DFL in infant formula. Given the specification for 2'-FL (> 75%) and DFL (< 20%) in Table 2.1-1 in the amendment to GRN 000815, please provide a statement that any infant formula manufactured with 2'FL/DFL meeting these specifications would not exceed DFL levels found in breastmilk.

Based on the proposed use level of 1.6 g/L and a specification of DFL of <20%, the maximum amount of DFL that would be in infant formula would be 0.32 g/L. This value is considered a conservative estimation as the typical level of DFL in the ingredient under current manufacturing conditions is consistently in the region of 11 ± 1% and would provide DFL concentrations of up to 0.18 g/L in infant formula. The average concentrations of DFL in pooled samples of human breast milk are presented in Table 3.1.3.2-1 of the Notice and range from 0.3 g/L in mature milk to 0.5 g/L in colostrum. Concentrations of up to 1.0 g/L have been reported in pooled samples from colostrum. Accordingly, it can be concluded that the potential levels of DFL in infant formula from the intended uses of 2'-FL/DFL fall within the normal biological variance that has been reported for human milk.

As reported on page 34 of Glycom's Notice:

> "*Using the range of average levels of DFL reported for breast milk over different times of lactation, of 0.2 to 0.5 g/L (see Table 3.1.3.2-1), combined with the estimated formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of DFL from breastfeeding can be estimated at between 52 and 130 mg/kg body weight/day*".

Dietary intakes of DFL from all intended food uses of 2'-FL/DFL have been estimated by Glycom using data provided by the 2013-2014 National Health and Nutrition Examination Survey (NHANES). Estimated intakes of 2'-FL/DFL in infants aged 0 to 6 months were 326 mg/kg body weight among 90th percentile consumers. For infants aged 7 to 12 months, intakes of 83.4 mg/kg body weight were estimated for 90th percentile consumers. Using a conservative assumption that up to 20% of 2'-FL/DFL may consist of DFL, the estimated intakes of DFL in 90th percentile infant consumers would be 65.2 and 16.7 mg/kg body weight for infants aged 0 to 6 months and 7 to 12 months respectively. These intakes are expected to overestimate dietary exposure to DFL as the usual concentration of DFL in 2'-FL/DFL manufactured by Glycom is typically below 20%; nevertheless, the estimated intakes of DFL will be within the normal range that can be estimated for infants consuming human breast milk.

FDA.2. *It is not explicitly stated in the notice whether or not 2'-FL/DFL will be added to infant formula that is to be supplemented with other substances that either mimic human milk oligosaccharides (HMOs) or are nearly identical to HMO (i.e. fructooligosaccharide, galactoooligosaccharide, inulin, lacto-N-neotetraose, etc.). If 2'-FL/DFL is intended to be added to infant formula or conventional foods containing other undigestible oligosaccharides, please comment on whether the total load of undigestible oligosaccharides present in the final formulation is expected to cause adverse effects for the intended subpopulation.*

The purpose of adding 2'-FL/DFL − and other human-identical milk oligosaccharides − to infant formula is to produce an infant formula that is compositionally representative of human breast milk. To this end, 2'-FL/DFL may be added together with other human-identical milk oligosaccharides [*e.g*., lacto-*N*-neotetraose (LNnT), lacto-*N*-tetraose (LNT), 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL)], to match the levels in breast milk, taking into account natural variation. The use of 2'-FL/DFL in infant formula will, however, be substitutional to other sources of 2'-FL that have GRAS status for use in infant formula (*e.g*., GRN 546, 571, 650, 735, 749). Glycom is a manufacturer of infant formula ingredients, not a manufacturer of infant formula; therefore, Glycom is not in a position to comment on the appropriateness of using 2'-FL/DFL with other resistant oligosaccharide products that are not endogenous to human milk such as fructo-oligosaccharides and galacto-oligosaccharides. However, Glycom notes that all infant formulas marketed in the U.S. must meet federal nutrient requirements and infant formula manufacturers must notify the FDA prior to marketing a new formula. Under Section 412(d)(1) of the Federal Food, Drug, and Cosmetic Act (FFDCA), a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula 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 2'-FL/DFL with galacto-oligosaccharides (GOS)] would be well tolerated as part of the Agency's 90-day notification procedure. It is therefore Glycom's view that existing regulations governing the pre-market clearance requirements for infant formula in the United States are sufficient to ensure that a particular combination of resistant oligosaccharides that may be used in a new infant formula product are safe and suitable for their intended use.

FDA.3. *Table 3.1.2.2-1 "Permitted Uses of 2'-FL" describes the Commission Implementing Regulation (EU) 2017/2470 establishing novel foods in the European Union (EU). Under the column "Other Requirements," it states:*

> *"The labelling of food supplements containing 2'-fucosyllactose shall bear a statement that the supplements should not be used if other foods with added 2'-fucosyllactose are consumed the same day," and*
>
> *"The labelling of food supplements containing 2'-fucosyllactose intended for young children shall bear a statement that the supplements should not be used if breast milk or other foods with added 2'-fucosyllactose are consumed the same day."*

Given these requirements by the EU, the implication is that there is a risk of overconsumption of 2'-FL that may result in potential adverse effects. Please provide a brief explanation as to why these EU requirements are not a safety concern for your intended uses and why such labeling is not needed.

The term "food supplements" in the European Union pertains to a category of food products, which in the United States are referred to as "dietary supplements". The GRAS status of 2'-FL/DFL described in GRN 815 applies to uses in infant formula and conventional food and beverage products, not dietary supplements. Dietary supplements are regulated separately from conventional food products and are subject to the provisions of Dietary Supplement Health and Education Act of 1994 (DSHEA); uses of 2'-FL/DFL in this category of food products would need to consider statutory requirements of DSHEA and its implementing regulations, considerations that are outside the scope of the GRAS procedure. Glycom notes that food supplement products containing various resistant oligosaccharides [*e.g*., inulin, fructooligosaccharides (FOS), GOS] that have GRAS status for food uses in the U.S. are widely marketed in dietary supplement products, and Glycom is not aware of safety concerns for such ingredients with respect to the potential for overconsumption and the need for cautionary labeling. As 2'-FL/DFL is not absorbed intact to any significant extent and is essentially a form of dietary fiber, any safety concerns with respect to overconsumption would be related to gastrointestinal tolerance and would be self-limiting. Although it may be instructive to label dietary supplement products containing 2'-FL/DFL with cautionary statements about overconsumption from potential occurrences of 2'-FL/DFL in food, there does not appear to be any generally recognized basis for concerns over excess dietary fiber intake in the U.S. population. For example, it has been reported that only 5% of U.S. adults consume adequate intakes of dietary fiber, which are 38 g per day for adult men and 25 g per day for adult women[1]. With average intakes of dietary fiber within the U.S. population being only 17 g per day, there is no basis to limit dietary fiber intakes of any kind through cautionary labeling practices. Notwithstanding these general comments, as the uses of 2'-FL/DFL in dietary supplements are subject to provisions of DSHEA and not the GRAS procedure, Glycom is unable to formally comment on the need for labeling of dietary supplements with cautionary labeling, as this question is not pertinent to the GRAS status of the ingredient in conventional food products.

[1] J Acad Nutr Diet. 2015 Nov;115(11):1861-70

FDA.4. *Please provide the mean and 90th percentile dietary exposure estimates for infants 0-6 months and infants 7-12 months. Although it is implicit, please confirm that the uses and use levels are substitutional and thus would not result in significant increases in overall exposure to 2'-FL/DFL for the intended subpopulations.*

The Agency is correct. This information should have been included in Glycom's Notification and we apologize for this oversight. The dietary intake of 2'-FL/DFL from all potential food uses was estimated for infants aged 0 to 6 and 7 to 12 using data provided by the 2013-2014 NHANES. On an absolute basis, the mean and 90th percentile consumer-only intakes of 2'-FL/DFL among infants aged 0 to 6 months were determined to be 1.77 and 3.78 g/person/day, respectively (Table 1). Among infants aged 7 to 12 months, the mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 1.53 and 2.96 g/person/day, respectively.

Table 1 Summary of the Estimated Daily Intake of 2'-FL/DFL[a] from Proposed Food-Uses in the U.S. by Infants (2013-2014 NHANES Data)

Population Group	Age Group (Months)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6	1.72	3.75	96.8	1,177	1.77	3.78
Infants	7 to 12	1.51	2.95	98.5	876	1.53	2.96

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

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

On a body weight basis, the mean and 90th percentile consumer-only intakes of 2'-FL/DFL among infants aged 0 to 6 months were determined to be 144 and 326 mg/kg body weight/day, respectively (Table 2). Among infants aged 7 to 12 months, the mean and 90th percentile consumer-only intakes of 2'-FL/DFL were determined to be 43.5 and 83.4 mg/kg body weight/day, respectively.

Table 2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL/DFL[a] from Proposed Food-Uses in the U.S. by Infants (2013-2014 NHANES Data)

Population Group	Age Group (Years)	*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	139	320	96.7	1,169	144	326
Infants	7 to 12	42.8	83.4	98.5	873	43.5	83.4

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

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

FDA.5. *In Table 2.1-1 in the amendment to GRN 000815, the specifications for 2'-FL/DFL include the parameters for Color, Identification (RT of Standard ± 3%), and pH. These parameters are not reported in section 2.3.2 Product Analysis (beginning on page 14) which discusses the analytical results of five independent production batches of 2'-FL/DFL. Were these parameters tested for the five batches?*

In regard to physico-chemical properties, the 2'-FL/DFL mixture can be described as white to off-white amorphous powder or agglomerate. Amorphous powders do not possess defined melting points. 2'-FL/DFL is readily soluble in aqueous solutions (max. 500 mg/mL, 25°C), with poor solubility in any organic solvents. The summary of batch results corresponding to the selected physico-chemical properties of the 2'-FL/DFL mixture referred to in the question is presented in Table 3. We apologize for the oversight.

Table 3 Batch Results for Selected Physicochemical Properties of 2'-FL/DFL Product

Parameters	Specification	Manufacturing Batch Numbers:						
		CPN6317 1000417 FD	CPN6317 1000517 FD	CPN6317 1000717 FD	CPN6317 1000917 FD	CPN6317 1001017 FD	AVE	± SD
Appearance	Powder or agglomerates	Complies	Complies	Complies	Complies	Complies	Complies	
Color	White to off white	Complies	Complies	Complies	Complies	Complies	Complies	
2'-FL/DFL Identification	RT of standard ± 3%	Complies	Complies	Complies	Complies	Complies	Complies	
pH (20°C, 5% solution)	4.0 to 6.0	4.5	4.4	4.6	4.1	4.8	4.5	± 0.3

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose; AVE = average; RT = retention time; SD = standard deviation.

FDA.6. *The notice does not cite any clinical studies on DFL. Please confirm that your search of the published scientific literature found no studies on DFL.*

Yes, Glycom confirms that comprehensive searches of the published scientific literature did not identify clinical studies on DFL.

FDA.7. *Please confirm that the use of the terms "follow-on formula" and "toddler formula" are used interchangeably in the notice.*

Yes, use of the terms "follow-on formula" and "toddler formula" in the Notice are used interchangeably.

***FDA.8.** As an amendment to GRN 000815, footnote (b) was included as part of Table 2.1-1 "Specifications for 2'-FL/DFL." Footnote (b) states:*

> *"[b]The following microbial specifications represent minimum limits for 2'-FL/DFL that is added to infant formula and toddler formula products during the wet-mix stage of the formula manufacturing process, and also is suitable for conventional food products used by the general population (i.e., non-infant formula and toddler formula food products): Aerobic mesophilic total plate count (<1000 CFU/g), Enterobacteriaceae (<10 CFU/g), Salmonella spp. (Absent in 25 g), Yeast (100 CFU/g), Molds (100 CFU/g)."*

These microbiological parameters are not as restrictive as those listed in Table 2.1-1. It is not clear what this footnote means. Does Glycom intend to manufacture two distinct versions of 2'-FL/DFL, one that is added to formula during the wet-mix stage of the formula manufacturing process and therefore, undergoes a thermal kill step, and one that is not expected to undergo a thermal kill step in the manufacture of the finished food?

Yes, the Agency is largely correct. Although Glycom will not manufacture two distinct versions of 2'-FL/DFL *per se*, Glycom has established two separate specifications for the ingredient depending on the food application. The specifications in Table 2.1-1 are considered by Glycom to be appropriate for use of 2'-FL/DFL in all infant formula applications including situations where the ingredient could be added at the dry-blending stage of infant formula manufacturing (*e.g.*, stage where addition of heat-sensitive vitamins or probiotics are added). The microbial specifications in the footnote are considered by Glycom to be appropriate for the use of 2'-FL/DFL in infant formula where the ingredient is added during the wet-blending stage of infant formula manufacturing and which includes a thermal treatment step. The specifications in the footnote are comparable to those previously established for the use of GOS in infant formula as described in GRN 620.

We hope this information adequately addresses the Agency's questions on GRN 815, and if there is any additional information or further clarification that is required, Glycom will happy to provide such information upon request.

Sincerely,

[redacted]

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

13 March 2019

Date

From:	Christoph Röhrig
To:	Harry, Molly
Cc:	Marta Hanna Miks; Ryan Simon Intertek
Subject:	Re: GRN 000815 - 2"-FUCOSYLLACTOSE AND DIFUCOSYLLATOSE
Date:	Monday, July 01, 2019 5:27:56 AM
Attachments:	image001.png

Dear Ms Harry,

Thanks for contacting me on behalf of Ms Anderson.

Before addressing the specific questions I would like to mention that the individual batch results for residual protein were presented in Table 2.3.3.2-1 on page 18 of the notice.

But to address the questions specifically:

R1) The method used to measure residual protein is the Bradford method, which is a total protein method and doesn't allow any differentiation of the type of protein in regards to potential allergenicity. However, with the exception of lactose, that may contain traces of cow milk protein, Glycom is sourcing all raw materials and processing aids with certification by Major Allergen Declarations, which are therefore ensured not to import any other source of allergenic protein into the manufacturing process. Since the proposed use of 2'-FL/DFL is currently limited to cow milk based non-exempt infant formulas (containing large amounts of lactose) we don't identify a risk of allergenicity connected to the ingredients.

R2) The batch results of Table 2.3.3.2-1 are expressed as < LOR (Level of Reporting), with the Level of Reporting being identical with the Level of Quantitation (LOQ). The LOQ has been validated to be at 0.0017 % (w/w).

Please don't hesitate to get back to us in case you need further clarification. Since I will be on extended Summer vacation / Parental leave after the end of this week (until August 19), I am copying my colleague Marta Hanna Miks who will stand-in for me during my absence, and our trusted and valued consultant Ryan Simon from Intertek. Please include them in any further communication until my return.

With kind regards,
Christoph

From: Harry, Molly <Molly.Harry@fda.hhs.gov>
Sent: Thursday, June 27, 2019 7:51:06 PM
To: Christoph Röhrig
Subject: RE: GRN 000815 - 2'-FUCOSYLLACTOSE AND DIFUCOSYLLATOSE

Dear Dr. Röhrig,

I am sending this e-mail on behalf of Ellen Anderson. Ellen is currently on outside assignment.

The review of GRN 000815 is coming to its conclusion. However, to enable us conclude the process, the review team would like you to provide some clarification regarding the allergenicity information

provided in the notice.

On page 14, Table 2.3.1-1 (Specification), you provide a specification for average residual protein in the product as <0.0017%. However, in the results of your batch analyses no information was provided for residual protein. Additionally, on page 58, Section 6.5 (Allergenicity), you state that "--the purification steps remove protein (i.e., potential allergen) to a level of <0.0017% (w/w)." Please clarify the following:

1. Please confirm if 2'-FL/DFL that is the article of commerce does (or does not) contain any detectable level of allergenic protein;
2. Confirm whether (or not) 0.0017% is the limit of detection for the residual protein.

Thanks,

Molly A. Harry
Regulatory Review Scientist/Acting Team Lead

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, Division of Food Ingredients
U.S. Food and Drug Administration
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